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dissertation entitled

INVESTIGATION OF THE MECHANISMS UNDERLYING
PHEROMONE-BASED MATING DISRUPTION

presented by

Lukasz Lech Stelinski

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Entomology

Major Professor's Signature

Date

INVESTIGATION OF THE MECHANISMS UNDERLYING PHEROMONE-BASED MATING DISRUPTION

By

Lukasz Lech Stelinski

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Entomology

2005

ABSTRACT

INVESTIGATION OF THE MECHACHANISMS UNDERLYING PHEROMONE-BASED MATING DISRUPTION

By

Lukasz Lech Stelinski

Laboratory and field investigations were conducted with four species of tortricid moth pests of apple to gain better understanding of the mechanisms mediating pheromone-based mating disruption. Electroantennogram studies revealed differences among the leafroller species, *Argyrotaenia velutinana* and *Choristonerura rosaceana*, in the onset and duration of an antennal adaptation termed long-lasting adaptation (LLA). Although this difference exists between these species, it is unlikely an important contributor to mating disruption given that the exposure concentration of pheromone required to induce LLA is well above that which can be achieved in the field. Direct observations of moth behavior in the field revealed that tortricids closely approach polyethylene-tube pheromone dispensers in untreated and pheromone-treated orchards. These dispensers are the dominant method of broadcasting pheromone for mating disruption in the U.S.A. Field-observed approaches were brief (less than 60 s) and close (within 100 cm of dispensers). Laboratory flight-tunnel studies revealed that exposures similar to those observed in the field affect subsequent behaviors of the three species investigated. However, a large proportion of each species retained capability to initiate anemotaxis and plume follow after pre-exposure. These results suggest that false-plume-following and competitive attraction between synthetic sources and authentic females are the dominant mechanism mediating mating disruption of the species investigated.

ACKNOWLEDGEMENTS

I thank my major professors and friends, Drs. Larry Gut and Jim Miller, for their teaching and supervision throughout the duration of my dissertation work. As their graduate student, I have become quite aware of how fortunate I am to have had Larry and Jim serve as major advisors during my Ph.D. They were both excellent teachers and inspiring colleagues. My productivity and numerous achievements are indebted to the mutual exchange of ideas during numerous scientific debates. During these heated meetings, Larry, Jim and I not only learned from and taught one another but also continually challenged each other taking the research to a higher level. I am also grateful for Larry and Jim's support during a challenging and sometimes unpleasant final year of my Ph.D. program.

I thank my committee members: Drs. Rufus Isaacs, Ke Dong, and Edward Walker for improving my dissertation, providing critical input during my work, and for attending and participating in the various meetings throughout my program. I also give special thanks to an honorary committee member, Dr. George Ayers. Dr. Ayers continually inspires me to be a teacher. Dr. Ayers is a master of experiencial learning and teaching by his side has vastly improved my capabilities as an educator. I also thank Dr. Ayers for his support during the final difficult year of my program.

I give special thanks to Dr. John Wise, Janice Howard and the entire staff of the Trevor Nichols Research Complex (TNRC) for their valuable assistance on this project. Based on their vast knowledge and experience, John and Janice offered crucial advice and also graciously provided me with materials and work space on virtually every summer's day I spent working in Fennville. I will always have fond memories of the

various experiences and adventures I encountered during the summers I spent working and living on the TNRC. I also than Mr. Peter McGhee for his assistance in obtaining research materials and advice.

I give my sincere thanks to my close friends and invaluable assistants Noah Ressa and Kevin Vogel. With their insight, enthusiasm, and never ending quest for efficiency, Noah and Kevin contributed greatly to the realization of the various projects discussed within this dissertation. In addition to their expert technical assistance in both the laboratory and in the field, Noah and Kevin offered their sense of humor in times of distress, advice in times of perplexity, and criticism always.

Finally, I would like to that the many undergraduate students who worked very hard in assisting me to realize this body of work. I recognize Kevin Vogel, Rachel Mallinger, Krista Brueher, Anna Pierzchala, Noah Ressa, Lissa Renick, Keyan Maccune, and Travis Reed for technical assistance with experiments. These excellent undergraduate assistants always went beyond my expectations contributing heavily to my successful completion of this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Practical application of sex-attractant pheromones

Sex-attractant pheromones are semiochemicals involved in long-range mate finding in various animals, both invertebrates to vertebrates. Synthetic copies of these chemicals released into a crop can disrupt normal mating and mitigate crop damage by key agricultural pest insect species (Cardé and Minks, 1995; Gut et al., 2004). Currently, this technique is most commonly refereed to as "mating disruption."

Sex pheromone communication among the Insecta occurs over distances of 10 meters or more and only minute quantities of pheromone, typically released by females, are required to attract males (Cardé, 2003). The resulting pheromone plume is a filamentous structure of varying concentration, which is detected by males as a series of stimulus pulses of varying duration and concentration (Murlis, 1986; Murlis et al., 1992). This information is received by the male's antennae and passed to higher processing centers in the brain that control the rate of moth casting and counter-turning behavior, flight speed, and orientation within the pheromone plume (Baker et al., 1985). This plume-following behavior brings males within close proximity of calling females, where high pheromone concentration and visual cues trigger arrestment. The male and female may then undergo courtship behaviors (Baker and Cardé, 1979) culminating in mating. Given that minute quantities of pheromone mediate the above-described mate-finding behavior, it was postulated that release and dispersion of small amounts of synthetic copies of natural pest pheromones into crops could interfere with the pest's normal mate finding (Gaston et al., 1967). In the late 1960s and early 1970s, advances in chemical

ecology resulted in the identification of hundreds of moth species' pheromones and led to the mass production of synthetic copies for field application (Arn et al., 2000).

The impetus for mating disruption programs has risen due to decreases in effectiveness of conventional pesticides due to physiological resistance and increasing government-imposed restrictions on pesticide use (Gut et al, 2004). While not all moth species have been successfully controlled by mating disruption programs, the various cases of success give credence to this approach and inspire pheromone researchers and practitioners to strive for further improvements (Gut et al., 2004). Research into new release technologies, which may increase field longevity of synthetic sex pheromones, as well as studies comparing effectiveness of blend completeness are underway to improve this approach. The variability of success for some pest species as well as the cost and labor required for mating disruption programs are some of the current limitations that must be overcome.

While mating disruption has been tested for a variety of insect pest taxa, there are few examples of successful disruption that are comparable to that of moths (Polavarapu et al., 2002). In pioneering experiments, Gaston et al. (1967) found that evaporating one component of the cabbage looper moth's (*Trichoplusia ni*) pheromone into the crop atmosphere significantly disrupted the male's ability to locate a calling female. Later tests with various pest moth species confirmed that releasing large amounts of synthetic sex pheromone into the atmosphere of a crop could interfere with mate location, thereby controlling the pest by delaying or preventing mating (Mitchell et al., 1974; Shorey et al., 1974; Taschenberg et al., 1974; Rothchild, 1975). Since then, numerous other cases of successful control of moth pest using synthetic pheromones have been described

(reviewed by Cardé and Minks, 1995). Other studies have shown that certain moth species are less likely to be controlled through pheromone-mediated disruption (Reissig et al., 1978; Sanders, 1982a; Audemard, 1988, Agnello et al., 1996; Lawson et al., 1996). The differing levels of success described above could be due to species or crop-specific variation in susceptibility to mating disruption. Furthermore, the mechanisms by which mating disruption operates may vary depending on the species or crop habitat in question. This may then translate into variation of effectiveness depending on the release formulation or pheromone blend used to control a specific moth species. The future prospects of the communication disruption tactic and the rapidity with which new insects are controlled by this method may depend on understanding the mechanisms or principles that underlie it. Such research may in turn improve the effectiveness of future formulations and dispensing systems (Cardé and Minks, 1995).

Several environmental and ecological factors also contribute to the level of success of mating disruption. For example, effectiveness is dependent on pest density, often succeeding at lower pest densities but failing at high or even moderate ones (Knipling et al., 1979). In addition, variable canopy structure and wind direction affect pheromone plume structure and retention within crops, potentially reducing effectiveness (Cardé and Minks, 1995). However, when successful, mating disruption offers many advantages over conventional controls: It is highly specific to the target pest, safe to workers, and non-damaging to the environment. Thus, recent research has focused on improving success of disruption programs for a wider variety of moth species, while attempting to minimize cost and labor inputs.

Mechanisms of mating disruption

To improve mating disruption, the physiological and behavioral mechanisms underlying this technique must be elucidated for a variety of key moth pests within their specific cropping systems (Stelinski et al., 2004 a, b). The major mechanisms thought to mediate mating disruption have been reviewed and articulated several times (Bartell, 1982; Minks and Cardé, 1988; Cardé, 1990; Cardé and Minks, 1995; Sanders, 1996). They include: 1) sensory adaptation of the pheromone receptors at the peripheral level or habituation of the central nervous system, both of which elevate the threshold of behavioral responsiveness to pheromone, 2) competition whereby males follow plumes of pheromone emanating from point source dispensers as opposed to locating a calling female (also referred to as false-trail-following or false-plume-following), 3) camouflage of a pheromone plume from a calling female amidst a higher background concentration of synthetic pheromone which prevents males from orienting to and locating a pheromone point-source, 4) arrestment of upwind flight toward a pheromone source, occurring when the concentration of synthetic pheromone is substantially higher than that of the authentic female plume resulting in behavioral inhibition rather than attraction 5) advancement of timing of male's sexual activity due to the omnipresence of synthetic pheromone so that it no longer correlates with and precedes female calling within the diel cycle. These mechanisms may work in concert, such as when false-trail following leads to a prolonged exposure to a high dosage of synthetic sex pheromone, resulting in sensory adaptation or habituation (Stelinski et al., 2004b).

Pheromones blends

Moth pheromones are commonly blends of two or more components (Tamaki, 1979; O'Connell, 1981). Although the major components of such blends often elicit some behavioral responses from males to calling females, usually the full complement and correct ratio of components are required to induce the normal sequence of male sexual behaviors (Linn et al., 1984). Such sensitivity to specific blend ratios is believed to function as a mechanism for maintaining species isolation (Linn and Roelofs, 1983). Therefore, the type of pheromone blend used may also influence the efficacy of mating disruption. It has been argued that exact replicates of the natural pheromone blends of moth species should result in the most efficacious disruption programs given that complete pheromone blends have the lowest threshold for response and in some cases are required to induce a response (Minks and Cardé, 1988). However, in certain cases incomplete blends are equally or more effective than complete ones (Flint and Merkle, 1983; Hiyori et. al, 1986). However, increased quantities of off-blends are usually required to mimic authentic pheromone (Roelofs, 1978). In both the smaller tea tortrix of Japan (*Adoxophyes honmai*) and the European grape moth (*Eupocilia ambiguella*), successful disruption was achieved using a partial pheromone blend (Mochizuki et al., 2002). In other studies, a complete blend of pheromone was more effective than a simpler blend (Valeur and Lofstedt, 1995). Furthermore, Evenden et al. (2000) documented greater inhibition of orientation for the oblique-banded leafroller (*Choristoneura rosaceana*) in a wind tunnel when applying disruption with a more complete four component pheromone blend compared with a less attractive two component blend. Thus, with respect to the effectiveness of a mating disruption program, the importance of blend

completeness is a species-specific characteristic. To this end, programs should be targeted to the particular behavior and physiology of the given pest.

Furthermore, combining pheromones and certain plant volatile compounds may produce more potent attractants for certain moth species compared with their pheromones alone. For example, combining the plant volatiles racemic linalool, (E)-ß-farnesene, or (Z)-3-hexen-1-ol with codlemone, the primary pheromone component of codling moth (*Cydia pomonella*), increased attraction of male moths over that to the pheromone alone (Yang et al., 2004). Such blends of pheromone and plant volatiles may feature prominently in future applications of attract-and-kill technologies, where a pheromone-plant volatile blend combined with an insecticide induces attraction to a point source and subsequently kills the target pest species.

Release technologies

Various dispensing technologies and formulations have been designed for releasing synthetic pheromone into crop atmospheres. Such formulations must be designed to reliably release an effective quantity of pheromone at night, when the moths are most active. They must also protect the pheromone components from degradation as a result of UV and/or other environmental exposure. Furthermore, the release rate of active ingredient should be relatively constant so that it lasts throughout the pest's seasonal flight period but is not wasted through an unnecessarily high release. Finally, the formulation should be easy to apply at a low cost.

Currently, hand-applied, polyethylene "rope" dispensers at *ca.* one to four per tree are the dominant technology for dispensing pheromone for mating disruption of moth pests in orchards (Nagata, 1989; Agnello et al., 1996; Knight and Turner, 1999; Knight et

al., 1998). Pheromones can also be encapsulated in semi-permeable polymeric membranes producing a formulation that is sprayed onto the crop using standard air-blast sprayers (Vickers and Rothchild, 1991). Sprayable microencapsulated formulations are characterized by 1st order release rates of active ingredient, which decays quickly over time. Thus they require multiple applications per season and each application should uniformly cover the crop canopy. Such microcapsules have a homogenous dispersion pattern and can be applied at a very high density on the order of hundreds of capsules per squared centimeter of leaf or bark (Waldstein and Gut, 2003). Other dispensers include plastic fibers (Scentry fibers, Doane and Brooks, 1981) and plastic flakes (Hercon flakes, Miller et al., 1990), which emit pheromone at rates similar to that of calling females and thus act as attractive point sources. These plastic formulations are designed to prevent pheromone degradation in the field and promote its gradual release into the atmosphere (Weatherston, 1990). A more recently developed technology involves the release of much larger quantities of pheromone from fewer point-sources, relying on wind to disperse pheromone throughout the crop (Shorey and Gerber, 1996; Fadamiro et al., 1999; Isaacs et al., 1999). These aerosol devices have been termed *puffers*, *misters*, or *Microsprayers* and are characterized by intermittent release intervals as well as predetermined release rates (eg. 4 μl) (Isaacs et al., 1999). In addition, they provide a stable environment for a large reservoir of pheromone.

Each formulation and dispenser type is deployed at a particular optimal density and has a specific release rate. The effects on male moth behavior may be directly dependent on the type of application technology employed and its characteristic release rate. For example, exposure to constant but low concentrations of pheromone may

produce a different effect on the male behavior compared with brief exposures to extraordinarily high or low concentrations. Formulations such as flakes, fibers, or ropes that do not disperse pheromone homogenously and act as attractive point sources should be more likely to elicit false-plume-following, whereas sprayable formulations and aerosol dispensers dispersing pheromone homogenously should be more likely to elicit camouflage and/or sensory adaptation. Therefore, uncovering the mechanisms by which moth species are disrupted will aid in formulating the most effective species-specific pheromone release device.

Pheromone trapping

The use of pheromone lures in association with sticky traps has provided effective and reliable devices for monitoring the presence of insect pests within crops. Although such trapping technologies have not been reliable in estimating population density or predicting possible crop damage (Gut et al., 2004), they can be effective tools for detecting pest phenology so as to appropriately time insecticide sprays. Furthermore, traps baited with multi-component pheromone blends attractive to specific species are often used as proxies for females when evaluating the effectiveness of mating disruption programs. Specifically, if an application of pheromone effectively "shuts down" male moth captures in such monitoring traps, pheromone practitioners infer that female location by males and subsequent mating may have also been diminished or entirely prevented. However, it appears that pheromone-mediated trap shut-down must be nearly complete (>99%) to correlate well with complete prevention of female mating (Stelinski et al., unpublished data).

Standardization of trap usage in orchards with mating disruption programs is necessary due to the presence of false negatives, or an absence of moths in traps despite fruit injury (Knight et al., 1999). Higher moth catches can be achieved by placing traps in the upper third of the tree canopy and using lures with high pheromone loading rates for certain species (>1mg codlemone for codling moth, *C. pomonella*) (Riedl et al., 1979). Also, trap placement relative to pheromone dispensers is important. Specifically, in cases where pheromone traps and mating disruption dispensers are placed less than 0.3m apart, moth catches are significantly reduced compared with schemes in which spacing between traps and dispensers exceed 0.3m (Knight et al., 1999). Finally, trap maintenance, spacing, and deployment density need to be tailored to the target species and mating disruption program employed (Gut et al., 2004).

Species under study:

The Oriental fruit moth

The Oriental fruit moth (OFM), *Grapholita molesta* (Busck), is a key pest of peaches and nectarines as well as an important recent pest of apples (Rothschild and Vickers, 1991). Development of mating disruption for OFM began in the 1970s (Gentry et al., 1974; 1975; Rothchild, 1975; Cardé et al., 1977; 1979) and since then, it has been repeatedly shown that OFM can be effectively controlled using pheromones. Direct control of OFM by mating disruption has been achieved in North America, Australia, and Africa (Pfeiffer and Killian, 1988; Audemard et al., 1989; Rice and Kirsh, 1990; Pree et al., 1994; Barnes and Bloomfield, 1997; Il'ichev et al., 2002).

Rumbo and Vickers (1997) showed that exposure of male OFM to extraordinarily high concentrations of their pheromone (3200 female equivalents) induces peripheral

adaptation. However, these excessively high levels of pre-exposure necessary to achieve significant levels of adaptation are well above those achieved in field applications and therefore peripheral adaptation is likely not a relevant mechanism (Rumbo and Vickers, 1997). Other research has suggested that false-plume-following may be an important mechanism for mating disruption for OFM (Stelinski et al., 2004b). Previous wind tunnel experiments have not provided a conclusive answer to explain how OFM are disrupted, supporting both adaptation and false-plume-following as potential mechanisms for disruption (Valeur and Löfstedt, 1996). The above study also indicated that a pheromone blend containing the three main components is more effective than a two-component blend in wind tunnel disruption tests, indicating the possible importance of blend completeness to achive false-plume-following for this species. Recent research has also focused on determining whether mating disruption is more effective for OFM with higher densities of lower-release dispensers versus lower densities of high-release dispensers (deLame, 2003).

The codling moth

The codling moth (CM), *Cydia pomonella* (L.), is a major pest of apples, pears, plums and walnuts world-wide (Krupke et al., 2002). The pheromone blend of CM determined thus far is comprised of eight components but only one major component, (*E*,*E*)-8,10-dodecadien-1-ol (codlemone), is required to attract male CM. Control of this pest by pheromones has been extensively investigated with mixed results (Cardé and Minks, 1995). Studies of CM for more than 25 years have established that mating disruption can be effective provided initial pest population densities are low and the treated orchards are isolated from sources of immigrating females (Charmillot, 1990;

Minks, 1996). In certain cases, it may be necessary to use supplementary insecticides in order to keep the population of CM at a manageable level and to mitigate unacceptable levels of crop damage (Gut and Brunner, 1998). Trimble (1995) concluded that mating disruption alone could not stabilize the damage to Ontario's organic apple orchards by CM after the first year of mating disruption treatments. However, an integrated program of pheromone-mediated mating disruption, post-harvest fruit removal, and tree-banding effectively controlled CM in organic apple orchards in British Columbia with damage averaging <0.7% over three years (Judd et al., 1996). Research efforts to improve mating disruption of CM are currently underway. This involves uncovering the relevant mechanisms underlying disruption for this species.

The leafrollers

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris), is native to North America and is widely distributed from British Columbia to Nova Scotia and south to Florida (Chapman et al., 1968). *C. rosaceana* has an extremely wide host range; however, the preferred hosts are woody plants including Rosaceae. *C. rosaceana* is an important pest of pome fruits in North America. The redbanded leafroller, *Argyrotaenia velutinana* (Walker), is sympatric with *C. rosaceana* and native to temperate Eastern North America (Chapman, 1973). This species also has a broad host range. It feeds on leaves of diverse plant species except conifers. The larvae feed on many unrelated plants, including most common fruits, vegetables, weeds, flowers, ornamentals and shrubs (Hull et al., 1995). Among the fruits, *A. velutinana* prefers apples and commonly occurs in the apple-growing areas of the Midwestern and Eastern United States and Eastern and Western Canada (Hull et al., 1995).

The obliquebanded and redbanded leafrollers share the major components of their pheromone blends; (*Z*)11-14:Ac and (*E*)11-14:Ac in a 98:2 ratio for *C. rosaceana* and 93:7 ratio for *A. velutinana.* (Roelofs and Arn, 1968; Roelofs and Tette, 1970; Cardé and Roelofs, 1977; Hill and Roelofs, 1978). *A. velutinana* is reported to be easily disrupted, in some cases using only the main pheromone component, (*Z*)11-14:Ac (Novak et al., 1978; Reissig et al., 1978; Novak and Roelofs, 1985). In contrast, *C. rosaceana* is often described as difficult to disrupt in the field as measured by lowered captures of males by synthetically baited traps, fruit and foliar damage, and mating reductions of tethered females (Novak et al., 1978; Reissig et al., 1978; Roelofs and Novak, 1981; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996) and possibly requiring the full natural blend of pheromone components. However, populations of *C. rosaceana* from Western Canada and Washington state, which are characterized by a slightly different blend of pheromone components compared with those from Central and Eastern North America (Vakenti et al., 1988; Thomson et al., 1991), have shown some potential for successful mating disruption in small-plot trials (Knight et al., 1998; Evenden et al., 1999b; Evenden et al., 1999c). Despite the close relatedness of these two species, the similarity of their pheromone blends, and their sympatric occurrence, the effectiveness of mating disrutption of these two species has differed drastically in the preponderance of studies conducted thus far.

Overall objectives

The overall objectives for this dissertation were to gain insight into the physiological and behavioral mechanisms mediating pheromone-based mating disruption. This task was undertaken by investigating the four tortricid moth species described above. The approach taken was multifaceted and incorporated laboratory and field electrophysiology, analytical chemistry, laboratory flight-tunnel investigations, field-trapping experiments, and direct observations of feral moth behavior. The specific objectives of each study are summarized in the introductions of the seven chapters that follow.

CHAPTER ONE

Presence of long-lasting peripheral adaptation in the obliquebanded leafroller, *Choristoneura rosaceana* and absence of such adaptation in the redbanded leafroller, *Argyrotaenia velutinana*

Also published as:

Stelinski, L. L., J. R. Miller, and L. J. Gut. 2003. Presence of long-lasting peripheral adaptation in the obliquebanded leafroller, *Choristoneura rosaceana* and absence of such adaptation in the redbanded leafroller, *Argyrotaenia velutinana*. *Journal of Chemical Ecology*. 29: 405-423.

ABSTRACT

Pre-exposure of male obliquebanded leafrollers, *Chroristoneura rosaceana* (Harris), to the main component of their pheromone blend and traces of its geometric isomer ((*Z*)11-14:Ac and (*E*)11-14:Ac, respectively) at 36 ± 12 ng / ml air for durations of 15 and 60 min in sealed Teflon chambers with continuous air exchange significantly reduced peripheral sensory responses to these compounds as measured by electroantennograms (EAGs). The EAG responses of *C. rosaceana* to all tested dosages of pheromonal stimuli and blank controls were lowered by 55-58 % and made a linear recovery to 70-100% of the pre-exposure amplitude within 12.5 min at a rate of 3-4 % / min. Exposures of 5 min were insufficient to maximally adapt *C. rosaceana*; however, exposures of 15 and 60 min reduced sensory responsiveness to the same minimum. In contrast, EAG responses of redbanded leafroller, *Argyrotaenia velutinana* (Walker), after identical pheromone exposure for 5 and 60 min yielded no long-lasting peripheral sensory adaptation as measured by EAGs, even though this species shares the same main pheromone components with *C. rosaceana.* We postulate that the long-lasting peripheral adaptation observed for *C. rosaceana* is a mechanism that impedes central nervous system habituation in this species. In contrast, *A. velutinana* may be more susceptible to central nervous system habituation because it lacks the capacity for minutes-long adaptation. We propose that long-lasting adaptation may be a mechanism explaining some of the variation in efficacy of pheromone-based mating disruption across taxa.

INTRODUCTION

Pheromone-based mating disruption for economically important lepidopteran pests has been successfully demonstrated and promises to make important contributions to biorational pest control (Cardé et al., 1975; Deland et al., 1994; Cardé and Minks, 1995). However, in certain cases, utility of pheromone mating disruption may be limited by such factors as: high population densities of moths (Schmitz et al., 1995a; Weissling and Knight, 1996; Suckling and Angerelli, 1996; Knight and Turner, 1999) which increase competition between calling females and pheromone dispensers; migration of mated females into treated areas; variable canopy structure; wind direction, which affects pheromone plume structure (Cardé and Minks, 1995) and retention; and the specific tuning of the pheromone blends employed (Pfeiffer et al., 1993; Knight et al., 1998; Knight and Turner, 1999; Evenden et al., 1999a). A challenge for pest managers is to determine which pests are most conducive to management by pheromones as opposed to other strategies.

The most popular hypotheses concerning mechanisms for disruption of pheromone-based communication are: 1) sensory adaptation at the peripheral level affecting olfactory receptors, 2) habituation affecting processing of and normal responsiveness to olfactory information reaching the central nervous system, 3) camouflage of female-produced plumes, and 4) false-trail-following of synthetic pheromone plumes by male moths (Rothchild, 1981; Bartell, 1982; Cardé, 1990).

The effects of short and prolonged exposures of moths to their species-specific synthetic pheromones and geometric isomers have been the targets of various

investigations (Bartell and Roelofs, 1973; Bartell and Lawrence, 1976a; 1976b; 1976c; Linn and Roelofs, 1981; Sanders, 1985). These and other studies established that prolonged exposure to pheromone decreased such male behavioral responses as: wing fanning and rapid walking (Bartell and Roelofs, 1973), recapture rates in mark-release studies, and orientation in wind tunnels (Rumbo and Vickers, 1997; Daly and Figueredo, 2000). For *Trichoplusia ni* (Hübner), these effects occurred with no corresponding decrease in responses of olfactory receptor neurons as measured by EAGs (Kuenen and Baker, 1981). In a later study, male *Cydia* (=*Grapholita*) *molesta* (Busck) exhibited days-long habituation after exposure to its pheromone (Figueredo and Baker, 1992). Such studies provide evidence that habituation of the central nervous system can be an important mechanism for observed decreases in males' responsiveness to female pheromones under conditions of mating disruption. In contrast, other studies suggest that adaptation and habituation, although inducible, had no influence on the effectiveness of mating disruption in the field (Novak and Roelofs, 1985; Schmitz et al., 1995a; 1995b; 1997). The main explanations proposed for mating disruption of these species are competition between females and pheromone dispensers as well as camouflage of female pheromone signals.

The recent review by Zufall and Leiders-Zufall (2000) has formally defined three distinct types of olfactory adaptation, using vocabulary likely to be adopted for animals generally. The categories are characterized by differing temporal dynamics. The two short-lived variants have onset times on the order of 100 ms and 4 s, and corresponding recovery times of 10 s and 1.5 min, respectively. The third type of adaptation is termed "long-lasting"; onset occurs after repetitive exposure for 25s and subsequent recovery

intervals in the vicinity of 6 min. As shown by Zufall and Leiders-Zufall (2000), these three types of adaptation are further distinguished by separate molecular mechanisms. For example, in salamander (*Ambystoma tigrinum*) olfactory receptor neurons, the long-lasting adaptive effect depends on the carbon monoxide (CO)/cGMP second messenger system, and can be uncoupled from excitation and completely eliminated by inhibitors of CO synthesis (Zufall and Leinders-Zufall, 1997). Likewise, elevated concentrations of cGMP were observed in moth antennae (*Heliothis virescens* (F.)), after application of high doses of pheromone (Boekhoff et al., 1993). Furthermore, Boekhoff et al., (1993) established that the IP_3 pathway mediates the primary transduction of pheromone signaling in antennal neurons, whereas activation of the cGMP-cascade is a secondary effect thought to be involved in adaptation and tuning of receptor neuron sensitivity.

Few studies have characterized sensory adaptation with the intention of distinguishing long-lasting vs. short-lived variants of peripheral adaptation. Kuenen and Baker (1981) documented a short-lived form of pheromonal adaptation in *T. ni* using EAG; full receptor cell recovery occurred within 1 min of exposure. Schmitz et al., (1997) characterized longer-lasting sensory adaptation in *Lobesia botrana* (Denis and Schiffermüller), from which EAG responses returned to 70% of their pre-treatment amplitude after 5 min.

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris), and the redbanded leafroller, *Argyrotaenia velutinana* (Walker), share the major components of their pheromone blends; (*Z*)11-14:Ac and (*E*)11-14:Ac in a 98:2 ratio for *C. rosaceana* and 93:7 ratio for *A. velutinana.* (Roelofs and Arn, 1968; Roelofs and Tette, 1970; Cardé and Roelofs, 1977; Hill and Roelofs, 1979). *A. velutinana* is reported to be easily

disrupted, in some cases using only the main pheromone component, (Z)11-14:Ac (Novak et al., 1978; Reissig et al., 1978; Novak and Roelofs, 1985; Miller et al., unpublished data). In contrast, *C. rosaceana* is often described as difficult to disrupt in the field as measured by lowered captures of males by synthetically baited traps, fruit and foliar damage, and mating reductions of tethered females (Novak et al., 1978; Reissig et al., 1978; Roelofs and Novak, 1981; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996; Miller et al., unpublished data) and possibly requiring the full natural blend of pheromone components. However, populations of *C. rosaceana* from Western Canada and Washington state, which are characterized by a slightly different blend of pheromone components compared with those from central and eastern North America (Vakenti et al., 1988; Thomson et al., 1991), have shown some potential for successful mating disruption in small-plot trials (Knight et al., 1998; Evenden et al., 1999b; Evenden et al., 1999c).

We seek to understand the mechanisms underlying the differences in susceptibility to mating disruption in these two sympatric tortricids. In this first paper of a following series, we characterize the differences in the capacity for "long-lasting" peripheral adaptation and disadaptation in *C. rosaceana* and *A. velutinana* using EAG measurements performed on moths before and after exposure to pheromone for various time intervals.

METHODS AND MATERIALS

Insect colonies.

C. rosaceana were drawn from a four-year-old laboratory colony originally collected as 1st and 2nd generation pupae from apple orchards in southwestern Michigan. *A. velutinana* came from a long-established laboratory colony maintained at Geneva, NY

by W. Roelofs. Both species were reared at 24°C on pinto bean diet (Shorey and Hale, 1965) under a 16 : 8 (L:D) photoperiod. Male pupae of each species were segregated in 1 L plastic cages containing a 5 % sucrose solution in plastic cups with dental cotton wick protruding from their lids.

Electroantennograms.

Our EAG system consisted of a data acquisition interface board (Type IDAC-02) and universal single ended probe (Type PRS-1) from Syntech (Hilversum, The Netherlands). The recording and indifferent electrodes consisted of silver coated wire in glass micropipettes (10 µl micro-hematocrit capillary tubes) containing 0.5 M KCl. Micropipettes were prepared by a Flaming/Brown micropipette puller (Model P-97, Sutter Instrument Co.). The pipettes were pulled at 308°C under time and velocity settings of 150 and 80, respectively. EAG data were recorded, stored, printed, and quantified using a Gateway 2000 (P-75) computer equipped with an interface card and software (PC-EAG version 2.4) from Syntech. The interface card contained a software-controlled amplifier, and an A/D conversion circuit; it operated with 12-bit resolution.

Male insects of both species were 2-4 d old when used for electroantennograms. EAGs were measured as the maximum amplitude of depolarization elicited by the applied stimulus. EAGs were conducted on live-insect preparations (Fig 1). Insects were restrained on a wax-filled, 3.5 cm diam Petri dish by placing clay (10 x 3 mm) over their thorax and abdomen. The terminal 2 segments of the antenna destined for recording were removed with fine scissors and the recording electrode was placed over the severed end. The reference electrode was inserted into the neck (Fig. 1).

Stimulus delivery.

This apparatus consisted of a glass Y-tube (each arm 2 cm in length, the base 1 cm long, 0.5 cm diam) positioned approximately 5 mm from the antennae (Fig 1). Carbon-filtered and humidified air (50 ml / min) was delivered continuously into one arm of the Y-tube via Tygon tubing, while pheromone stimuli were delivered through the second arm of the Y-tube. (*Z*)11-14:Ac (lot # 10010) was obtained from Shin Etsu (Tokyo, Japan) and purity was determined with GLC (96.1 % (*Z*)11-14:Ac and 3.9% (*E*)11-14:Ac). Various concentrations of this mixture of pheromone in hexane (20 µL total solution) were pipetted onto 1.4 x 0.5 cm strips of Whatman No. 1filter paper. After 5 min in a fume hood for solvent evaporation, treated strips were inserted into disposable glass Pasteur pipettes, sealed with Parafilm, and allowed to equilibrate for 24 hr prior to use. A given stimulus pipette (cartridge) was inserted into the Y-tube such that its tip was positioned at the junction of the Y-tube and 1.5 cm from antennal preparations (Fig. 1). Stimulus puffs (1 ml) were generated through the cartridges with a clean hand-held 20 ml

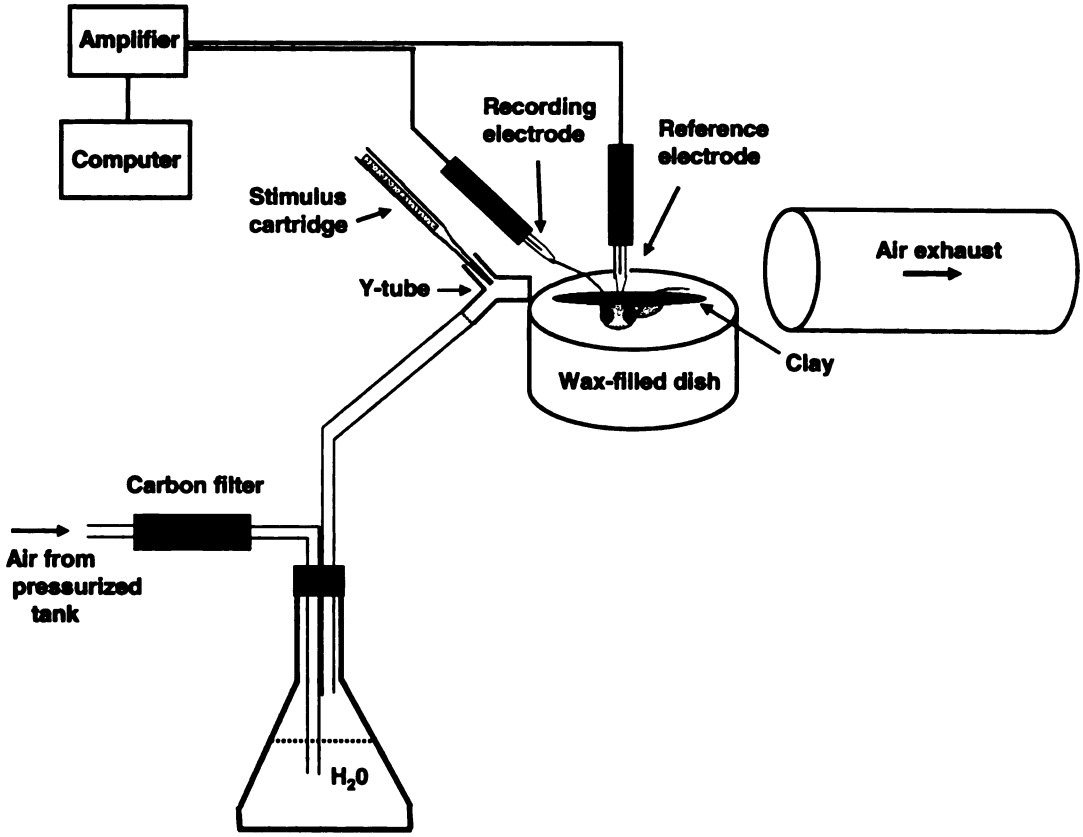


Figure 1. Design of electroantennogram recording and stimulus delivery apparatus. Insect and electrodes are enlarged.

glass syringe connected to the pipettes with a 1 cm piece of Tygon tubing. The time interval to expel 1 ml of stimulus odor or clean air from the syringe was quantified with video-cinematography and slow-motion playback. The 1ml puff of air was expelled from the syringe within 120 ± 0.02 (S. D.) ms (n = 20).

Adaptation experiments.

Prior to adaptation experiments, dose-response curves were obtained for both moth species. Pheromone dosages were delivered to individual moths ($n \geq 10$) either in ascending or descending order. Four puffs of each dosage spaced 10 sec apart were administered to yield duplicate depolarization amplitudes at each dosage. Appropriate dosages were chosen for further experimentation and the ascending order of stimulus-dose application was employed in all later studies.

To test for inducement of adaptation, males of both species were placed in adaptation chambers consisting of 1 L Teflon transfer containers (Jensen, Coral Springs, FL) equipped with two 0.64 cm ports in their lids (Fig. 2). Glass inlets and outlets were affixed to the lids, allowing for carbon-filtered air (30 ml / min) to pass through the chambers. Chambers were divided with wire mesh (Fig. 2), such that insects were confined in upper halves while a rubber septum impregnated with 5 mg (solvent free) of the same pheromone blend used in EAG cartridges was placed in the lower half (Fig. 2). This arrangement was designed to reduce variation in pheromone exposure relative to that possible in a static container, where moths might touch the dispenser.

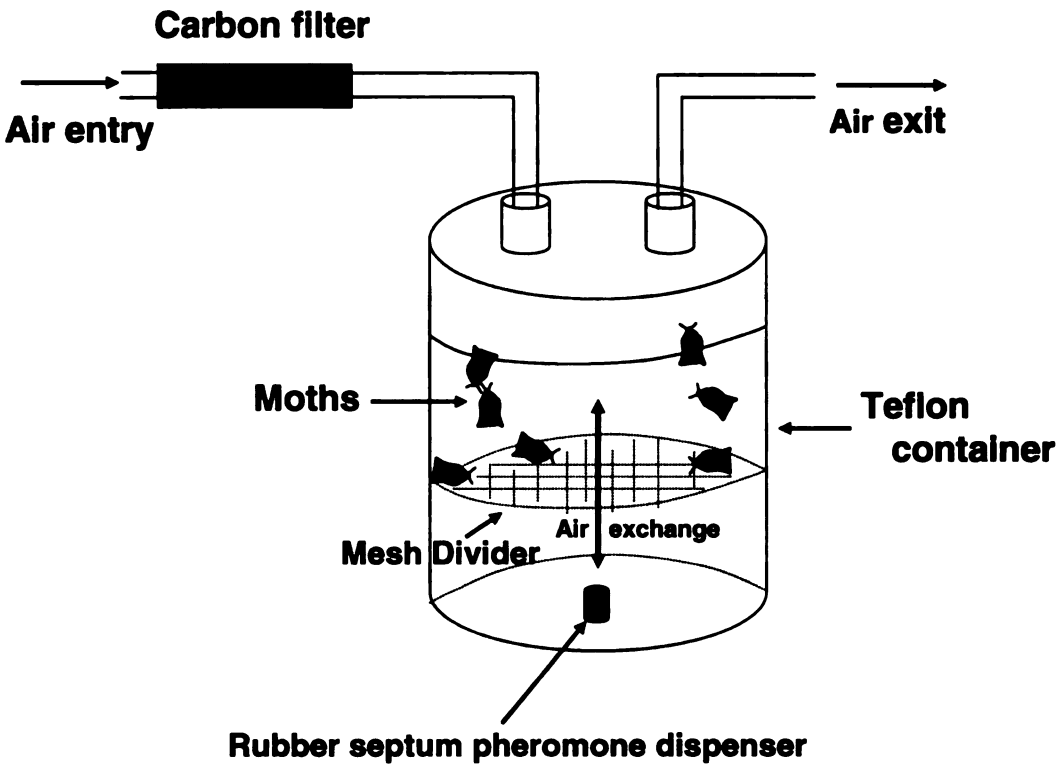


Figure 2. Adaptation chamber: 1 L Teflon container with glass inlet and outlet. Wire mesh divider prevents insects from contacting pheromone dispenser but allows exchange of air at throughput of 30 ml/min.

All chambers were allowed to equilibrate for 15 min prior to insertion of insects. To assay the onset of peripheral adaptation, EAGs were performed on both species ($n \geq 18$) after confinement for 5, 15, or 60 min. Sham treatments ($n \geq 18$) were administered in separate, pheromone-free chambers. EAGs were performed on all insects prior to confinement, exactly 1 min after confinement, and 12.5 or 60 min post-confinement.

Disadaptation study.

Because minutes-long adaptation did not occur in *A. velutinana* (Results below), only *C. rosaceana* was used for characterization of disadaptation. EAGs were performed on groups of six male *C. rosaceana* prior to confinement in adaptation chambers for 15 min of continuous pheromone exposure. After exposure, entire groups were removed from adaptation chambers and placed into clean-air containers. Individuals were assayed 1, 2.5, 5, 7.5, 10, 12.5, and 30 min thereafter. A total of 7 groups of six individuals was assayed in this manner.

Pheromone concentration in adaptation chamber.

Rubber septa impregnated with 5 mg of pheromone were placed into adaptation chambers and allowed to equilibrate for 15 min with air flowing through at a rate of 30 ml / min, as previously described. After equilibration, the exhaust port was replaced with a port sealed with a clean rubber septum. Immediately following port replacement, 15 ml of air was withdrawn from adaptation chambers through the septum-sealed port into a 20 ml glass syringe fitted with a 22 gauge needle. In rapid succession, 5 ml of hexane wash containing an internal standard of saturated 14:Ac at 6.4 ng / µl was drawn into the syringe. The solution within the syringe was carefully shaken for 30 sec then expelled

into a gas chromatography vial. This entire procedure was repeated 5 times with separate pheromone-impregnated septa. Prior to analysis, samples were concentrated under nitrogen by a factor of 50. One µl samples were analyzed by gas chromotography (HP-6890, Hewlett-Packard Co) with flame ionization detection (FID) to reveal the concentration of pheromone present in adaptation chambers. The GC was fitted with a DBWAXETR polar column (model 122-7332, J and W Scientific, Folsom, CA) of length 30 m and internal diam. 250 µm. The initial GC temperature was held at 100 °C for 3 min and the program ran at a rate of 10°C/1 min, 100-250°C and was held for 3 min; the carrier gas was He. We calculated the concentration of pheromone present in the 15 ml of adaptation chamber air by multiplying the ratio of peak areas of the target compound ((Z)11-14:Ac) over the standard (saturated 14:Ac) by: 1) the concentration of internal standard present in the hexane wash, and 2) 100 to account for GLC analysis of only 1 % of the total concentrated sample.

The dosage of pheromone in adaptation chambers relative to pheromone cartridges was also compared to that of EAG cartridges. Adaptation chambers containing rubber septa impregnated with 5 mg of pheromone and equilibrated for 15 min were used as stimulus cartridges in EAG-assays of both *C. rosaceana* and *A. velutinana;* n=10 for both species. EAGs were also carried out on individuals of both species (n=10) using control chambers lacking pheromone. Chambers were modified by replacing the glass exhaust tube with a 5 cm Teflon tube. The incurrent port was disconnected from the continuous air-flow 1 min prior to assay and connected to a 20 ml glass syringe via a 25 cm piece of Tygon tubing. The Teflon exhaust tube was positioned *ca.* 1 cm from antennal preparations and 1 ml puffs were delivered through the chambers.

Statistical Analysis.

Data were subjected to analysis of variance (ANOVA) and differences in pairs of means over time and between treatments were separated using Tukey's multiple comparisons test (SAS Institute, 2000). The relationship between percent recovery from adaptation and time interval post-exposure to pheromone was analyzed using linear regression. All ± values are SEM. unless otherwise designated. Images in this dissertation are presented in color.

RESULTS

EAG dose-response curves. Profiles for both species are presented in Fig. 3 as unnormalized mV responses in both ascending and descending orders of stimulus-dosage applied. Under both regimes, responses of the two species overlapped for pheromone dosages 0-2.0 μg (Fig. 3). Beginning at 20 μg, the dosage-response curves diverged; *A. velutinana* consistently produced higher EAG amplitudes at the high dosages than did *C. rosaceana.* Under the ascending regime, the mean responses reached a plateau of *ca.* 6 and 5 mV for *A. velutinana* and *C. rosaceana*, respectively, at the 200 μg dose. The mean EAG amplitudes were significantly ($P<0.05$) different at the 200 μg and 2 mg dosages. Quite linear dosage-response curves were observed when dosages were applied in descending order, and the divergence in EAG amplitude between species was significantly ($P<0.05$) different at the 200 μg, 2mg, and 20 mg dosages. The maximum mean mV response for a given species was consistent irrespective of the order for dosage presentation (Fig. 3).

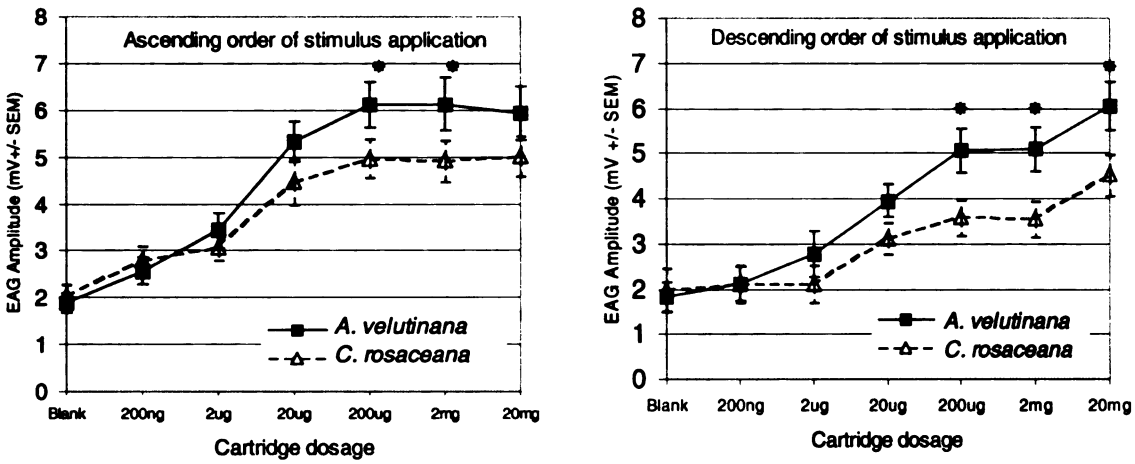


Figure 3. Dosage-response relationships for *Choristoneura rosaceana* and *Argyrotaenia velutinana* live-insect antennal preparations in both ascending and descending orders of stimulus application. Significant ($p<0.05$) differences between pairs of means are indicated by *.

Pheromone exposure studies.

EAG amplitudes of male *C. rosaceana* prior to and 1 min after 5 min of confinement in adaptation chambers were not significantly different (Fig. 4 a, Fig. 5 a). However, EAG responses to the 200 µg and 2 mg dosages were significantly ($P<0.05$) lower in *C. rosaceana* assayed 1 min after 15 min of confinement compared with their pre-exposure responses (Fig. 4 b, Fig. 5 b). The mean EAG responses of these individuals returned to their pre-exposure amplitudes within 30 min post-exposure (Fig. 4 b, Fig. 5 b). *C. rosaceana* exposed for 60 min in adaptation chambers produced significantly lower

($P<0.05$) mean EAG amplitudes 1 min post-confinement compared with their pre-exposure responses to the 2 μg, 200 μg, and 2 mg doses (Fig. 4 c, Fig. 5 c). After a 60 min post-exposure interval in clean air, the mean EAG responses of *C. rosaceana* exposed for 60 min were not significantly different from their pre-exposure amplitudes for all dosages except 2 mg (Fig. 4 c, Fig. 5 c).

EAG responses of *A. velutinana* prior to exposure in adaptation chambers and after exposure for 5 and 60 min durations were not significantly different at any of the stimulus dosages applied (Fig. 4 e, f, Fig. 5 d).

Disadaptation study.

C. rosaceana EAG amplitudes post 15 min of exposure were significantly ($P<0.05$) lower compared with pre-exposure amplitudes for the 200 μg, and 2 mg doses at 1, 2.5, and 5 min post-exposure (Fig. 4 d). At 7.5 min post-exposure, the mean EAG amplitudes were no longer significantly different from pre-exposure amplitudes and responses returned to amplitudes nearly equal to pre-exposure responses within 12.5 minutes after exposure (Fig. 4 d). The mean EAG amplitudes elicited by the blank and 2 μg doses also decreased after 15 min of exposure and followed a similar trend of increase post-exposure; however, the mean differences were not significant in simple pair-wise comparisons. A significant ($P<0.05$) linear relationship was found between the percent recoveries of the mean EAG responses elicited by each dosage including the blank when regressed over time post 15 min exposure in adaptation chambers (Fig 6).

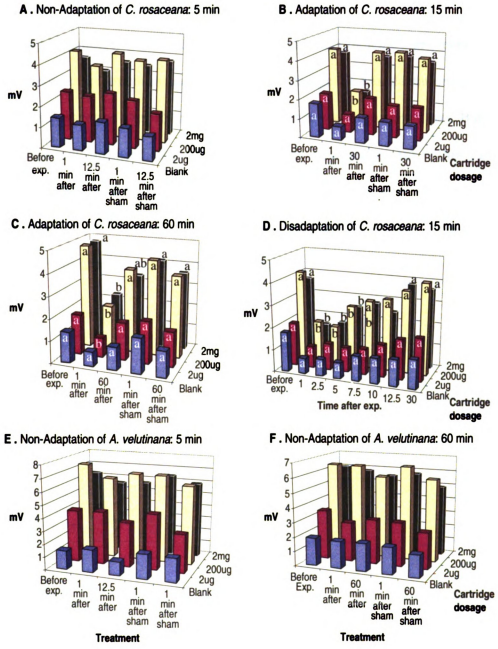
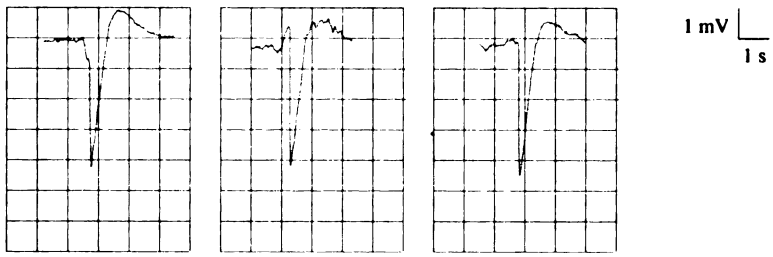


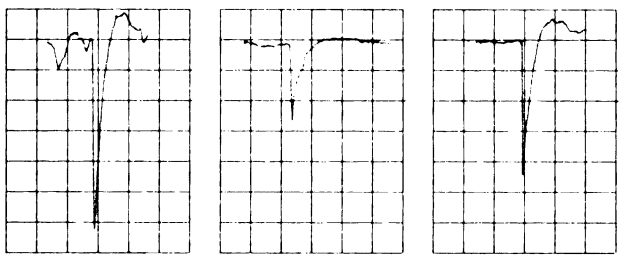
Figure 4. A. Effect of 5 min of confinement of *Choristoneura rosaceana* in adaptation chambers. There were no significant differences between treatment means for responses within or across dosages. B. Effect of 15 min of confinement of *C. rosaceana* in adaptation chambers. Bars with different letters indicate significant differences between

treatment means within a given dosage. C. Effect of 60 min of confinement of *C. rosaceana* in adaptation chambers. Bars with different letters indicate significant differences between treatment means within a given dosage. D. Disadaptation of *Choristoneura rosaceana* after 15 min of confinement in adaptation chambers. Bars with different letters indicate significant differences between treatment means within a given dosage. E and F. Effect of 5 and 60 min, respectively of confinement of *Argyrotaenia velutinana* in adaptation chambers. There were no significant differences between treatment means for responses within a given dosage.

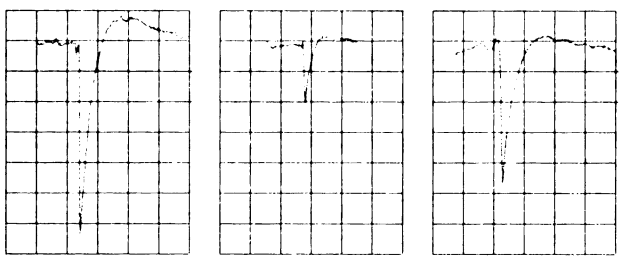
A) *C. rosaceana*: 5 minutes exposure



B) *C. rosaceana*: 15 minutes exposure



C) *C. rosaceana*: 60 minutes exposure



D) *A. velutinana*: 60 minutes exposure

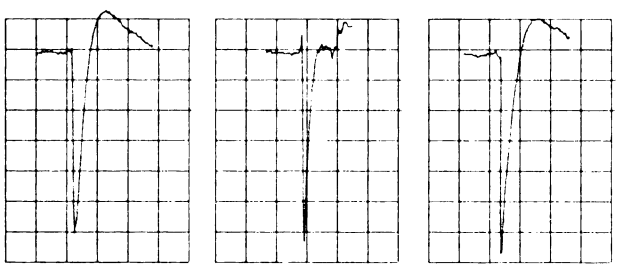


Figure 5. Representative EAG tracings of *Choristoneura rosaceana* and *Argyrotaenia velutinana* responding to 1 ml puffs from pheromone cartridges loaded with 200 μg of pheromone. Tracings on the left within each row are responses of *C. rosaceana* or *A. velutinana* prior to exposure in adaptation chambers. Center tracings within each row are responses of the same individuals after various exposure intervals within adaptation chambers. Tracings on the right within each row were measured after 30-60 min intervals within pheromone-free air after exposure in adaptation chambers. Each horizontal tick mark represents 1 s and each vertical tick mark represents 1 mV of depolarization. Percent recovery was calculated as the ratio of the mean EAG amplitude at the various time intervals at which individuals were assayed post-exposure over the mean pre-exposure amplitude. The process of recovery was decidedly linear; recovery rate was a constant 3-4 % / min and was nearly complete within 12.5 min (Fig. 5).

Pheromone concentration in adaptation chamber.

The retention times for the 14:Ac (internal standard) and (*Z*)11-14:Ac were 13.7 ± 0.012 and 14.3 ± 0.008 min, respectively. The trace amount of (*E*)11-14:Ac present in the pheromone used to impregnate the rubber septa was undetected by FID in the air samples taken from adaptation chambers. The mean peak areas in thousands for the 14:Ac standard and (*Z*)11-14:Ac were 454 ± 28 and 8 ± 3, respectively. The calculated concentration of (*Z*)11-14:Ac present in our adaptation chambers was 36 ± 12 ng / ml of air.

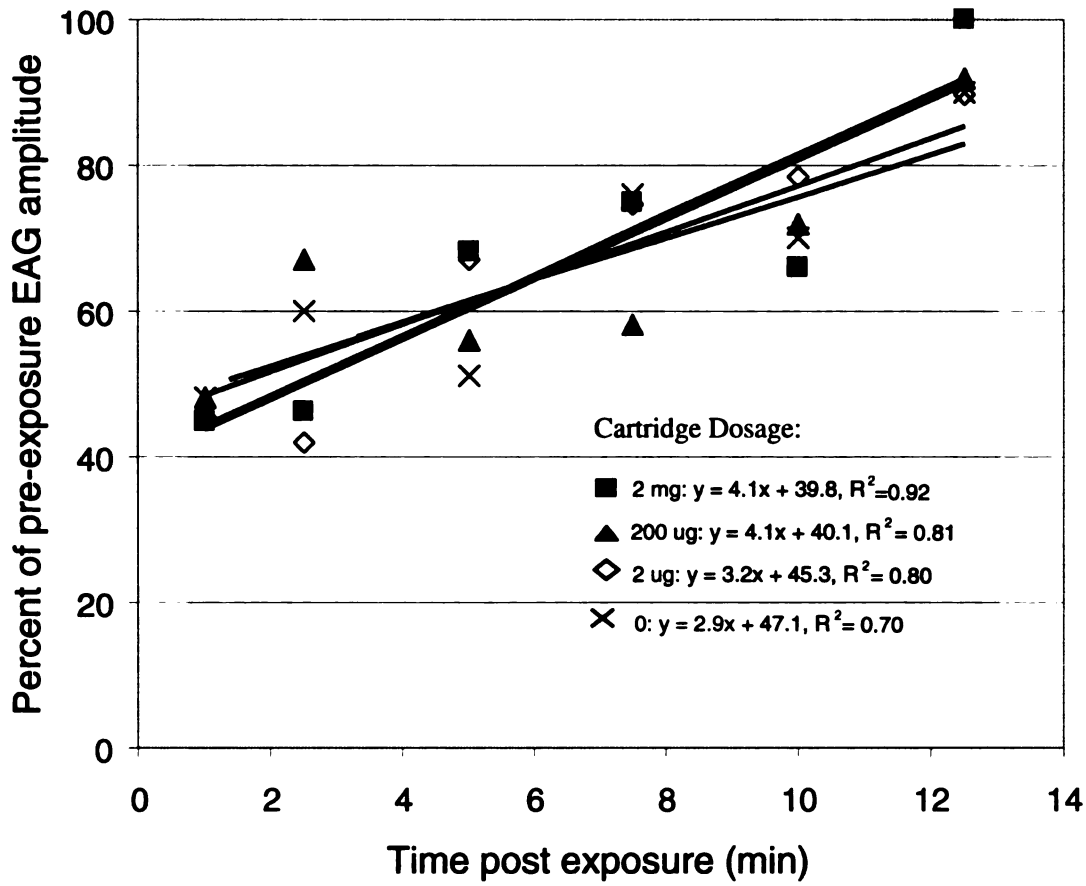


Figure 6. Linear regressions of percent recovery of EAG responses of *Choristoneura rosaceana* post 15 min of confinement in adaptation chambers over time. Percent recovery was calculated as the ratio of the mean EAG amplitude at the various time intervals at which individuals were assayed post-exposure over the mean pre-exposure amplitude. All regressions were significant at $p<0.01$.

For *C. rosaceana*, adaptation chambers containing pheromone elicited significantly ($P<0.05$) higher EAG responses (2.5 ± 0.24 mV) compared with depolarizations elicited by control chambers lacking pheromone (1.1 ± 0.13 mV). The mean amplitude elicited by the adaptation chamber closely corresponded to the mean response generated by our stimulus cartridges at the 200 ng dosage (Fig. 3). For *A. velutinana*, a response of 4.1 ± 0.4 mV was generated by the adaptation chamber with pheromone, which was also significantly ($P<0.05$) higher than the responses elicited by control chambers lacking pheromone (1.0 ± 0.13). For this species, the adaptation chambers induced a response that closely corresponded to responses generated by stimulus cartridges charged with the 2 µg dosage (Fig. 3).

DISCUSSION

Dynamics of long-lasting adaptation in C. rosaceana.

Pre-exposure of male *C. rosaceana* to the main components of its pheromone blend ((*Z*)11- and (*E*)11-14:Ac) decreased EAG responses to the pheromone for up to 10 min post exposure (Fig. 4 d). Assuming that an EAG consists of a summed depolarization of receptor potentials summed across the antennal olfactory neurons (Roelofs, 1984), the observed decrease in response by *C. rosaceana* can be attributed to decreased sensitivity of pheromone receptor neurons. The EAG response of pre-exposed *C. rosaceana* to our stimuli was reduced by 55-58 % for all cartridge dosages tested including the blank (control). Responses returned in a linear fashion to 70-100% of the pre-exposure response within 12.5 min (Fig. 6). These results establish that the process of recovery from adaptation took place at a constant rate. At our exposure dosage of 36.2 ± 11.7 ng / ml, exposures of only 5 min were not sufficient to induce adaptation in *C. rosaceana*;

however, exposures of 15 and 60 min reduced sensory responsiveness to the same degree (60 %). Given that exposure duration of 60 min did not increase adaptation, it seems that a plateau was reached at or even before 15 min. Currently, we have no physiological explanation for why long-lasting adaptation in *C. rosaceana* peaks at 60 %. Perhaps the titer of some inhibitory signaling agent (see below) is set so as not to turn off signaling transduction completely. Alternatively, we have not ruled out the possibility that certain populations of receptors adapt completely while others do not. Given the adaptive significance of male sensitivity to female pheromone, it would seem disadvantageous for male *C. rosaceana* to become completely anosmic to its pheromone. Characterization of the dose-response relationship and threshold for long-lasting adaptation in *C. rosaceana* will be treated further in a subsequent publication.

Similarities of C. rosaceana adaptation response to those of other moth species.

By performing recordings from single antennal neurons, Baker et al., (1989) showed that male *Agrotis segetum* (Schiffermüller) olfactory receptor neurons adapted when they were exposed to high pheromone concentrations known to cause in-flight arrestment of progress toward the source. Using the same technique, they also showed that antennal neurons from *H. virescens* failed to adapt regardless of concentration. Baker et al., (1989), proposed that, given the low emission rate of (*Z*)11-16:Ald from the rubber septa employed in their study, it was unlikely that *H. virescens* neurons were challenged to the same degree as *A. segetum* had been by the more volatile pheromone of that species. Alternatively, we suggest that *A. segetum* and *H. virescens* may differ in their susceptibility to peripheral sensory adaptation, as we have observed with *C. rosaceana* and *A. velutinana.* Specifically, certain species such as *C. rosaceana* and *A. segetum* may

exhibit greater capacity for sensory adaptation at the peripheral level than others such as *A. velutinana* and possibly *H. virescens*. Other electrophysiological studies have also demonstrated differential degrees of peripheral adaptation among moth species (Kuenen and Baker, 1981; Schmitz et al., 1997).

Molecular bases of long-lasting adaptation.

Adaptation of moth olfactory receptor neurons has long been recognized to occur following intense pheromonal stimulation, be it constant or pulsed (e.g., Baker et al., 1989; Figuerdo and Baker, 1992); however, delineation of the particular types of adaptation as recently defined by Zufall and Leinders-Zufall (2000) has just begun. Both invertebrate and vertebrate animal models reveal that both rapid and slower, longer-lasting forms of odor adaptation exist (Marion-Poll and Tobin, 1992; Getchell, 1986). The molecular basis and temporal dynamics of some of these differences has recently been described (Zufall and Leinders-Zufall, 2000). The rapid forms of adaptation result from Ca^{2+}-dependent cyclic nucleotide-gated (CNG) ion channel modulation and Ca^{2+}/calmodulin kinase II-dependent phosphorylation, respectively. In contrast, long-lasting adaptation is mediated by the carbon monoxide (CO)/cGMP second messenger system. Thus, odor adaptation is a complex phenomenon mediated by diverse molecular processes; these may differ within and among taxa depending on the nature of the signal-transduction cascades mediating olfaction in those species. Boekhoff et al., (1993) showed that in moth antennae cGMP formation is a consecutive reaction to pheromone-induced elevation in IP_3 concentration. Consistent with this interpretation, exogenously applied cGMP abolished the phasic but not tonic component of the pheromone-stimulated IP_3 signal in *H. virescens* (Boekhoff et al.,'s, 1993, Fig. 5 b). Sustained pheromonal

stimulation is also known to induce cGMP signals in *Antheraea polyphemus* as well as *Bombyx mori* (Ziegelberger et al., 1990), and, pheromone-activated cation channels sensitive to cGMP have been found in insect olfactory cilia (Zufall and Hatt, 1991). Furthermore, an IP_3 –mediated increase in intracellular Ca^{2+} could be the direct stimulus for shifts in Ca^{2+}/calmodulin interactions or alternatively could activate NO-synthase, leading to the generation of NO and subsequently activating cytoplasmic guanylyl cyclase (Steinlen et al., 1990; Boekhoff et al., 1993). Thus, as proposed by Zufall and Leinders-Zufall (2000), there is good reason to believe that insects and vertebrates share parallel mechanisms yielding long-lasting adaptation. The NO/cGMP pathway has been implicated in odor processing in vertebrates and invertebrates (Breer et al., 1992; Boekhoff et al., 1993) and should be considered along with the (CO)/cGMP second messenger system as possible mechanisms leading to the long-lasting form of adaptation such as that observed in *C. rosaceana* in the present study. Pharmacological inhibitors of CO and NO formation (Zufall and Leinders-Zufall, 1997) will be useful in testing this hypothesis.

Adaptation to blank air puffs.

Throughout this study, blank (negative control) puffs of clean air elicited EAG responses of 1-2 mV from both tortricids when applied from either clean disposable Pasteur pipettes or clean adaptation chambers (Fig. 3), and adaptation resulted in reduced responses to both pheromonal and clean air (control) stimulus puffs (Fig. 4). We took utmost care to assure that there was no pheromone contamination in controls. Pioneer studies on *B. mori* showed that EAG depolarizations occur in response to blank (control) puffs of clean pheromone-free air (Schneider 1962, Figs. 2, 3) and more contemporary

studies using *A. velutinana* also showed EAG responses to 1 ml puffs of clean air (Baker and Roelofs 1976, Fig. 3); however, these responses were smaller than in the present study. Mayer et al., (1984) also obtained pronounced EAG responses to their blank control stimulus puffs and proposed that stimuli such as water vapor, extraneous room contaminants, or delivery-line plasticizers may have been responsible for eliciting these responses. If the blank responses in our study were due to such extraneous contaminants, then adaptation to pheromone may have resulted in cross-adaptation to other unknown chemical or physical stimuli. In addition, the 1 ml puffs of air momentarily trembled the filiform antennae of both species and such movement of the antennae may have added to the apparent EAG depolarization.

Proposed impacts of long-lasting adaptation on susceptibility to pheromone disruption.

Exposure of *A. velutinana* to the components of its pheromone blend results in distortion and inhibition of the normal sexual response (Bartell and Roelofs, 1973). However, our results indicated that long-term exposure of *A. velutinana* to the main component of its pheromone blend and the geometric isomer had no effect on peripheral sensitivity even 1 min post exposure. Our results with *A. velutinana* are similar to those of Kuenen and Baker (1981), who showed that adaptation of receptor response in *T. ni* was also short-lived and returned to control levels 1 min after cessation of pheromone stimulation. In both cases, sensory processes at the peripheral level appear not to have declined after pheromone exposure, whereas central nervous system habituation appears to be the longer lasting and fundamental cause of sexual response inhibition. Long-term habituation has also been shown to occur with *C. molesta* in wind-tunnel trials (Figueredo and Baker, 1992) and in field experiments (Rumbo and Vickers, 1997). Similarly, wind-

tunnel and field experiments using *H. virescens* implicated central nervous system habituation, lasting as long as 96 h, as the major mechanism for modulating male moth response to female pheromone and as the underlying means for pheromone-based mating disruption (Daly and Figueredo, 2000).

Bartell and Lawrence (1977) suggested that male moth exposures to pulsed pheromonal stimuli would result in greater reductions of sexual response compared with constant stimulation, because peripheral adaptation would be circumvented so as to allow for greater central habituation. Kuenen and Baker (1981) obtained data supporting this hypothesis for *T. ni* by showing that pulsed rather than constant pre-exposure resulted in greater disorientation. Also, they demonstrated decreased EAG amplitudes with concurrent exposure, indicating that receptor adaptation was taking place. They concluded that receptor adaptation might have been an impediment for central nervous system habituation. Therefore, we postulate that the long-lasting peripheral adaptation documented in this study for *C. rosaceana* could be a mechanism precluding central nervous system habituation in this species and reducing susceptibility to pheromone-based mating disruption, as observed in field studies (Novak et al., 1978; Reissig et al., 1978; Roelofs and Novak, 1981; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996; Gut and Miller, unpublished data). In contrast, *A. velutinana*, which is easily disoriented in lab and field studies (Bartell and Roelofs, 1973; Novak et al., 1978; Reissig et al., 1978; Cardé et al., 1975; Novak and Roelofs, 1985; Gut and Miller, unpublished data), may be more susceptible to central nervous system habituation, as it appears to lack the capacity for long-lasting peripheral adaptation. In addition to the above hypothesis, it is possible that the onset of long-lasting adaptation in *C. rosaceana* may result in a

decrease in perception of the active space of synthetic pheromone point-sources and therefore a decreased inclination for false-trail-following. The opposite effect would be expected for *A. velutinana*, resulting in sustained false-trail-following or pheromone-induced excitation and arrestment precluding movement into zones of pheromone free air.

The concentration of pheromone per ml of air in the adaptation chambers was judged high as it was within the range of pheromone present per abdominal tip extract of female *A. velutinana* (Roelofs et al., 1975; Miller and Roelofs, 1980). Future studies must be extended into the field to document whether similar adaptation takes place at pheromone concentrations realized within a pheromone-treated crop. Finally, studies employing intracellular recordings from central nervous system processing centers in the olfactory lobe (Gadenne et al., 2001; Anton and Gadenne, 1999), obtained from pheromone-exposed *C. rosaceana* and *A. velutinana*, may illuminate whether adaptation actually precludes habituation.

CHAPTER TWO

Concentration of Air-Borne Pheromone Required for Long-Lasting Peripheral Adaptation in the Obliquebanded Leafroller, *Choristoneura rosaceana*

Also published as:

Stelinski, L. L., L. J. Gut., and J. R. Miller 2003. Concentration of air-borne pheromone required for long-lasting peripheral adaptation in the Obliquebanded Leafroller, *Choristoneura rosaceana*. *Physiological Entomology*. 28: 97-107.

ABSTRACT

Electroantennogram (EAG) responses of male obliquebanded leafrollers, *Choristoneura rosaceana* (Harris), to the main component of its pheromone blend and traces of geometric isomer ((*Z*)11-14:Ac and (*E*)11-14:Ac, respectively) were recorded before and after 1 h of continuous exposure to pheromone in laboratory experiments, and 24 h of exposure under field conditions. Concentrations of pheromone ranging from 56 to below 1 ng / ml air in Teflon chambers with regulated air exchange reduced peripheral sensory responses by 40-60 % as measured by amplitudes of the EAG. Adaptation did not increase in a dosage-dependent fashion over most of this range; an identical reduction of responsiveness was observed at each exposure to an effective concentration. Exposure of *C. rosaceana* at a loading dosage of 1 ng of pheromone in 100 µl of mineral oil (air concentration below the GLC detection limit) did not induce measurable adaptation. Caging *C. rosaceana* in apple trees adjacent to 1, 2 or 4 Isomate OBLR/PLR Plus polyethylene pheromone dispensers for 24 h resulted in long-lasting adaptation similar to that of laboratory experiments. Adaptation was not observed for *C. rosaceana* caged at a distance of 2 m from Isomate dispensers in 1 ha plots treated with 500 dispensers per ha. Whenever observed, this type of adaptation was expressed for more than 5 min after exposure to pheromone ceased. Collectively, this adaptation phenomenon in *C. rosaceana* is consistent with the third of Zufall & Leinders-Zufall's (2000) types of olfactory adaptation that is "long-lasting." Although the dosage of pheromone required to induce long-lasting adaptation in this moth is judged high relative to that for normal sexual communication, we suggest this type of adaptation may come into play for some but not all moths under pest-control regimes using the tactic of pheromone-disruption,

particularly those using high-dosage release technologies like pheromone rope dispensers or Microsprayers.

INTRODUCTION

Under natural conditions, adaptation of pheromonal and other olfactory neurons is thought to enable an animal's sensory system to adjust levels of sensitivity to allow appropriate behavioural responses at varying stimulus intensities (Zufall and Leinders-Zufall, 2000). Moreover, adaptation may preclude saturation of cellular transduction processes and/or overwhelming of sensory processing centers during continuous high levels of stimulation. In some cases, adaptation is reported to improve the detection of signal in a background of noise, e.g., in lobsters (Atema et al., 1989).

Sensory adaptation and central nervous system habituation are also thought to be important under unnatural conditions, e.g., when semiochemicals like sex attractant pheromones are broadcast throughout a crop to control insects by disrupting mate-finding (Cardé, 1990; Cardé and Minks, 1995). For example, Baker et al. (1988; 1989) reported that adaptation of antennal neurons was responsible for stopping the flight of male *Agrotis segetum* (Schiffermüller) up a pheromone plume. It has become clear that insights into processes of adaptation will be important from both the applied as well as basic perspective.

The molecular basis and temporal dynamics of three distinct types of adaptation have recently been elucidated in vertebrate olfactory neurons (Zufall and Leinders-Zufall, 2000). They include: Ca^{2+} influx through cyclic nucleotide-gated channels and Ca^{2+} - dependent ion channel modulation; Ca^{2+}/calmodulin kinase II-dependent phosphorylation; and activation of the carbon monoxide (CO)/cGMP second messenger

system. These different types of adaptation can be distinguished on the basis of their onset and recovery times. Two short-lived variants exhibit onset times on the order of 100 ms and 4 s and corresponding recovery times of 10 s and 1.5 min, respectively. The third type of adaptation is characterized as 'long-lasting'; onset requires repetitive exposures of at least 25 s and recovery is seen after 6 min.

Morphologically and physiologically, the olfactory neurones of vertebrates and insects are similar both peripherally and in the initial steps of central processing (Lancet, 1986; Anholt, 1987; Boeckh and Ernst, 1987; Homberg et al., 1989; Zufall and Leinders-Zufall, 2000). Moreover, insects and vertebrates are thought to share many olfactory transduction mechanisms (Stengl et al., 1992). Thus, it is reasonable to search in insects for types of sensory adaptation already established for vertebrates.

We recently discovered (Stelinski et al., 2003 a,b) in a moth of the family Tortricidae a form of pheromonal sensory adaptation corresponding to the 'long-lasting' adaptation reported by Zufall and Leinders-Zufall (2000) for the tiger salamander, *Ambystoma tigrinum.* Pre-exposure of male obliquebanded leafrollers, *Choristoneura rosaceana* (Harris), to components its pheromone blend ((*Z*)11-14:Ac and (*E*)11-14:Ac) at a concentration of 36 ± 12 ng / ml air for durations of 15 or 60 min significantly reduced peripheral sensory responses to these compounds as measured by electroantennograms (EAG). The EAG amplitudes of *C. rosaceana* were reduced by 55-58 % and recovered linearly to 70-100% of the pre-exposure response within 12.5 min at a rate of 3-4 % / min. Exposures of 5 min were insufficient to elicit maximal adaptation in *C. rosaceana*; however, exposures of 15 or 60 min reduced sensory responsiveness to the same minimum. In contrast, EAG responses of redbanded leafroller, *Argyrotaenia*

velutinana (Walker), after identical pheromone exposure for 5 or 60 min, yielded no long-lasting peripheral sensory adaptation as measured by EAGs, even though this species shares the same main pheromone components with *C. rosaceana.*

The concentration of pheromone (*c.* 40 ng of per ml of air) used to induce long-lasting adaptation in *C. rosaceana* in our initial study was well above levels used in normal sexual communication by these tortricid moths (Miller and Roelofs, 1980). The objectives of the current study were to characterize the dose-response relationship for long-lasting adaptation in *C. rosaceana*, define its threshold, and determine whether this type of adaptation could be coming into play under certain regimes of pheromone-based mating disruption in the field.

MATERIALS AND METHODS

Insect Source.

C. rosaceana were drawn from a four-year-old laboratory colony collected originally as 1st and 2nd generation pupae from apple orchards in Southwest, Michigan. Moths were reared at 24°C on pinto bean diet (Shorey and Hale, 1965) in a L:D 16:8 h photocycle. Male pupae of each species were segregated in 1 L plastic cages containing a 5 % sucrose solution in plastic cups with dental cotton wick protruding from their lids.

Laboratory Electroantennograms.

The EAG system and test protocols were identical to those detailed by Stelinski *et al.*, (in press). Briefly, our EAG system consisted of a data acquisition interface board (Type IDAC-02) and universal single ended probe (Type PRS-1) from Syntech (Hilversum, The Netherlands). The recording and indifferent electrodes consisted of silver-coated wire in glass micropipettes (10 µl micro-hematocrit capillary tubes)

containing 0.5 M KCl. Moths were 2-4 d post-eclosion when used for electroantennography. EAGs were measured as the maximum amplitude of depolarization elicited by 1 ml puffs of air through EAG cartridges of various pheromone loadings directed over live-insect preparations. The time interval to expel 1 ml of stimulus odor or clean air from the syringe was 120 ± 0.02 (S. D.) ms (n = 20) (Stelinski et al., 2003a).

Pheromone Source and Purity.

(*Z*)11-14:Ac (lot # 10010) was obtained from Shin Etsu (Tokyo, Japan); its purity was determined with gas chromatography to be 96.1 % (*Z*)11-14:Ac and 3.9% (*E*)11-14:Ac.

Laboratory Adaptation Experiments.

Moths were placed in adaptation chambers consisting of cylindrical, 1 L Teflon transfer containers (Jensen, Coral Springs, FL) equipped with two 64 mm ports in their lids. Glass inlets and outlets were affixed to the lids, allowing pressurized air that had been filtered through carbon to pass through the chambers at 30 ml / min. Chambers were divided with wire mesh such that insects were confined in upper halves, while one 2 cm diam. x 0.5 cm deep stainless steel planchette loaded with a given dosage of pheromone was placed in the lower half. This arrangement reduced variation in pheromone exposure relative to that in a static container, where distance of the moth from the pheromone source might influence pheromone uptake. The highest dosage of pheromone tested was 100 µl of neat pheromone in the planchette. Successively lower dosages were achieved by serially diluting 100 µl of mineral oil (Aldrich Chemical Company; Milwaukee, WI);

loadings ranged in decade steps from 10 mg to 1 ng of pheromone. Chambers always equilibrated for 60 min prior to insertion of insects.

Twelve male *C. rosaceana* were assayed at each pheromone concentration. EAGs were performed on all insects (left antenna) prior to confinement, exactly 1 min after the 60 min confinement (right antenna), and after 5 or 15 min post-confinement intervals in clean air (left antenna 2nd time). Two control treatments were performed on additional groups of moths (n = 12). In the first control, we employed adaptation chambers containing planchettes with 100 µl mineral oil only, and followed all other procedures as described above. In the second control, we sought to establish a baseline for consistency of EAG readings between antennae for a given individual. We chose to perform EAGs on the left antenna, and then right antenna 1 h later without exposure in adaptation chambers, followed by a second reading from the left antenna 15 min later.

In previous work (Stelinski et al., 2003a), *C. rosaceana* exhibited adaptation lasting up to 7.5 min after 15-60 min constant exposures to its pheromone at a concentration of 36 ± 12 ng / ml air achieved by loading adaptation chambers with single rubber septa impregnated with 5 mg of pheromone. In the present study, we sought to determine whether decreasing the loading dosage in adaptation chambers would alter the longevity of adaptation to pheromone in *C. rosaceana*, which was previously established at an arbitrary and high pheromone dosage.

Measurement of pheromone concentration in adaptation chamber.

Planchettes containing each dosage of pheromone tested above were placed one at a time in adaptation chambers. After equilibration for 60 min, the exhaust port was replaced with a port sealed with a clean rubber septum. Immediately thereafter, 15 ml of

air was withdrawn from adaptation chambers through the septum-sealed port into a 20 ml glass syringe fitted with a 22 gauge stainless steel needle. In rapid succession, 5 ml of hexane wash containing 14:Ac at 6.4 ng / µl (used as an internal standard) was drawn into the syringe. The solution within the syringe was carefully shaken for 30 s then expelled into a 2 ml glass vial designed for gas liquid chromatography (Amber Crimp Vial, Hewlett-Packard Co). This entire procedure was replicated 5 times at 23° C and *c.* 35 % RH with separate planchettes for each pheromone dosage tested. Prior to analysis, samples were concentrated under nitrogen by a factor of 50 (10 µl final volume). Samples were analyzed by a gas liquid chromatograph (GLC) (HP-6890, Hewlett-Packard Co) with flame ionization detection to reveal the concentration of pheromone present in adaptation chambers. The GLC was fitted with a DBWAXETR polar column (model # 122-7332, JandW Scientific, Folsom, CA) of length 30 m and internal diameter 250 µm. The initial oven temperature was held at 100 °C for 3 min and then programmed to increase 10°C / min up to 250°C where it was held for 3 min; the carrier gas was He. We calculated the concentration of pheromone present in the 15 ml of adaptation chamber air by multiplying the ratio of peak areas of the target compound ((Z)11-14:Ac) over the standard (saturated 14:Ac) by the concentration of internal standard present in the hexane wash and then multiplying by 100 to account for GLC analysis of only 1 % of the total concentrated sample.

Field adaptation experiments.

All field experiments were conducted in June of 2002 at Michigan State University's Trevor Nichols Research Complex, Fennville, MI. Experiments were conducted within a 15 year-old planting of Red Delicious apples spaced 3 m within and 6

m between rows. Male *C. rosaceana* (2-4 days post-eclosion), taken from the same colony as used in laboratory experiments, were placed in 6 x 4 x 2 cm wire mesh cages (3 per cage) containing a 2 cm piece of dental wick moistened with sugar-water. Cages were hung in branches of apple trees at 1.5-2 m above ground under various pheromone exposure regimes for 24 h (Table 1). Isomate OBLR/PLR Plus pheromone rope dispensers (Pacific Biocontrol Co., Vancouver, WA) containing 227 mg of (Z)11-14:Ac were used to deliver pheromone in all field exposure trials. Treatments included cages hung in untreated 1 ha plots of: trees containing no dispensers; 2 m from 1 pheromone dispenser within the same tree; adjacent to 1 dispenser; adjacent to 2 dispensers; and adjacent to 4 dispensers surrounding the cage. The final treatment consisted of cages hung 2 m from pheromone dispensers within 1 ha plots treated with pheromone at the recommended label rate of 500 dispensers per ha.

After 24 h of field exposure under the various pheromone regimes, male *C. rosaceana* were assayed by EAG in the field to measure the possible onset of adaptation. The field-EAG system and stimulus-delivery methods were as similar as possible to those used in the laboratory setup. Briefly, the micromanipulators used to maneuver the reference and recording electrodes were mounted on a 1 m x 1 m x 2 cm steel plate placed in the rear compartment of a Minivan automobile. A 0.75 x 1 x 1 m Faraday cage covered the electrodes, micromanipulators and a dissecting microscope, all situated on the steel plate. A Syntech data acquisition interface board (Type IDAC-02, Hilversum, The Netherlands) and computer used to record and store data were placed adjacent to the Faraday cage. Power outlets located throughout the research orchard supplied electricity. The van was always positioned *c.* 12-15 m from test plots. The field EAG system differed

from our laboratory arrangement in that a constant stream of charcoal-filtered and humidified air was not continuously delivered over antennal preparations.

One live *C. rosaceana* out of a possible three was randomly removed from each wire mesh cage and immediately mounted for EAG analysis. Cases in which more than 1 min elapsed between cage retrieval and mounting the individual for EAG recording were discarded because of possible onset of disadaptation. As in laboratory experiments, the tip of an EAG cartridge was positioned *c.* 5 mm from antennal preparations. Stimulus puffs (1 ml) were generated through pheromone cartridges (200 µg loading dosage) with a clean hand-held 20 ml glass syringe (Stelinski et al., 2003a). *C. rosaceana* responding at least 1 mV below the mean amplitude obtained from individuals having no pheromone exposure were left mounted in pheromone-free air for 10 min and EAG-assayed a second time to quantify disadaptation.

Statistical Analysis.

Data were subjected to analysis of variance (ANOVA) and differences in selected pairs of means over time and between treatments for laboratory and field experiments were separated using Tukey's multiple comparisons test (SAS Institute, 2000). The relationship between loading dosage of pheromone diluted in mineral oil and pheromone concentration per ml of air in adaptation chambers was analyzed using the trendline feature of Microsoft Excel.

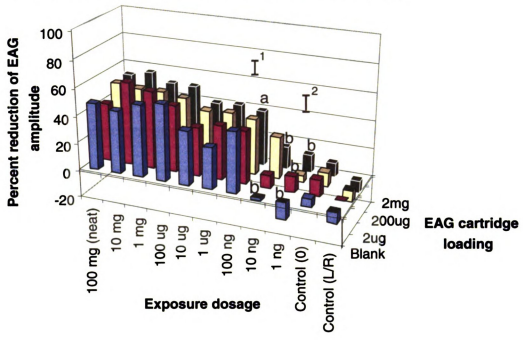
RESULTS

Adaptation Experiments in the Laboratory.

The EAG responses of male *C. rosaceana* to the 2 µg, 200 µg, 2 mg, and blank cartridges were significantly ($P < 0.05$) reduced (between 40-60 %) after 60 min of

exposure at pheromone dosages ranging from the maximum loading of 100 µl neat pheromone to 100 µl of mineral oil containing 100 ng pheromone (0.0001% by volume) (Fig 7 A). A slight reduction in EAG amplitude was recorded for *C. rosaceana* exposed in chambers containing the 10 ng pheromone dosage when tested with 1 ml puffs of air through EAG stimulus-cartridges loaded with 200 µg of pheromone (Fig. 7 A). No adaptation was recorded for moths exposed in chambers containing the 1 ng dosage. Adapted *C. rosaceana* recovered their pre-exposure EAG amplitudes within 15 min post-exposure (Fig. 7 B), but not by 5 min post-exposure (Fig. 8). The change in amplitude was the only notable shift in EAG profile visible for moths exposed to dosages that caused adaptation (Fig. 9, A-G) versus dosages of pheromone that did not (Fig. 9, H and I). On average, responses to blank cartridges ranged from 0.9-1.7 mV; Stelinski et al., (2003a) have discussed such responses to blank cartridges.

a. EAG response 1 min after 1 hr of constant exposure



b. Percent recovery of EAG response after 15 min of clean air

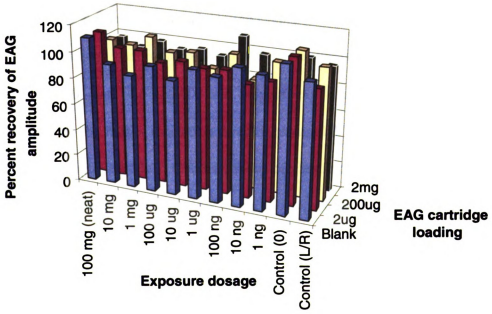


Figure 7. A. Effect of 60 min of confinement of *Choristoneura rosaceana* (n=12 per treatment) in adaptation chambers with various pheromone-loading dosages. Bars with

different letters indicate significant ($P<0.05$) differences between treatment means within a given cartridge dosage. B. Effect of 15 min of recovery of *C. rosaceana* in pheromone-free air after 60 min confinement in adaptation chambers. There were no significant differences between treatment means for responses within or across dosages. Exposure dosages of 10 ng / planchette and lower were non-adapting; these became the reference point for calculating percent recovery. [1]Average standard error of the mean percentages of reduction of EAG amplitude for all exposure dosages ranging from 100 mg to 100 ng and assayed by the 2 mg EAG cartridge loading. [2]Average standard error of the mean percentages of reduction of EAG amplitude for 10 and 1 ng exposure dosages and the two control treatments assayed by the 2 mg cartridge loading. The average standard errors for treatments assayed by the other cartridge loadings were lower than those shown here for the 2 mg cartridge loading.

Pheromone Concentration in the Adaptation Chamber.

The retention times for the unsaturated 14:Ac (internal standard) and (Z)11-14:Ac were 13.7 ± 0.001 and 14.5 ± 0.003 min, respectively. Sample detection and quantification by the flame- ionization detector required 0.1 ng of pheromone per 1 µl of sample injected onto the GLC. Concentrations of pheromone in adaptation chamber air were detectable down to but not below the 1µg loading (Fig. 10). The air-borne concentration of pheromone in adaptation chambers reached a plateau at the 1 mg loading dosage; there were no significant ($P > 0.05$) differences between the measured concentrations of pheromone achieved by the three highest loading dosages (1, 10, and 100 mg) (Fig. 10) Although not statistically different with the current small sample size, we found a surprising trend toward a drop in atmospheric concentration when the

planchette was loaded with neat compound. Nevertheless, the pheromone concentration in air was well approximated ($R^2 = 0.8$) by the expression: $y = 3.42 \text{ Ln}(x) + 11.5$. On this basis, we estimate that the air concentration required for full adaptation (100 ng loading in 100 µl mineral oil) was *c.* 0.5 ng / ml air.

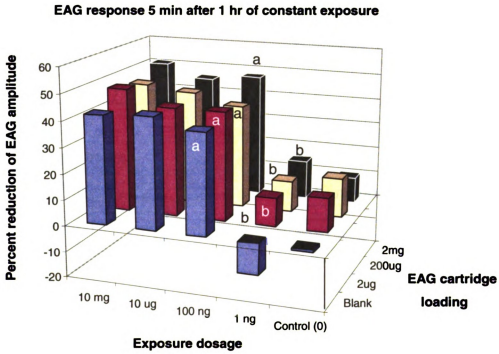
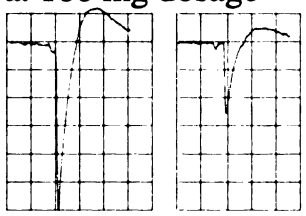
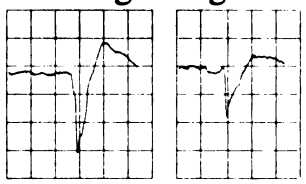


Figure 8. Lack of effect of 5 min of recovery of *Choristoneura rosaceana* in pheromone-free air after 60 min confinement in adaptation chambers. Bars with different letters indicate significant ($P<0.05$) differences between treatment means within a cartridge loading.

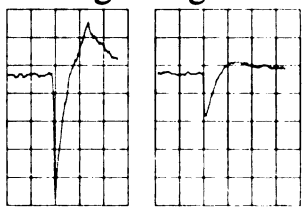
a. 100 mg dosage



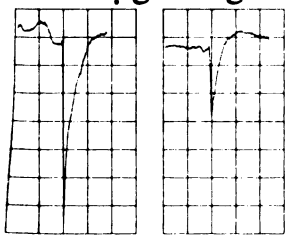
b. 10 mg dosage



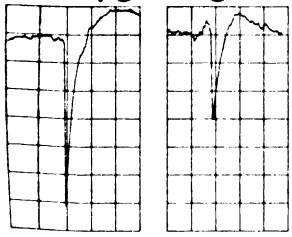
c. 1 mg dosage



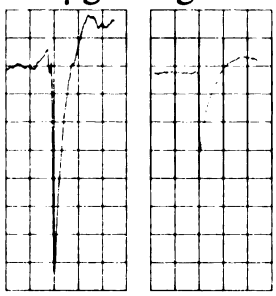
d. 100 µg dosage



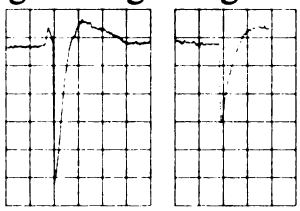
e. 10 µg dosage



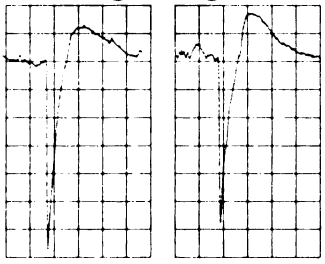
f. 1 µg dosage



g. 100 ng dosage



h. 10 ng dosage



i. 1 ng dosage

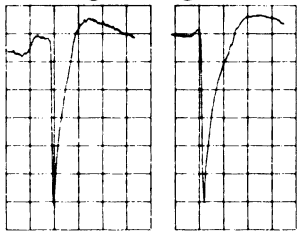


Figure 9. Representative EAG tracings of *Choristoneura rosaceana* responding to 1 ml puffs from pheromone cartridges loaded with 200 µg of pheromone. Tracings on the left within each column are responses of *C. rosaceana* prior to exposure in adaptation chambers. Tracings on the right side within each column are responses of the same individuals after 60 min of exposure within adaptation chambers containing the above-mentioned loading dosages of pheromone. Each horizontal tick mark represents 1 s and each vertical tick mark represents 1 mV of depolarization.

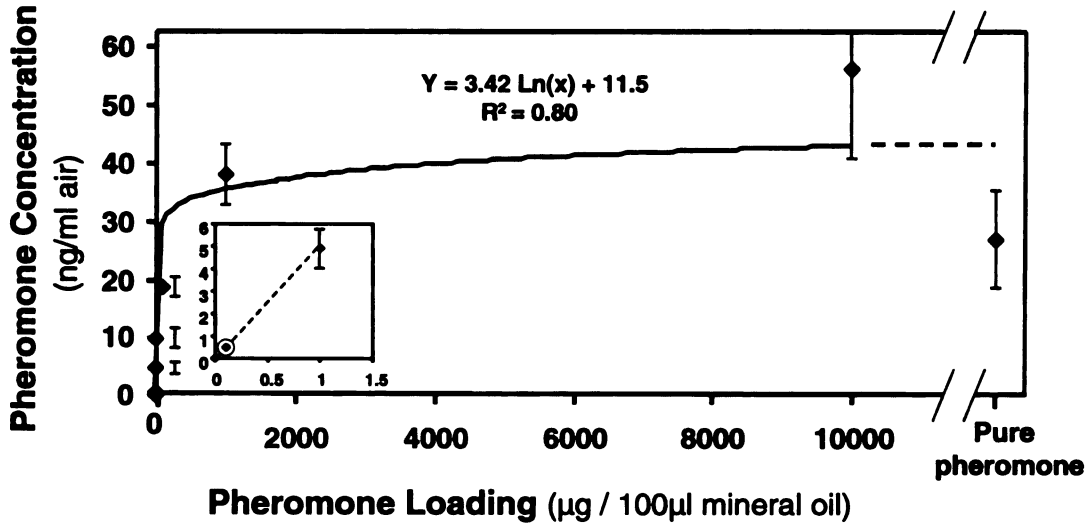


Figure 10. GC-quantified pheromone concentration in adaptation chambers in relation to loading dosage of pheromone in planchettes. The circled datum within the box expansion is an estimation of the threshold concentration causing long-lasting adaptation in *Choristoneura rosaceana*. Error bars indicate standard error of the mean.

Adaptation Experiments in the Field.

No long-lasting adaptation, as measured by decreased EAGs, was observed in the field for male *C. rosaceana* caged for 24 h at a distance of 2 m from Isomate pheromone dispensers in otherwise untreated plots (Table 1). Also, no adaptation was found for *C. rosaceana* caged 2 m from Isomate dispensers in 1 ha plots treated with pheromone at a rate of 500 Isomate dispensers per ha (Table 1). However, adaptation was observed in *C. rosaceana* caged adjacent to 1, 2, or 4 Isomate dispensers in otherwise untreated plots (Table 1). The frequency with which adaptation was observed increased as the number of Isomate pheromone dispensers placed adjacent to cages increased. Most individuals adapted at the 4-dispenser treatment (Table 1). As observed in the laboratory, the EAG response of adapted individuals returned to pre-exposure levels within 10 min after pheromone exposure (Table 1, Fig 11).

Table 1. Prevalence and degree of 'long-lasting' adaptation in laboratory-reared *Choristoneura rosaceana* upon differing levels of exposure to Isomate OBLR/PLR Plus pheromone dispensers in the field.

Pheromone Exposure (24 h)	n	Number adapted	EAG Amplitude (mean mV ± SE) upon stimulation with 1 ml of air through pheromone cartridge (200 µg loading dosage)		
			Non-adapters	Adapters	
				Pre-recovery[1]	Post-recovery[2]
Directly from lab, no pheromone	17	0	3.26 ± 0.23a[3]	_	_
Field exposure, no pheromone	16	0	3.41 ± 0.33a	_	_
1 rope @ 2 m, single treated tree	16	0	3.28 ± 0.27a	_	_
1 dispenser @ 2 m, treated plot	16	0	3.14 ± 0.25a	_	_
1 dispenser adjacent	22	5	3.51 ± 0.25a	2.00 ± 0.25b	3.62 ±0.47a
2 dispensers adjacent	16	7	3.46 ± 0.36a	1.69 ± 0.28b	3.70 ± 0.30a
4 dispensers adjacent	16	12	3.48 ± 0.30a	1.78 ± 0.15b	3.33 ± 0.25a

[1] Mounted and assayed within 1 min of field exposure.
[2] 10 min after pheromone exposure and first EAG assay
[3] Means not followed by the same letter are significantly different ($P<0.05$) by ANOVA followed by Tukey's multiple comparisons test

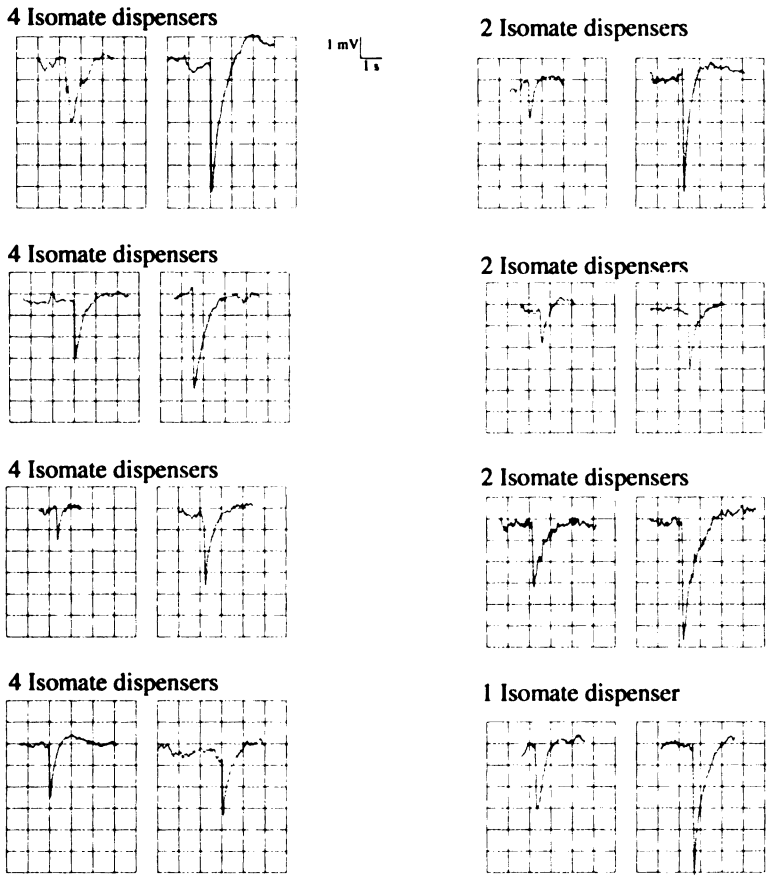


Figure 11. Representative EAG tracings of adapted *Choristoneura rosaceana* responding to 1 ml puffs from pheromone cartridges loaded with 200 μg of pheromone. Tracings on the left within each column are responses of *C. rosaceana* 1 min after 24 h of field exposure under various pheromone exposure treatments. Tracings on the right side within each column are responses of the same individuals after 10 min of recovery in pheromone-free air.

DISCUSSION

Cumulative characteristics of long-lasting adaptation in C. rosaceana.

Adaptation of moth olfactory receptor neurons has long been recognized to occur following intense pheromonal stimulation, be it constant or pulsed (e.g., Baker et al., 1988; Marion-Poll and Tobin, 1992; Figueredo and Baker, 1992); however, delineation of the particular types of adaptation as recently defined by Zufall and Leinders-Zufall (2000) has just begun. The 'long-lasting' variant of adaptation to sex pheromone we report for *C. rosaceana* has this emerging set of characteristics: as reported previously (Stelinski et al., 2003a) it can be measured by EAGs as a reduction in summated depolarization across unexcised antennal mounts in response to pheromone puffs of various dosages following sustained pheromone exposure of whole moths; its onset occurs after > 5 min exposure to pheromone and, like the adaptation reported for some other moths [*Trichoplusia ni* (Kuenen and Baker, 1981); *Lobesia botrana* (Schmitz et al., 1997)], it plateaus at *c.* 40-60% response reduction regardless of longer exposures; it is a 'long-lasting' (*sensu* Zufall and Leinders-Zufall, 2000) phenomenon measurable in full for up to 5 min after pheromone exposure ceases; moreover, the effect decays linearly over a period of 12.5 min.

The current study extends this list to include the following features: the maximal 40-60% EAG amplitude reduction was evident for all concentrations of pheromone at which adaptation occurred [from *c.* 0.5 – 50 ng / ml (Fig. 7 a)]; and concentrations only slightly lower than 0.5 ng / ml produced no long-lasting adaptation. Rather than a typical dosage-response phenomenon with a graded effect extending across orders of magnitude, this dosage pattern suggests a distinct threshold concentration above which long-lasting

adaptation occurs and is immediately maximal, albeit never total. In that sense this long-lasting adaptation as measured in *C. rosaceana* is more quantal than gradual. We estimate the threshold concentration for onset of long-lasting adaptation (*c.* 500 pg / ml air) to be at least 5 orders of magnitude higher than the threshold for positive anemotactic flight by tortricidae in a wind tunnel (Miller and Roelofs, 1978), as estimated by response to known release rates of pheromone from microcapillary tubes (J. Miller, unpublished data). Wide separation between the dosage of pheromone triggering long-lasting adaptation and that for normal sexual communication makes sense, as it is difficult to envision any circumstance where it would be advantageous for a male moth to dampen sensitivity and responsiveness for minutes at a time when competing for a mate.

Mechanisms of long-lasting adaptation in vertebrates

In vertebrates, long-lasting adaptation is mediated by the carbon monoxide (CO)/cGMP second messenger system (Zufall and Leinders-Zufall, 1997). The onset of long-lasting adaptation results in reduced amplitude and prolonged kinetics of the cAMP-mediated excitatory odor response and the generation of a persistent current-component that lasts several minutes; these effects are attributed to cyclic nucleotide-gated channel activation by cGMP. Therefore, cGMP mediates this type of olfactory adaptation by modulating the signaling properties of olfactory receptor neurons and thus controlling the sensitivity of the excitatory cAMP cascade (Zufall and Leinders-Zufall, 1997).

Proposed mechanisims of long-lasting adaptation in relation to moth olfactory cellular signaling.

Transduction in insect olfactory neurons is mediated via an IP_3 (rather than cAMP) second-messenger cascade (Boekhoff et al., 1990; Boekhoff et al., 1993);

otherwise, the process has many similarities to that of vertebrates (Stengl et al., 1992). In *Heliothis virescens*, the concentration of IP_3 peaks 50 ms after nanomolar applications of pheromone (Boekhoff et al., 1993). Applications of larger dosages of pheromone yield rapid and transient increases in IP_3 followed by a rise in cGMP sustained over 10 s. Based on these results, Boekhoff et al., (1993) postulated that cGMP is involved in adaptation. Consistent with this interpretation, exogenously applied cGMP abolished the phasic but not tonic component of the pheromone-stimulated IP_3 signal in *H. virescens* (Fig. 5 b in Boekhoff et al., 1993). Importantly, there was a good match between the time-course of this phasic to tonic shift and the kinetics of the biochemical interaction between IP_3 and cGMP. Sustained pheromonal stimulation is also known to induce cGMP signals in *Antheraea polyphemus* as well as *Bombyx mori* (Ziegelberger et al., 1990), and, pheromone-activated cation channels sensitive to cGMP have been found in insect olfactory cilia (Zufall and Hatt, 1991). Thus, as proposed by Zufall and Leinders-Zufall (2000), there is good reason to believe that insects and vertebrates share parallel mechanisms yielding long-lasting adaptation.

Some but not all electrophysiological recordings from pheromone-sensitive single-sensilla of insects clearly reveal long-lasting adaptation. As recorded by Kaisling (1986), *A. polyphemus* pheromonal sensillae transitioned from phasic bursts of action potentials to a much-reduced and more steady-state tonic response within the first 100 ms of stimulation at 10^{-2} μg of bombykol. The normal phasic response returned only after a 10 and 30 min resting interval following 10 s and 10 min stimulation, respectively.

Our data on *C. rosaceana* appear to be consistent with cGMP-mediated long-term adaptation. To date, we have measured this adaptation only by EAG; nevertheless, we

believe this effect may be correlated with a phasic to tonic shift in action potential output if measured at the single-sensillum level. Such shifts are well documented in the insect pheromonal literature e.g., *A. polyphemus*, *A. pernyi* (Strausfeld and Kaissling, 1986) *Grapholita molesta* , *A. segetum* (Baker et al., 1988), *Trichoplusia ni* (Borroni and O'Connell, 1992; Grant et al., 1997), and *H. virescens* (Almaas and Mustaparta, 1991). However, long-lasting vs. short-term variants of adaptation have not yet been fully differentiated.

Possible significance of long-lasting adaptation under pheromone disruption regimes in the field.

Currently, hand-applied rope dispensers deployed at *c.* one per tree are the dominant method of dispensing pheromone for mating disruption of moth pests in orchards (Nagata, 1989; Agnello et al., 1996; Knight et al., 1998; Knight and Turner, 1999). The release rate for ropes marketed for leafroller moths averages *ca.* 11 ng / s (Knight et al., 1998; Knight and Turner, 1999). Moths within the treated crop can be exposed to various spatial distribution of pheromone: a 'cloud' of pheromone resulting from a coalescence of plumes emanating from the many dispensers; a localized plume down-wind of a nearby dispenser; or, at the highest level, a moth could be attracted onto a dispenser. In the current field tests, *C. rosaceana* exhibited long-lasting adaptation upon exposure to pheromone ropes, but only when held within a few cm of the dispenser. Nevertheless, these results demonstrate that this phenomenon can occur under field conditions. Use of low-density, high-release dispensers like puffers (Shorey and Gerber, 1996) or Microsprayers (Isaacs et al., 1999) offers even greater opportunity for male moths to be exposed to very high concentrations of pheromone; the pheromone solution

emitted in an aerosol spray falls onto foliage and droplets of pure pheromone accumulate over time on the source tree. Moreover, large and highly concentrated plumes are thought to waft great distances downwind of the source trees.

C. rosaceana, a long-lasting adaptor, has the reputation of a pest moth that is difficult to disrupt (Novak et al., 1978; Reissig et al., 1978; Roelofs and Novak, 1981; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996; Miller et al., unpublished data) relative to the closely related redbanded leafroller, *A. velutinana*, which does not exhibit long-lasting adaptation (Stelinski et al., 2003a). In certain cases, populations of *C. rosaceana* from Western Canada, which are characterized by a slightly different blend of pheromone components compared with those from central and eastern North America (Vakenti et al., 1988; Thomson et al., 1991), have shown some potential for successful mating disruption in small-plot trials (Evenden et al., 1999a; Evenden et al., 1999b). We speculate that under pheromone mating-disruption regimes, moths capable of long-lasting adaptation like that of *C. rosaceana* may be advantaged relative to those who cannot. For example, perhaps moths experiencing long-lasting adaptation might sufficiently suppress overt sexual responses so as to allow them to depart extraordinarily high-dosage pheromone sites where the likelihood of finding and mating with a female is nil. If they then happen to arrive in a location of low pheromone, disadaptation would occur within 10 min and their ability to discriminate and orient to a natural pheromone plume would be restored, provided the possible effects of central nervous system habituation were shielded (Bartell and Lawrence, 1977; Kuenen and Baker, 1981). Alternatively, long-lasting adaptation might preclude normal orientation and act to arrest flight so that the responder is not attracted to an abnormally high pheromone dosage. Notably, the work of

Grant et al. (1997) supports the idea that pheromone receptors need to be capable of phasic responses for normal plume-following behavior. When shifts in wind direction bring pockets of pheromone-free air (Cardé and Minks, 1995), a long-lasting adaptor would soon escape that location, possibly shielded from CNS fatigue.

Testing the hypothesis that absence of long-lasting adaptation is correlated with ease in pheromone disruption across moth taxa is the next step in this study. Another intriguing puzzle is why long-lasting adaptation to sex attractant pheromones exists when it is difficult to envision a natural context in which selection would have rewarded it. Perhaps this phenomenon is a generalized physiological response to high dosages of chemostimuli set in place (and broadly retained) to handle high dosages of other natural products, e.g., plant volatiles. This idea begs cross-adaptivity tests spanning broad classes of compounds. Answers to such questions, generated by these early characterizations of long-lasting olfactory adaptation in insects, may reveal fundamental principles of insect physiology and behavior that can be exploited for improved management of important pests.

CHAPTER THREE

Increased EAG Responses of Tortricid Moths after Prolonged Exposure to Plant Volatiles: Evidence for Octopamine-Mediated Sensitization

Also published as Published as:

Stelinski, L. L., J. R. Miller, N. E. Ressa, and L. J. Gut 2003. Increased EAG responses of tortricid moths after prolonged exposure to plant volatiles: evidence for octopamine-mediated sensitization. *Journal of Insect Physiology*. 49: 845-856.

ABSTRACT

As measured by electroantennograms (EAG), both male and female obliquebanded leafrollers, *Choristoneura rosaceana* (Harris), and redbanded leafrollers, *Argyrotaenia velutinana* (Walker), were similarly sensitive to host-related plant volatiles: *trans*-2-hexenal, benzaldehyde, 1-hexenol, *cis*-3-hexen-1-ol, geraniol, linalool, (+)-limonene, hexenal and *trans*-2-hexenol. Females of both species were similarly sensitive to the shared major component of their sex attractant pheromone ((*Z*)11-14:Ac). Continuous 60 min pre-exposure of male and female *C. rosaceana* and *A. velutinana* to successively higher concentrations of a mixture of the nine plant volatiles in Teflon chambers with continuous air exchange caused a dosage-dependent increase in subsequent responsiveness (sensitization) to green leaf volatiles, as measured by EAGs. In addition, 60 min of pre-exposure of male *C. rosaceana* to certain individual volatiles ((+)-limonene, geraniol, benzaldehyde) increased EAGs nearly as much as did the mixture of nine volatiles. Pre-exposures to the nine plant-volatile mixture at concentrations achieved by 100 μg and 1 mg loading dosages in 100 μl of mineral oil significantly increased EAG depolarization to pheromone (cross-sensitization) in males but not females of both moth species. Antennae of male *C. rosaceana* pre-injected with 100 μg of octopamine (OA) without volatile pre-exposure exhibited sensitization nearly identical to that induced by pre-exposing moths to sensitizing concentrations of the plant volatile mixture. Moreover, injection of the OA antagonist chlorpromazine (CP) blocked sensitization by the plant-volatile pre-exposure. Collectively, these findings suggest that exposures of tortricid moths to certain host-plant related volatiles may modulate

subsequent olfactory sensitivity to behaviorally relevant chemical cues and that plant-volatile induced sensitization may be octopamine mediated.

INTRODUCTION

Recently, we reported (Stelinski et al., 2003a) that pre-exposure of male *Choristoneura rosaceana* (Harris), to the synthetic pheromone ((*Z*)11-14:Ac and (*E*)11-14:Ac) at 36 ± 12 ng / ml air for durations of 15 and 60 min in Teflon chambers with regulated air exchange reduced peripheral sensory responses to these compounds by 40-60 % in subsequent EAG assays. The EAG responses of male *C. rosaceana* to their sex-attractant pheromone recover linearly to 70-100% of their pre-exposure amplitude within 12.5 min at a rate of 3-4 % / min (Stelinski et al., 2003a). Furthermore, an identical reduction of responsiveness was observed at each effective exposure concentration ranging from 56 to below 1 ng / ml air (Stelinski et al., 2003b). In contrast, no corresponding long-lasting adaptation was found in identically treated *Argyrotaenia velutinana* (Walker), despite its close taxonomic relatedness and similarity of pheromone blend to *C. rosaceana* (Stelinski et al., 2003a). The current study originally aimed at determining whether exposure of leafroller moths to EAG-active plant volatiles would induce auto-adaptation of antennal responses to these chemicals and/or reciprocal cross-adaptation to pheromone as measured by EAG. As the study progressed, sensitization rather than adaptation was the predominant phenomenon uncovered and documented herein.

There is precedent for interactions between plant volatiles and insect sex-pheromone at the level of individual olfactory receptor neurons (ORNs) as measured by electrophysiological recordings. For example, geraniol, nerol, linalool, and eugenol

reduced the frequency of action potentials of male *Adoxophyes orana* ORNs to their sex pheromone (Den Otter et al., 1978). Also, a corresponding inhibition was induced by geraniol, *trans*-2-hexenal, butanal, and benzaldehyde in female *A. orana* co-stimulated with the male sex pheromone. Similar studies demonstrated reductions in spike frequencies of pheromone receptors also exposed to various plant volatiles in male *Antheraea pernyi, Yponomeuta vigintinpunctatus, Y. irrorellus, Y. cagnagellus Y. rorelus,* and *Bombyx mori.* (Schneider et al., 1964; Kaissling, 1977; Priesner, 1979; Van der Pers, 1980; Van der Pers, 1982; Kaissling, 1987). Increased spike frequencies of pheromone ORNs were also induced by the plant volatile linalool in males of various moth species (*Y. cagnagellus, Y. irrorellus, Y. rorellus, and Y. plumbellus,* and *B. mori*) (Van der Pers, 1980; Kaissling, 1987; Kaissling et al., 1989). Linalool at 51 ng / puff, presented as a mixture with the major pheromone component ((*Z*)11-16:Ald at 0.04 ng / puff) of male corn earworms, *Helicoverpa zea*, synergized the responses of ORNs tuned specifically to (*Z*)11-16:Ald (Ochieng et al., 2002). When presented alone, the plant volatile did not cause significant firing of pheromone-specific ORNs. However, when presented with (*Z*)11-16:Ald, the plant volatile significantly increased the firing rate of the ORNs compared with the response to (*Z*)11-16:Ald alone.

Recordings from sensilla trichodea of *Y. evonymellus*, under simultaneous application of geraniol and the sex-attractant (*Z*)11-14:Ac, established that the cells' response to the sex pheromone was directly inhibited by 1 s 'puffs' of the plant volatile (Van der Pers, 1980). Furthermore, simultaneous EAG and single-sensillar recordings from *Y. rorellus* proved that hexenal, benzaldehyde, and linalool inhibited pheromone receptors in the sensilla trichodea, while simultaneously generating excitatory EAGs

(Van der Pers, 1980). These results established a direct interaction between plant odors and pheromones at the receptor level and implicated sensilla other than sensilla trichodea for plant-odor detection.

Sensitization of ORNs in the housefly, *Mucsa domestica* L. after exposure to background concentrations of an attractive odorant has also been measured by EAG (Kelling et al., 2002). Constant atmospheric background concentrations of 1-octen-3-ol at 1.7×10^{-7} M (22 ng / ml air) and 2.4×10^{-7} M (33 ng / ml air) increased EAG responses (sensitization) to 1-octen-3-ol and (+)-limonene when these odorants were puffed at low dosages (0.001 and 0.01 mg) and decreased EAG responses (adaptation) to those compounds when puffed at higher (1 and 10 mg) dosages.

In other studies, injection of the biogenic amine octopamine (OA) into the head cavity of moths increased nerve impulse frequencies of stimulated pheromone-sensitive moth ORNs (Pophof, 2000; Grosmaitre et al., 2001; Pophof, 2002) and/or increased receptor potentials (Pophof, 2002). OA is also known to improve the detection and behavioral reactivity to pheromone blends in male moths (*Grapholita molesta* and *Trichoplusia ni*) (Linn and Roelofs, 1984; 1986).

The objectives for the current study were: 1) to characterize the EAG responses of male and female *C. rosaceana* and *A. velutinana* to a series of green leaf plant and fruit volatiles, 2) to determine the effect of sustained pre-exposure to various concentrations of a nine plant-volatile mixture or the sex-attractant pheromone of the two leafroller moth species on subsequent EAG responses to those compounds by both sexes of those species 3) to determine whether the effect of pre-exposure to the mixture of nine plant volatiles (nine-mix) varied from that of individual volatiles comprising that mixture, 4) test

whether injection of octopamine induced sensitization of EAG responses in leafroller moths, and 5) determine whether plant-volatile induced sensitization was suppressed by the octopamine antagonist, chlorpromazine.

MATERIALS AND METHODS

Insect colonies.

C. rosaceana were drawn from a four-year-old laboratory colony originally collected as 1st and 2nd generation pupae from apple orchards in Southwest Michigan. *A. velutinana* came from a long-established laboratory colony maintained at Geneva, NY by W. Roelofs. Both species were reared at 24°C on pinto bean-based diet (Shorey and Hale, 1965) under a 16:8 (L:D) photoperiod. Pupae sorted by species and sex emerged in 1 L plastic cages containing 5 % sucrose in plastic cups with cotton dental wick protruding from their lids.

Chemical sources and purities.

(*Z*)11-14:Ac (lot # 10010) was obtained from Shin Etsu (Tokyo, Japan). Purity was determined by gas chromatography (GLC) to be 96.1 % (*Z*)11-14:Ac and 3.9% (*E*)11-14:Ac. The plant volatiles *trans*-2-hexenal, benzaldehyde, 1-hexenol, *cis*-3-hexen-1-ol, geraniol, linalool, (+)-limonene, hexenal and *trans*-2-hexenol were purchased from Aldrich Chemical Company (Milwaukee, WI) and were ≥ 98 % pure (confirmed by our gas chromatographic analysis). Octopamine and the octopamine antagonist, chlorpromazine, were also purchased from Aldrich.

Electroantennogram apparatus.

The EAG system and test protocols were identical to those detailed by Stelinski et al., (2003a). Briefly, our EAG system consisted of a data acquisition interface board

(Type IDAC-02) and universal single ended probe (Type PRS-1) from Syntech (Hilversum, The Netherlands). The recording and indifferent electrodes were silver-coated wire in pulled glass micropipettes (10 µl micro-hematocrit capillary tubes) containing 0.5 M KCl. Moths were 2-4 d post-eclosion when used for electroantennography. EAGs were measured as the maximum amplitude of depolarization elicited by 1 ml puffs of air through EAG cartridges loaded with various concentrations of pheromone or plant volatiles and directed over antennae of whole-insect preparations. Stimulus puffs were generated through the cartridges with a clean, hand-held 20 ml glass syringe connected to the pipettes with a 1 cm piece of Tygon tubing. The time interval to expel 1 ml of stimulus odor or clean air from the syringe was quantified with video-cinematography and slow-motion playback. The 1 ml puff of air was expelled from the syringe within 120 ± 0.02 (S. D.) ms (n = 20) (Stelinski et al., 2003a).

EAG dose-response characterizations.

Prior to sustained exposure experiments, dose-response profiles for each plant volatile were obtained for males and females of both moth species. EAG cartridges were made by pipetting various concentrations (200 µg - 20 mg) of plant volatiles or pheromone (2 µg – 2 mg) in hexane (20 µL total solution) onto 1.4 x 0.5 cm strips of Whatman No. 1 filter paper. After 5 min in a fume hood for solvent evaporation, treated strips were inserted into disposable glass Pasteur pipettes, sealed with Parafilm, and allowed to equilibrate for 24 hr prior to use. Plant volatile and pheromone dosages were delivered to individual moths ($n \geq 12$) in ascending order. Four 1-ml puffs spaced 12 s apart were administered to each antenna at each dosage. Dose-response curves for males

of both species responding to pheromone have been previously reported (Stelinski et al., 2003a).

EAG analysis after constant exposure to a plant-volatile mixture or pheromone.

Moths were placed in pre-exposure chambers consisting of cylindrical, 1 L Teflon transfer containers (Jensen, Coral Springs, FL) equipped with two 64 mm ports in their lids. Glass inlets and outlets were affixed to the lids, allowing pressurized, carbon-filtered air to pass through the chambers at 30 ml / min. Chambers were divided with wire mesh, such that insects were confined in upper halves, while 2 cm diam. x 0.5 cm deep stainless steel planchettes loaded with a given dosage of odorant in mineral oil (Aldrich Chemical Company; Milwaukee, WI) were placed in the lower half. This arrangement reduced variation in odorant exposure relative to that in a static container, where distance of the moth from the odor source might influence compound uptake. In plant-volatile pre-exposure trials nine separate planchettes, each loaded with a given dosage of an individual plant-volatile, were placed into an exposure chamber. The highest dosage of plant volatiles tested was 100 µl of neat compound per planchette. Successively lower dosages were achieved by serially diluting 100 µl of mineral oil with loadings ranging in decade steps from 10 mg to 1 ng of each chemical. The control treatment consisted of exposing moths in clean chambers containing planchettes with mineral oil only.

In pheromone pre-exposure trials, the treatment was a single planchette loaded with either 1 µg or 10 mg of pheromone (diluted in 100 µl of mineral oil). These two loading dosages of pheromone are known to induce long-lasting peripheral adaptation to pheromone in male *C. rosaceana* (Stelinski et al., 2003a; Stelinski et al., 2003b). The air-

borne concentrations achieved by these two loading dosages were previously measured by GLC at 4.9 ± 1.5 and 56.2 ± 15.4 ng of pheromone per ml air in exposure chambers, respectively (Stelinski et al., 2003b). Both pre-exposure concentrations reduced subsequent EAG amplitudes in male *C. rosaceana* to pheromone by 40-60 % of normal. Females of both species were pre-exposed at the 1 μg loading dosage. Chambers always equilibrated for 60 min prior to insertion of insects. All insect pre-exposures were conducted in the exhaust hood of a laboratory remote from that housing the electroantennogram apparatus.

Initially, EAGs were measured from all insects exactly 1 and 60 min after the 60 min pre-exposure to volatiles. A minimum of twelve insects (1 per day) of each species and sex were exposed in the Teflon chambers for every volatile loading dosage tested. A total of six plant-volatile pre-exposure dosages were tested ranging in decade steps from 1μg – 100 mg. After the exposure treatments, moths were assayed by EAG with puffs of each of the nine plant volatiles as well as pheromone. Odorants were presented to each moth in a random order. In this series of tests, EAG cartridges were loaded either with 20 mg of each plant volatile or 2 mg of pheromone. We chose these particular cartridge loadings, after having determined the EAG dosage-response characteristics for these compounds (see results), because they consistently produced marked EAG responses.

After determining that exposure to plant volatiles produced a similar effect on subsequent EAG responses in both sexes of both species of moths (see results), we chose to use *C. rosaceana* males as our experimental animals for the remainder of this study.

Recovery time from sensitization in male C. rosaceana.

In a separate experiment measuring the time-course of recovery from sensitization (see results), male *C. rosaceana* were assayed 5, 10 and 15 min after 60 min of confinement in pre-exposure chambers containing all nine volatiles at the 1 mg loading dosage, which produced maximal sensitization in previous experiments (see Fig. 3 A). Ten male *C. rosaceana* were EAG-assayed with each of the nine plant volatiles or pheromone after each post-exposure recovery interval as described in section 2.5.

EAG analysis after constant exposure to individual plant volatiles.

This experiment determined whether increased EAG responses after pre-exposure to the nine-compound mixture (see results) were due to a synergistic effect of the mixture or whether single plant volatiles could induce the same overall effect. Male *C. rosaceana* were assayed 1 min after 60 min of pre-exposure to each of the nine volatiles individually (dissolved in mineral oil as described previously) at the 1 mg loading dosage. This loading dosage induced maximal sensitization when *C. rosaceana* were pre-exposed to the nine-compound mixture (see Fig. 14 A). Ten male *C. rosaceana* were EAG-assayed with each of the nine plant volatiles or pheromone at 1 min post-exposure as described in section 2.5.

Measurement of plant volatile concentration in exposure chambers.

Nine planchettes, each separately containing a common dilution of one of the nine plant volatiles tested above, were placed into one pre-exposure chamber. After equilibration for 60 min, the exhaust port was replaced with a port sealed with a rubber septum. Immediately thereafter, 15 ml of air was withdrawn through the septum into a 20

ml gas-tight glass syringe fitted with a 22 gauge stainless steel needle. In rapid succession, 5 ml of hexane wash containing nonanol at 6.4 ng / µl (used as an internal standard) was drawn into the syringe. The solution within the syringe was carefully shaken to avoid any spillage for 30 sec, then expelled into a GLC vial. This entire procedure was replicated 5 times at 23° C and *ca.* 35 % RH for each loading dosage (chemical diluted in mineral oil) tested. Prior to GLC analysis, samples were concentrated by a factor of 50 under N_2. Target volatiles were quantified by GLC (HP-6890, Hewlett-Packard Co) using flame ionization detection. The GC was fitted with a DBWAXETR polar column (model # 122-7332, JandW Scientific, Folsom, CA) of length 30 m and internal diam. 250 µm. The initial oven temperature was held at 50 °C for 5 min and then programmed to increase 30°C / min up to 250°C where it was held for 5 min; the carrier gas was He. We calculated nanograms of plant volatiles present in the 15 ml of exposure chamber air by multiplying the ratio of peak areas of the target compound (Z11-14:Ac) over the standard (nonanol) by: 1) nanograms of internal standard present in 1 µl of concentrated hexane wash, and 2) 100 to account for GLC analysis of only 1% of the total concentrated sample.

Effects of injected octopamine and chlorpromazine on sensitization as measured by EAG.

All injected compounds were dissolved in Haemolymph Ringer (HR) (Kaissling and Thorsson, 1980). DL-Octopamine hydrochloride (OA) and chlorpromazine (CP) were diluted to 100 µg/µl (527 nM) and 35.5 µg/µl (100 nM), respectively, as per Grosmaitre et al. (2001). OA and CP were injected into the heads of male *C. rosaceana* near the base of an antenna using a fine tipped capillary (*ca.* 0.1 mm tip opening) tightly joined to a 10 µl Hamilton syringe. The syringe tip of the capillary was carefully inserted into the insect

with a micromanipulator and approximately 1 µl of test solution was injected per insect. EAG recordings were performed on *C. rosaceana* 5 min after injection of HR alone, OA, and CP (n=10 per treatment). Finally, 16 male *C. rosaceana* were pre-injected with CP and subsequently exposed to the plant volatile mixture for 60 min at a concentration known to induce sensitization (1 mg loading dosage, see results), which was followed by EAG assay 1 min after pre-exposure as described in section 2.5.

Statistical Analyses.

Data were subjected to analysis of variance (ANOVA) and differences in selected pairs of means were separated using Tukey's multiple comparisons test, P=0.05 (SAS Institute, 2000).

RESULTS

EAG dose-response curves for plant volatiles and pheromone.

Response profiles for males and females of both moth species are presented in Fig. 12 as unnormalized EAG responses in ascending orders of stimulus-dosage applied. At the highest cartridge dosages, the mean EAG responses to the various volatiles ranged from 1 to 2 mV. Generally, at the highest cartridge dosages, hexanal, *trans*-2-hexenal, and geraniol produced higher responses (*ca.* 1.8-2 mV) than (+)-limonene, linalool, and *trans*-2-hexenol (*ca.* 1 mV). For male *C. rosaceana*, maximal responses to hexenal were significantly higher than for geraniol, *trans*-2-hexenol, benzaldehyde, linalool, and (+)-limonene. For male *A. velutinana*, responses elicited by hexenal were significantly higher than for geraniol, *trans*-2-hexenol, benzaldehyde, linalool, (+)-limonene, and *trans*-2-hexenal. For female *C. rosaceana* and *A. velutinana*, maximal responses elicited by *trans*-2-hexenal, hexenal, benzaldehyde, and geraniol were significantly higher than those

from 1-hexenol, linalool, (+)-limonene, *trans*-2-hexenol, and *cis*-3-hexen-1-ol. Females of both species responded to their own pheromone; EAGs averaged 2.5 ± 0.33 mV for *A. velutinana* and 2.0 ± 0.20 mV for *C. rosaceana* in response to the 2 mg EAG-cartridge dosage of pheromone (Fig. 13). These responses did not differ statistically from those elicited by *trans*-2-hexenal, hexenal, benzaldehyde, and geraniol, but were significantly greater than those elicited by 1-hexenol, linalool, (+)-limonene, *trans*-2-hexenol, and *cis*-3-hexen-1-ol (Fig. 13).

EAG response to plant volatiles and pheromone after pre-exposure to the plant-volatile mixture.

Up to and including the 1 mg loading, pre-exposure of male *C. rosaceana* to successively higher concentrations of the nine plant volatiles yielded a dosage-dependent increase in subsequent responsiveness to those volatiles, as measured by EAGs 1 min after exposure (Fig. 14 A). Pre-exposures at the 100 µg and 1 mg loading dosages significantly increased EAG responses to pheromone compared to pheromone-induced EAGs recorded after any other exposure treatment including the control (Fig. 14 A). Also, EAG responses to several of the plant volatiles were significantly ($P < 0.05$) larger 1 min after pre-exposure to the nine plant-volatile mixture at 10 µg, 100 µg, and 1 mg loading dosages (Fig. 14 A). By contrast, pre-exposure of male *C. rosaceana* at the two highest concentrations (10 and 100 mg loading dosages), significantly reduced subsequent EAG responses to all plant volatiles and pheromone. At 60 min post-exposure, the mean EAG responses of male *C. rosaceana* pre-exposed at every loading dosage were not significantly different from the control treatment, i.e. recovery to normal was complete.

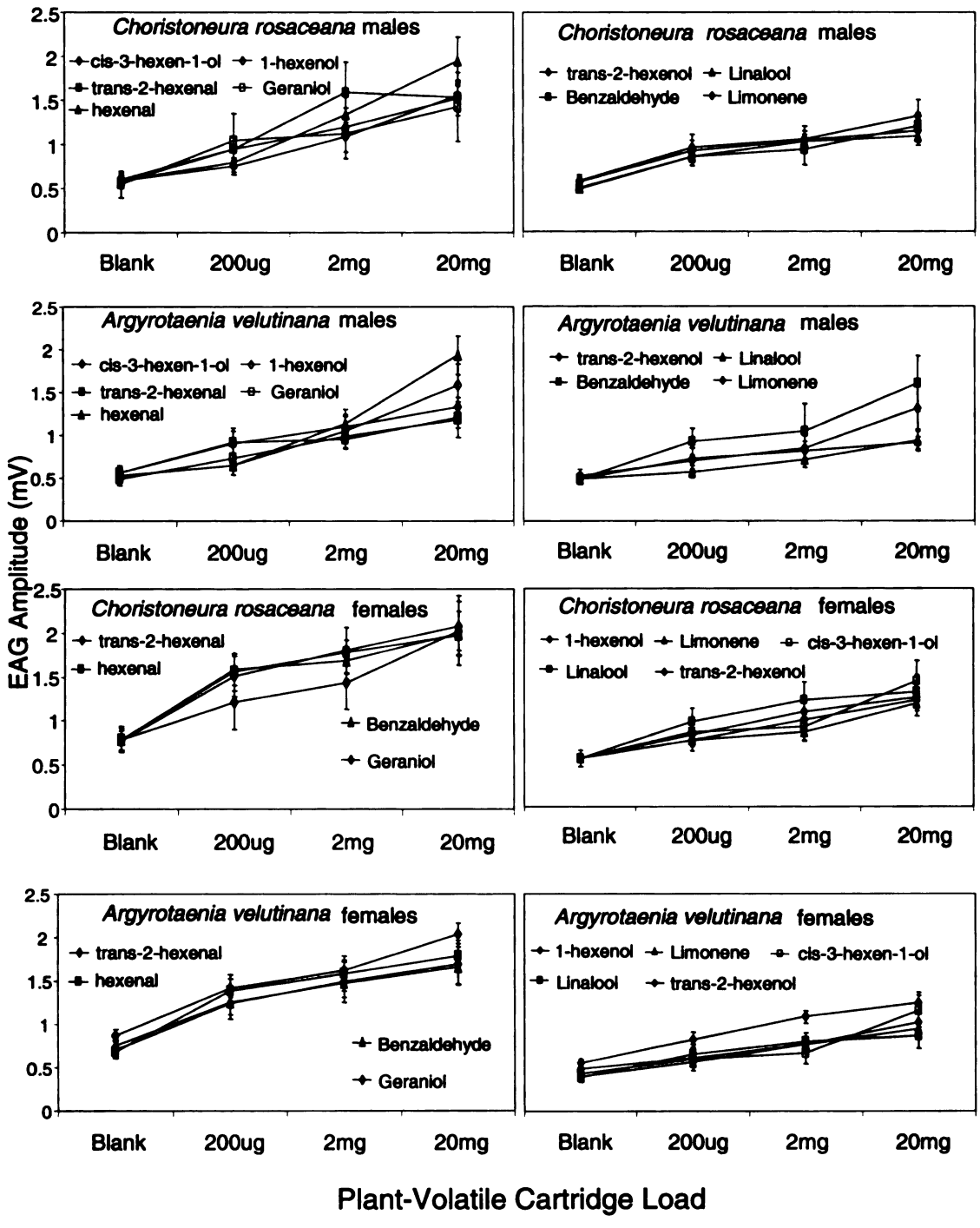


Figure 12. EAG dose-response profiles of male and female *Choristoneura rosaceana* and *Argyrotaenia velutinana* to nine plant volatiles (mean ± SEM, n=12 for each volatile

dosage). Cartridge load indicates the amount of compound loaded onto a piece of filter paper in a Pasteur pipette.

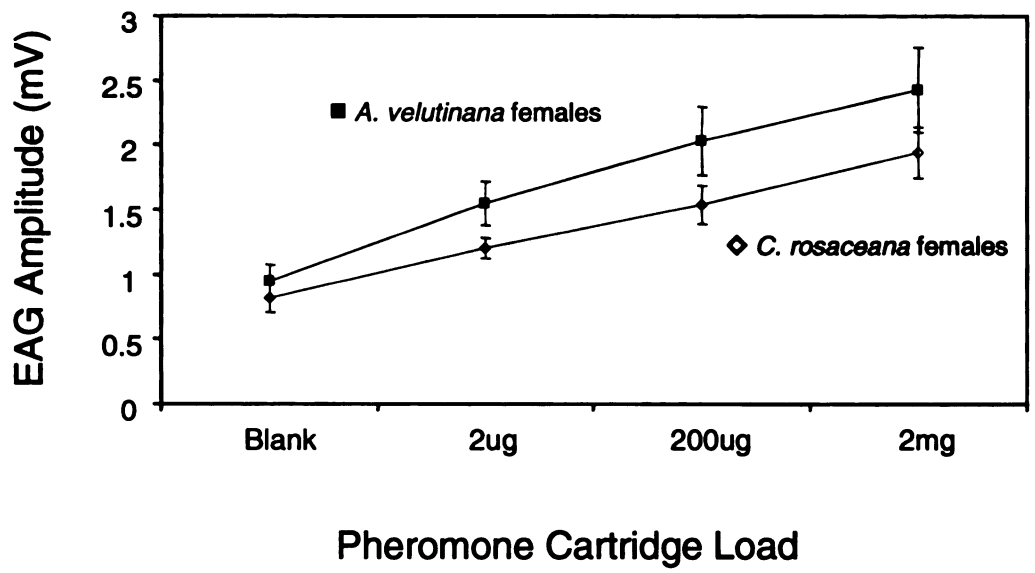


Figure 13. EAG dose-response profiles of female *Choristoneura rosaceana* and *Argyrotaenia velutinana* to their major pheromone components (mean ± SEM, n=12 per volatile). Cartridge load indicates the amount of compound loaded onto a strip of filter paper in a Pasteur pipette odor cartridge.

A similar pattern was observed in *A. velutinana* males pre-exposed to plant volatiles. EAG responsiveness to plant volatiles also increased in a dosage-dependent fashion with increasing pre-exposure loadings, up to the 10 mg dosage (Fig. 14 B). A

significant ($P < 0.05$) cross-sensitization to post-exposure pheromone puffs and sensitization to puffs of all nine volatiles were observed after pre-exposure loadings ranging from 100 μg through 1 mg. For *A. velutinana*, significant desensitization to all plant volatiles and pheromone occurred only at the highest (100 mg) loading dosage (Fig. 14 B).

The effects of plant-volatile pre-exposure were similar on female *C. rosaceana* and *A. velutinana* (Fig. 14 C and D). Pre-exposure to the 100 μg and 10 mg loadings of the nine volatiles significantly increased subsequent responsiveness to some volatiles in both *C. rosaceana* and *A. velutinana* (Fig. 14 C and D). Also, pre-exposure at the two highest loadings (10 and 100 mg), significantly reduced subsequent EAG responses to all plant volatiles and pheromone for females of both species. A notable difference between the sexes was that females were not significantly cross-sensitized to pheromone after pre-exposure to the plant volatiles.

EAG response to plant volatiles and pheromone after pre-exposure to individual plant volatiles.

The EAG responses of male *C. rosaceana* 1 min after 60 min of exposure to *cis*-3-hexen-ol and *trans*-2-hexenol were not significantly different from control responses (Fig. 15 A). Pre-exposures (60 min) to hexenol, hexenal and *trans*-2-hexenal significantly increased responsiveness to hexenal, *trans*-2-hexenal, and geraniol (Fig. 15 A). EAG responses of male *C. rosaceana* after pre-exposure (60 min) to (+)-limonene, geraniol, and benzaldehyde were significantly elevated for many of the plant volatiles and for pheromone 1 min after exposure (Fig. 15 B). None of the pre-exposures to individual plant volatiles induced subsequent sensitization of EAG responses to all of the plant

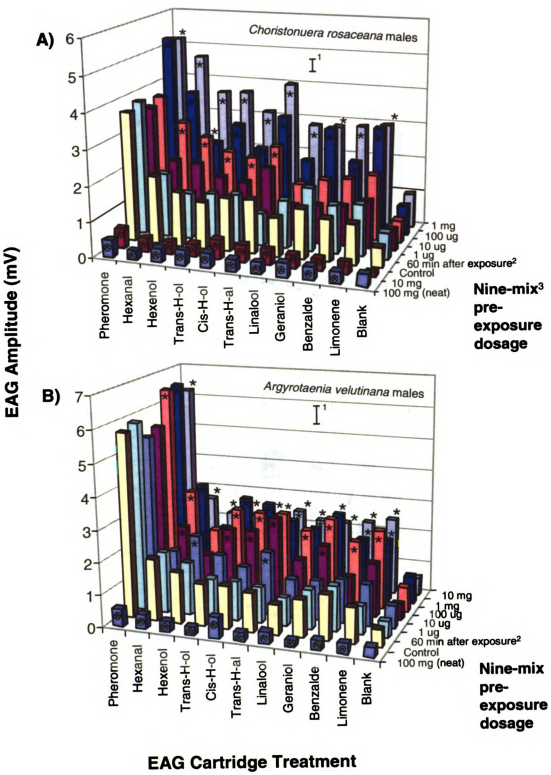
volatiles and pheromone as was seen after pre-exposure to the plant volatile nine-mix (Fig. 15 A and B).

EAG response to plant volatiles and pheromone after pre-exposure to pheromone.

As previously reported and confirmed here (Stelinski et al., 2003a and b; hence data not shown here), pre-exposure of male *C. rosaceana* to pheromone at 1 μg and 10 mg loading dosages significantly reduced EAG responsiveness to pheromone one min post-exposure, while such as effect was not seen for *A. velutinana*. After 10 min of recovery in clean air, the mean EAG responses of pre-exposed *C. rosaceana* to pheromone were no longer significantly different from control amplitudes. However, in the reciprocal test, no significant changes in EAG responses occurred for any of the plant volatiles tested 1 and 10 min after pheromone pre-exposure in male *C. rosaceana* and male *A. velutinana* (data not shown). Also, there were no significant effects of pheromone pre-exposure on EAG amplitudes of female *C. rosaceana* and female *A. velutinana* to any of the plant volatiles and pheromone 1 and 10 min after the pre-exposure interval.

Recovery time from sensitization in male C. rosaceana.

EAG responses of male *C. rosaceana* pre-exposed for 60 min to the plant-volatile mixture at the 1.0 mg loading dosage were significantly ($P < 0.05$) elevated for all puffs of plant volatiles and pheromone 1 and 5 min after exposure (Fig. 16). At 10 min after pre-exposure, the EAG responses of *C. rosaceana* males were no longer significantly different from control responses recorded from unexposed *C. rosaceana* (Fig. 16).


A)
Choristonuera rosaceana males
B)
Argyrotaenia velutinana males
EAG Amplitude (mV)
Pheromone
Hexanal
Hexenol
Trans-H-ol
Cis-H-ol
Trans-H-al
Linalool
Geraniol
Benzalde
Limonene
Blank
1 mg
100 ug
10 ug
1 ug
60 min after exposure²
Control
10 mg
100 mg (neat)
Nine-mix³ pre-exposure dosage
EAG Cartridge Treatment

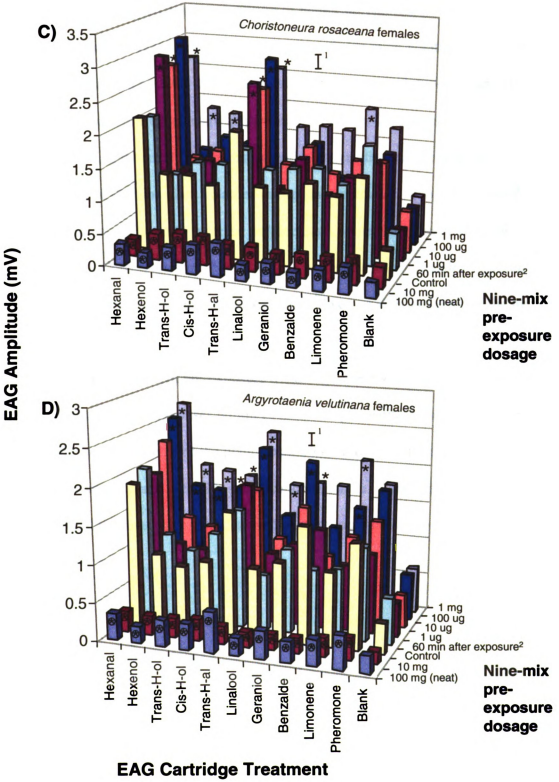

C)
Choristoneura rosaceana females
D)
Argyrotaenia velutinana females
EAG Amplitude (mV)
0
0.5
1
1.5
2
2.5
3
3.5
Hexanal
Hexenol
Trans-H-ol
Cis-H-ol
Trans-H-al
Linalool
Geraniol
Benzalde
Limonene
Pheromone
Blank
1 mg
100 ug
10 ug
1 ug
60 min after exposure²
Control
10 mg
100 mg (neat)
Nine-mix pre-exposure dosage
EAG Cartridge Treatment

Figure 14. A. Effect of 60 min pre-exposure of male *Choristoneura rosaceana* (n=12 per treatment) to various loading dosages of a mixture of nine plant volatiles. X-axis displays the compounds tested by EAG 1 min after the 60 min exposure interval. Trans-H-ol, Cis-H-ol, Trans-H-al, and Benzalde are abbreviations for *trans*-2-hexenol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, and benzaldehyde, respectively. Y-axis displays the loading concentrations of the nine plant volatiles in exposure chambers. Bars with * and ⊖ indicate significant ($P < 0.05$) increase and decrease, respectively, relative to the control treatment. B-D. Corresponding results for *Argyrotaenia velutinana* males, *C. rosaceana* females, and *A. velutinana* females, respectively. [1]Average standard error of the mean for EAG amplitude across all treatments. [2]Pooled mean mV responses to EAG cartridge treatments 60 min after each pre-exposure treatment. There were no significant differences in the mean responses within EAG cartridge treatments assayed 60 min after pre-exposure to each dosage of the plant-volatile mixture. Therefore, these data were pooled into a single grand mean for statistical comparison. [3]Nine-mix refers to the combination of the nine plant volatiles used in moth exposures.

A)
Choristonuera rosaceana males
EAG Amplitude (mV)
Pheromone
Hexanal
Hexenol
Trans-H-ol
Cis-H-ol
Trans-H-al
Linalool
Geraniol
Benzalde
Limonene
Blank
Nine-mix
hexenol
trans-2-hexenal
hexenal
cis-3-hexen-1-ol
trans-2-hexenol
Control
Pre-exposure treatment
B)
Choristonuera rosaceana males
limonene
geraniol
benzaldehyde
linalool
Cartridge treatment

Figure 15 A and B. EAG responses of male *Choristoneura rosaceana* after 1 min of clean-air recovery (n=10 per treatment) post 60 min of confinement in pre-exposure chambers with 1 mg loadings of nine plant volatiles individually and as a mixture. Bars with different letters indicate significant ($P < 0.05$) differences between treatment means within a cartridge treatment. Trans-H-ol, Cis-H-ol, Trans-H-al, and Benzalde are abbreviations for *trans*-2-hexenol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, and benzaldehyde, respectively. [1]Average standard error of the mean EAG amplitude across all treatments.

Plant-volatile concentration in exposure chamber.

Concentrations of eight of the nine plant volatiles in adaptation chambers were detectable down to the 1μg loading (Fig. 17). Because of interfering peaks at its retention time, geraniol could not be reliably quantified in this experimental context. Under our conditions where air moved through the pre-exposure chambers at 30 ml / min, the concentration of all compounds in the air rose linearly with the log of loading concentration in mineral oil. Although loading dosages were identical at each increment, the concentrations of plant volatiles in the air inside the pre-exposure chamber varied widely, as expected, given the wide range in known vapor pressures of these compounds (http://hazard.com/msds/gn). The highest concentrations in exposure chambers were reached by the aldehydes (hexenal and *trans*-2-hexenal) and the terpene (+)-limonene (Fig. 17). None of the other compounds (mostly alcohols) reached mean concentrations above 100 ng / ml of air.

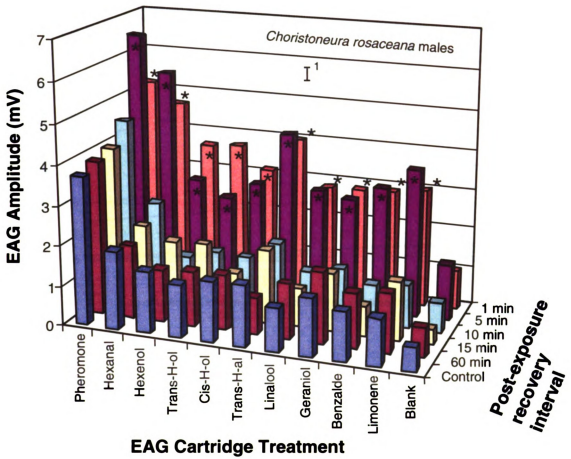


Figure 16. EAG responses of male *Choristoneura rosaceana* after various intervals of clean-air recovery (n=12 per treatment) post 60 min of confinement in pre-exposure chambers with 1 mg loadings of nine plant volatiles. Bars with asterisks indicate significant ($P < 0.05$) differences between treatment means and the control treatment. Trans-H-ol, Cis-H-ol, Trans-H-al, and Benzalde are abbreviations for *trans*-2-hexenol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, and benzaldehyde, respectively. [1]Average standard error of the mean EAG amplitude across all treatments.

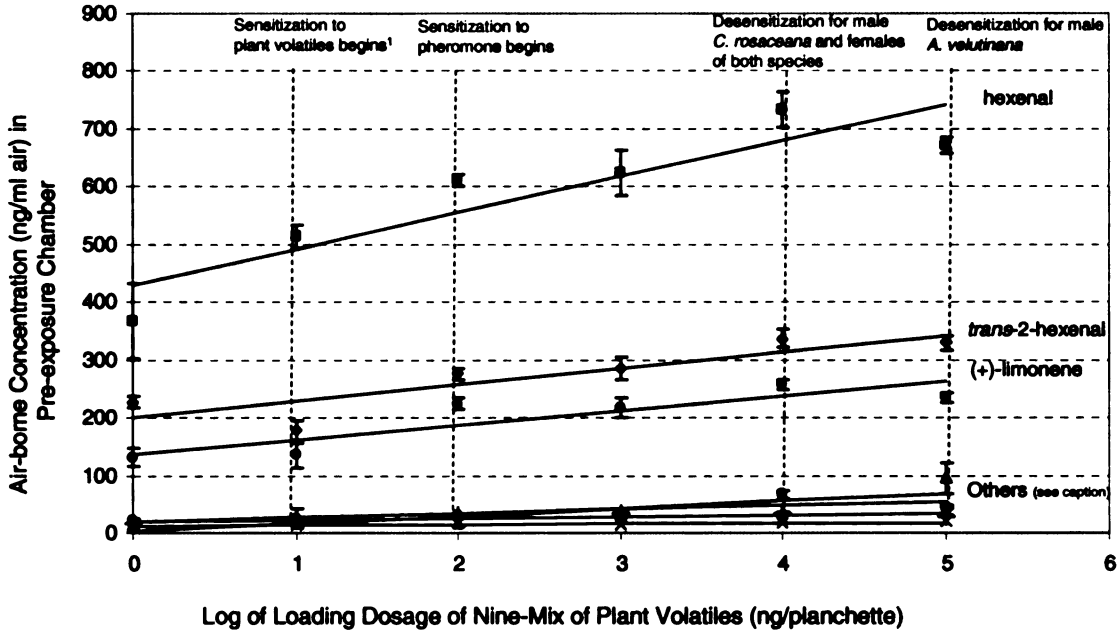


Figure 17. Relationship between GLC-quantified plant volatile concentrations in exposure chambers and loading dosage of chemicals in planchettes. Hexenal: $y = 62.8x + 429.4$, $R^2 = 0.81$; *trans*-2-hexenal: $y = 28.4x + 200.8$, $R^2 = 0.77$; (+)-limonene: $y = 25.2x + 136.8$, $R^2 = 0.76$; benzaldehyde: $y = 13.1x + 2.6$, $R^2 = 0.66$; 1-hexenol: $y = 6.9x + 20.5$, $R^2 = 0.61$; *cis*-3-hexen-1-ol: $y = 3.0x + 20.0$, $R^2 = 0.64$; linalool: $y = 1.2x + 12.4$, $R^2 = 0.80$; *trans*-2-hexenol: $y = 7.0x + 20.5$, $R^2 = 0.61$. [1]These notes highlight the cartridge loading dosages at which various changes in EAG responsiveness were observed.

Effects of octopamine and chlorpromazine on sensitization in male C. rosaceana.

The mean EAG responses of OA-injected *C. rosaceana* to all of the plant volatiles and pheromone were significantly ($P < 0.05$) greater than responses recorded from control insects or those injected with either HR or CP (Fig. 18). There were no significant differences in the responses of *C. rosaceana* five min after injection of OA compared to those assayed one min after 60 min of pre-exposure to the plant-volatile nine-mix for several of the volatiles and pheromone (Fig. 18). The EAG responses of *C. rosaceana* injected with CP prior to pre-exposure to the plant volatiles were not significantly different from the controls after the pre-exposure period except for responses to pheromone; these were significantly lower than those recorded from untreated moths. The EAG responses of moths injected with HR were significantly ($P < 0.05$) greater than control responses for hexenal and *trans*-2-hexenal (Fig. 18).

DISCUSSION

Mechanisms of olfactory sensitization to plant volatiles.

The enhanced antennal sensitivity of leafroller moths following continuous exposure to plant volatiles described herein was measured up to five min after the pre-exposure terminated and was no longer evident 10 min after the pre-exposure, indicating that the dynamics of recovery are rapid. In addition, plant-volatile and pheromone stimulation of whole-antennae of live male *C. rosaceana* five min after injection of OA increased depolarization of olfactory receptors similarly to that induced by pre-exposing moths to a mixture of nine plant volatiles at a loading dosage of *ca.* 1 mg / 100 µl mineral

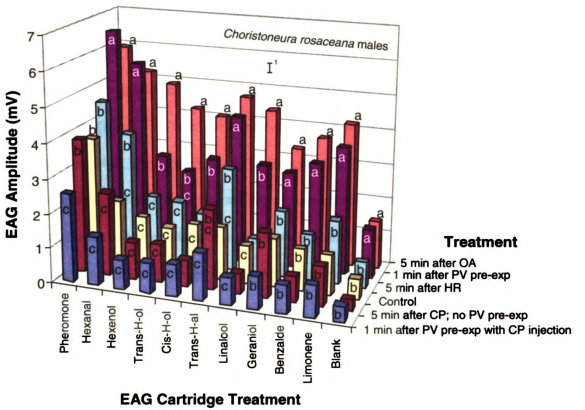


Figure 18. EAG responses of male *Choristoneura rosaceana* 5 min after injection of octopamine (OA), chlorpromazine (CP), haemolymph ringer (HR), 60 min of pre-exposure to the nine-mix of plant volatiles (PV), 1 min after 60 min of pre-exposure to the nine plant volatile mixture for moths pre-injected with CP, an untreated (control) moths. Bars with different letters indicate significant ($P < 0.05$) differences between treatment means within cartridge treatment. Trans-H-ol, Cis-H-ol, Trans-H-al, and Benzalde are abbreviations for *trans*-2-hexenol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, and benzaldehyde, respectively. [1]Average standard error of the mean EAG amplitude across all treatments.

oil (Figs. 14 A and 18). Moreover, injection of the OA antagonist, chlorpromazine (CP), blocked the sensitizing effect of plant-volatile exposure as evidenced by normal EAG amplitudes immediately following sustained pre-exposure to sensitizing concentrations of plant volatiles (Fig. 18). Therefore, we postulate that pre-exposure to plant volatiles may have increased OA titre, subsequently sensitizing ORNs.

Octopamine (OA) is a major biogenic amine of insects and known to function as a neurotransmitter, neuromodulator, and neurohormone (Evans, 1985). Injecting OA into male *B. mori* significantly increased the amplitude of receptor potentials and action potential frequencies elicited by the pheromone components bombykol and bombykal (Pophof , 2002). OA also affected spontaneous oscillations of the transepithelial potential in *Manduca sexta* (Dolzer et al., 2001). However, in *Antheraea polyphemus* and *Mamestra brassicae*, OA injections only increased action potential frequencies of pheromone-sensitive ORNs in moths and did not affect transepithelial potentials (Pophof, 2000; Grosmaitre et al., 2001).

Pophof (2002) suggested the mechanisms underlying the modulatory action of OA differ between species and advanced two possible modes of action; OA could act directly on the receptor neurons by affecting a transduction pathway involved in spike generation. Alternatively, OA could act via auxiliary cells through modulation of the V-ATPase located in the membrane of auxiliary cells; this would influence transepithelial potential. The latter mechanism is supported by our results, given that EAG measures a depolarization of receptor potentials summed across the antennal olfactory neurons (Roelofs, 1984).

The rapid recovery time-course from sensitization after exposure to plant volatiles measured in this study corresponds with rapid catabolic rates for insects degrading various amines including OA. After injection of 2 μg of 5-HT (serotonin) into *Perplaneta americana*, measured titres of the amine decreased from 5 ng / μl to 200 pg / μl within 10 min (Sloley and Downer, 1990). NA5-HT was also metabolized rapidly, returning to undetectable levels within 15 min. Rapid metabolism of OA following injection has also been documented in *P. americana*, *Schistocera gregaria*, and *T. ni.* (Goosey and Candy, 1982; Downer and Martin, 1987; Linn et al., 1994). In a pilot study that guided the current work, EAG responses of *C. rosaceana* returned to normal levels 20 min after injecting OA. The similarity between the time-course for desensitization after plant-volatile exposure and that for metabolism of injected OA is an additional line of evidence implicating OA as a mediator of the current sensitization phenomenon.

Pophof (2002) found that injected OA significantly increased amplitude of receptor potentials and nerve impulses in male *B. mori* with no corresponding effect on homologous female ORNs. We also found that sensitization of the peripheral olfactory system was greater for males than females and cross-sensitization to pheromone only occurred in males. Pophof (2002) suggested that the OA-dependent modulatory pathway may be absent or less sensitive in females or there may be differences in metabolic rates across the sexes.

Essential oils, which include terpenoids and phenols, constitute a family of substances naturally occurring in the vegetative tissue of a number of plant families including: Myrtaceae, Lauraceae, Rutaceae, Limiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae, and Piperaceae. The terpenoids are thought to

function as insect attractants, repellants, and oviposition cues (Karr and Coats, 1992; Hori, 1999; Landolt et al., 1999). Recent studies aimed at screening essential oils and essential oil component extracts for insecticidal activity have shown that some natural terpenes (identified as ZP-51 and SEM-76) (Kostyukovsky et al., 2002) have appreciable insecticidal activity against several stored-product pests including *Sitophilus oryzae, Rhizopertha dominica, Tribolium castaneum,* and *Oryzaephilus surinamensis*. Other essential oils (eugenol and α-terpeniol) are toxic to cockroaches (*P. americana* and *Blattella germanica*) and carpenter ants (*Camponotus pennsylvanicus*) (Enan, 2001). Exposure to these terpene extracts or essential oils changed intracellular cAMP levels biphasically with increasing exposure concentrations (Enan, 2001; Kostyukovsky et al., 2002). Moreover, treatment with OA induced identical fluctuations of cAMP to those induced by exposure to the terpenes and the OA-antagonist, phentolamine, inhibited such fluctuations indicating competitive activation of octopaminergic receptors by the terpenoid essential oil constituents (Enan, 2001; Kostyukovsky et al., 2002). Additionally, OA receptors were blocked under exposure to essential oils (eugenol and α-terpeniol) as measured by decreased binding activity of [^{3}H]octopamine to its receptors. The above studies clearly implicate the octopaminergic system as the target for several monoterpenoids as substantiated by the similarities between activity of OA and terpenoid-exposure, common blocking action of OA-antagonists, and competitive OA-receptor binding. In the present study, the monoterpenes limonene and geraniol were two constituents of the plant-volatile mixture used in exposure trials. Pre-exposure of male *C. rosaceana* to these volatiles individually markedly increased EAG responsiveness, albeit not identically to that observed after exposure to the mixture of nine plant-volatiles. Our

results are congruent with those discussed above in that certain terpenes (limonene, geraniol) induced an effect similar to that observed by OA-injection. Therefore, plant-volatile induced sensitization of olfactory receptors may have also taken place via competitive binding of octopaminergic receptors by certain terpene compounds tested in this study.

Desensitization of EAG responses following pre-exposure to highest loading dosages of plant volatiles.

Pre-exposure at the two highest loading dosages of the nine plant-volatile mixture markedly desensitized antennae of male and female *C. rosaceana* and female *A. velutinana* to subsequent puffs of the nine volatiles and of pheromone (Fig. 14 A, C, D). This effect occurred in male *A. velutinana* only at the highest loading dosage (Fig. 14 B). It appears that a threshold pre-exposure was reached between the 1 and 10 mg loading dosages above which there was marked and broad desensitization. The effect was completely reversible and no longer measurable by EAG 60 min after the exposure treatment (Fig. 14, A-D). According to the GLC quantifications, the concentration of volatiles in air inducing desensitization were < 2 fold greater than those inducing maximal sensitization (Fig. 17). In fact, maximal sensitization was observed at a combined estimated concentration of all of the volatiles (excluding geraniol) of *ca.* 1.28 µg / ml of air, while desensitization was induced at a combined concentration of *ca.* 1.52 µg / ml of air. Notably, the dosage of these volatiles required to cause sensitization was on the order of tens of nanograms per ml of air if as few as one particular volatile were the causative agent. If all of the compounds in the mixture were contributing, the dosage for sensitization could have been *ca.* 1 µg / ml.

The desensitization we observed by EAG is reminiscent of the inhibitory action of volatiles such as linalool on pheromone receptor potentials in *B. mori* (Kaissling, 1977), geraniol and other terpenes on *A. pernyi* (Schneider et al., 1964), and of various other green leaf volatiles on *A. orana* (Den Otter et al., 1978) and the yponomeutid species discussed previously. It has been suggested that the inhibitory action of these compounds could be due to a disturbance of the cell membrane, possibly caused by inculcations of the plant volatiles into the lipid matrix, indirectly affecting the transduction process (Kaissling, 1977). Alternatively, certain plant volatiles may directly inhibit signal transduction. The diacylglycerol analog, 1,2-di-octanoyl-sn-glycerol (DOG), activates transduction as measured by increased nerve impulses similar to those elicited by stimulation with odorants (Pophof and Van der Goes van Naters, 2002). This DOG-mediated activation is directly inhibited by linalool and 1-heptanol, providing evidence for a linalool- or 1-heptanol-dependent inhibition of the diacylglycerol-dependent part of the transduction cascade, possibly related to the opening of ion channels (Pophof and Van der Goes van Naters, 2002).

Given the completeness and breadth of the desensitization observed at the two highest exposure concentrations, we cannot judge whether the effect was due to some type of adaptation resulting from an exhaustion of available odorant-binding proteins, saturation of receptor sites, a decrease in membrane conductance due to altered membrane structural integrity, or some other type of toxicity.

Possible significance of sensitization in response to plant-volatile exposure.

Most of the previously discussed studies have focused on the effect of single compounds or binary mixtures of a plant volatile and pheromone on single antennal

sensilla. To our knowledge, this is the first study examining the effect of prolonged pre-exposure to both a complex mixture of plant volatiles as well as the individual volatiles comprising that mixture on subsequent responsiveness of whole antennae of live moths to plant volatiles and pheromone. Our results support the idea that consequential interactions do occur between host-plant odors and sex attractant pheromones in moths; however, they must be followed up with behavioral studies to substantiate this hypothesis. The current study has shown that antennal sensillae of *C. rosaceana* and *A. velutinana* are sensitive to a wide array of green leaf and fruit volatiles that might serve as cues in host-plant finding for these polyphagous herbivores. Continuous pre-exposure to a mixture of plant compounds and certain individual volatiles enhanced the antennal sensitivity of both sexes to subsequent presentations of these chemicals as well as cross-sensitized male antennae to the female sex-pheromone. These findings suggest that exposures of tortricid moths to certain host-plant related volatiles may modulate subsequent olfactory sensitivity to behaviorally relevant chemical cues. Based on these results, it will be informative to conduct behavioral studies to determine whether certain plant volatiles could improve practical applications of semiochemicals, such as pheromone-based mating disruption (Ochieng et al., 2002).

CHAPTER FOUR

Behaviors of naïve vs. pheromone-exposed leafroller moths in plumes from high-dosage pheromone dispensers in a sustained-flight wind tunnel: implications for mating disruption of these species.

Also published as:

Stelinski, L. L., K. J. Vogel, L. J. Gut, and J. R. Miller. 2004. Behaviors of naïve and pheromone pre-exposed leafroller moths in plumes of high-dose pheromone dispensers in a sustained-flight wind tunnel: implications for pheromone-based mating disruption of these species. *Journal of Insect Behavior.* 17: 533-554.

ABSTRACT

Brief exposures of male *Choristoneura rosaceana* and *Argyrotaenia velutinana* to the plumes generated by lures releasing 3-component pheromone blends specifically tuned for each species or by commercially distributed Isomate OBLR/PLR Plus pheromone 'rope' dispensers induced markedly different subsequent behavioral responses to pheromone. A greater proportion of *C. rosaceana* males took flight and successfully oriented toward lures 24 h after pre-exposure to a lure, a rope, or the lure-rope combination in a sustained-flight wind tunnel compared to naïve moths. Flights were also longer for pre-exposed than naïve moths. Pre-exposed male *C. rosaceana* were not more likely to fly toward ropes 24 h after pre-exposure. By contrast, fewer male *A. velutinana* oriented to lures 24 h after pre-exposure than did naïve moths. Those pre-exposed *A. velutinana* successfully locking onto plumes from lures flew for significantly shorter intervals than did unexposed moths. Electroantennograms revealed no changes at the periphery 15 min and 24 h after pre-exposure. For *A. velutinana*, the long-lasting effect was decreased attraction to a lure and increased attraction to a rope. For *C. rosaceana*, pheromone pre-exposure increased responsiveness to its authentic blend. This behavioral evidence is sufficient to explain why sexual communication of *C. rosaceana* is more difficult to disrupt than that of *A. velutinana*. Furthermore, it suggests a more complete blend of pheromone may be necessary to disrupt the former species but not the latter when using rope dispensers.

INTRODUCTION

Much research has been aimed at understanding the physiological and behavioral mechanisms involved in mating disruption of moths by synthetic pheromone formulations (Sanders, 1985; Sanders, 1996; Cardé et al., 1997; Cardé et al., 1998; Sanders, 1998; Evenden et al., 2000; and others). An understanding of the behavioral and physiological mechanisms underlying pheromone-based mating disruption is crucial to the successful development, deployment, and optimization of efficacious pheromone formulations, release devices, and deployment strategies (Cardé and Minks, 1995; Sanders, 1996). Although mating disruption through application of synthetic pheromone formulations has been successfully achieved and adopted for various moth species (Cardé and Minks, 1995), ideal levels of population control and accompanying crop protection have not always been attained for certain species under certain disruption regimes (Seabrook and Kipp, 1986; Deland et al., 1994; Agnello et al., 1996; Lawson et al., 1996). It has been suggested that physiological variation in response to pheromone (i.e. varying degrees of peripheral adaptation) (Stelinski et al., 2003 a, b) along with differences in chemical characteristics of pheromones (degree of "stickiness" related to molecular chain length) (Gut et al., 2004) may explain some of the variation in the degree of success achieved in controlling moth pests by mating disruption.

The major mechanisms thought to underlie mating disruption (Bartell, 1982; Cardé, 1990; Cardé and Minks, 1995; and others) are: (1) camouflage of female-produced plumes amongst a constant background concentration of synthetic pheromone may impede successful anemotactic orientation, (2) false-plume-following of synthetic pheromone plumes by male moths may decrease the time available for finding authentic

females, and (3) adaptation of peripheral receptors or habituation of the central nervous system (CNS) may impair or eliminate normal responses to pheromone. Other proposed mechanisms include advancement of the male's diel rhythm of response (Cardé et al., 1998) and premature arrestment of male's response under high pheromone concentrations (Baker and Cardé, 1979). Cardé et al., (1997; 1998) caution that these mechanisms need not be mutually exclusive; rather they may act additively or synergistically.

A plausible scenario for mating disruption proposed by Cardé et al. (1998), and embellished upon here reasons that an initial bout of false-plume-following by a male moth may result in an eventual encounter with a high-dose releaser of synthetic pheromone (e.g., polyethelene tube dispensers, referred to as 'ropes'). In the short term, such an encounter may cause that male's peripheral receptors to adapt, decreasing or eliminating his ability to perceive pheromone. Given the reversibility and short-lived nature of peripheral adaptation (Kuenen and Baker, 1981; Stelinski et al., 2003 a, b), it is plausible that an encounter with the rope dispenser may be more likely to decrease the male's responsiveness to pheromone via habituation. Such males may then preferentially orient to plumes emanating from high-dosage pheromone dispensers over the lower levels emitted by females. Further visits by pre-exposed and habituated males to dispensers such as ropes may compound a male's inability to find a female; e.g., greater levels of habituation, physiological exhaustion, and decreased fecundity with age.

Responses by male moths to calling females or surrogate-female lures in the presence of background concentrations of pheromone have been quantified in laboratory wind tunnels. Prolonged pre-exposure (1-4 d) of *Choristoneura fumiferana* males to plumes of pheromone (95:5 *E*:*Z*-11-tetradecenal) emanating from rubber septa in a wind

tunnel decreased male orientation by 27 - 95 % in a dosage dependent manner; longer exposures were positively correlated with greater inhibition of orientation. A time-averaged atmospheric concentration of 20 ng/m^3 of pheromone was necessary to highly disrupt this species (Sanders, 1996). In further studies that employed a sustained-flight wind tunnel, the proportion of *C. fumiferana* males sustaining flights for 4 min or longer decreased from > 50 % to < 10 % as the concentration of background pheromone increased in the tunnel (Sanders, 1998). There was considerable variability in the responsiveness of males under simulated conditions of mating disruption (Sanders, 1998). The longest sustained flight recorded (53 min) was by a male orienting in a background concentration of *ca.* 20 pg/m^3 of the binary 95:5 blend of *E*:*Z*-11-tetradecenal. Sanders (1998) suggested that complete disruption of a population of *C. fumiferana* by this binary pheromone blend may be very hard to achieve given that a small proportion of males was able to lock onto female-produced plumes for prolonged durations despite high background concentrations of synthetic pheromone.

In similar wind tunnel investigations of the mechanisms of pheromone communication disruption using *C. rosaceana* as a study system, Evenden et al. (2000) documented that reduction of successful orientations to calling females was most pronounced in a background of the complete 4-component pheromone blend (Thomson et al., 1991) rather than a less attractive 2-component formulation. In addition, pre-exposing *C. rosaceana* for 30 min to pheromone 30 min prior to bioassay did not alter the proportion of males successfully contacting calling females. These authors concluded that disruption of *C. rosaceana* in their wind tunnel bioassay was due to a combination of

peripheral adaptation, camouflage of the female-produced plume, and false-plume-following rather than by habituation.

Sanders' (1995) conclusions from work on *C. fumiferana* and that of Evenden et al. (2000) on *C. rosaceana* suggest that sensory fatigue after pre-exposure to naturally-produced and synthetic pheromone may be less important than false-trail-following in disrupting orientation of males to calling females. Interestingly, brief pre-exposures of male *C. fumiferana* to calling females dramatically increased the percentage of males successfully locking onto and flying to sources of pheromone off-blends. Similar results were obtained by pre-exposing males to a 95:5 blend of *E*:*Z*-11-tetradecenal (Sanders, 1984). This "priming" effect was thought possibly to increase false-plume-following by male moths in mating disruption regimes using off-blends.

Sanders' (1984; 1995) results are reminiscent of an earlier study by Linn and Roelofs (1981) documenting that minutes-long pre-exposures of male *Grapholita molesta* to *E*-8-dodecenyl acetate enhanced subsequent responses to selected off-blends of pheromone (blends containing high % *E*) that normally elicited few completed flights from naïve males. More recently, Anderson et al. (2003) found that male *Spodoptera littoralis* briefly pre-exposed to a female-produced plume responded more vigorously to subsequent presentations of the female sex-pheromone. This increased responsiveness to pheromone lasted up to 27 h and was not associated with a change in the sensitivity of sex-pheromone receptors. The authors suggested that the brief pre-exposure induced a change in the CNS that increased male responsiveness to the female's pheromone.

In electrophysiological studies aimed at understanding the mechanisms underlying the differences in susceptibility to mating disruption among two sympatric

tortricid moths, *C. rosaceana* and *Argyrotaenia velutinana* (Stelinski et al., 2003 a), differences were found at the level of peripheral receptors. Specifically, the antennae of *C. rosaceana* exhibited a long-lasting adaptation (*ca.* 12.5 min) after 5 min exposure to pheromone, while the antennae of *A. velutinana* dis-adapted within 1 min after identical exposure. A consistent reduction in responsiveness to pheromone of 40-60 % occurred for *C. rosaceana* after exposure to air-borne concentrations of pheromone ranging from 56 to below 1 ng / ml air (Stelinski et al., 2003 b).

The current study compared the behavioral responses of *C. rosaceana* and *A. velutinana* to high doses of pheromone delivered from rope dispensers in a sustained-flight wind tunnel to determine whether previously documented physiological differences between these species would result in measurable behavioral differences. The specific objectives were to: (1) compare the responses of *C. rosaceana* and *A. velutinana* to rope dispensers of pheromone used for mating disruption and lures used in monitoring traps in a sustained-flight wind tunnel and (2) determine whether/how brief pre-exposures to high-dose pheromone releasers modulate subsequent responses to those releasers 15 min and 24 h after exposure.

MATERIALS AND METHODS

Insect Colonies

C. rosaceana were drawn from a five-year-old laboratory colony originally collected as 1st and 2nd generation pupae from apple orchards in Southwest Michigan. *A. velutinana* came from a long-established laboratory colony maintained at Geneva, NY by W. Roelofs. Both species were reared at 24°C and 60 % RH on pinto bean-based diet (Shorey and Hale, 1965) under a 16:8 (L:D) photoperiod. Pupae sorted by species and sex

emerged in 1 L plastic cages containing 5 % sucrose in plastic cups with cotton dental wicks protruding from their lids.

Chemicals and Release Devices

The behavioral responses of *C. rosaceana* and *A. velutinana* were quantified in a sustained-flight wind tunnel using two types of pheromone dispensers. First, pheromone blends specific to each species were formulated in red rubber septa (Suterra, Bend, OR). The optimally-attractive lures in experiments with *A. velutinana* were rubber septa loaded with 0.93 mg (*Z*)- and 0.07 mg (*E*)-11-tetradecenyl acetates (93 : 7 ratio of *Z* : *E*) and 2.0 mg dodecyl acetate (Roelofs et al., 1975). For *C. rosaceana*, rubber septa were loaded with 0.485 mg of (*Z*)- and 0.015mg (*E*)-11-tetradecenyl acetates (92.2 : 3.0 ratio of *Z* : *E*) and 0.026 mg of (*Z*)-11-tetradecenol (Hill and Roelofs 1979). Pheromone blend solutions used to load rubber septa were prepared in HPLC grade hexane and stored at -18 ° C. Henceforth, such rubber septum dispensers formulated specifically for each species will be referred to as 'lures'. Second, we assayed the responses of both moth species to Isomate OBLR/PLR Plus ropes (Pacific Biocontrol Co., Vancouver, WA) containing 274 mg of 93.4 % (*Z*)-11-tetradecenyl acetate, 5.1 % (*E*)-11-tetradecenyl acetate, and 1.5 % (*Z*)-9-tetradecenyl acetate. Such pheromone release devices are currently the dominant method of dispensing pheromone for mating disruption of leafroller pests in commercial orchards (Nagata, 1989; Agnello et al., 1996; Knight et al., 1998; Knight and Turner, 1999). All dispensers were aged for 2 wk in a laboratory fume hood prior to use in behavioral assays to allow dissipation of pheromone that might have built up on their surfaces during shipping and freezer storage.

Wind Tunnel General Description

Behavioral assays with male *C. rosaceana* and *A. velutinana* adults were conducted in a recently constructed Plexiglas sustained-flight wind tunnel patterned after that of Miller and Roelofs (1978). The rectangular wind tunnel measured 1.3 x 0.8 m in cross section and 2.4 m long. It was housed in a temperature-controlled room maintained at 50-70 % RH and 15-16 ° C to stimulate moth behavioral responsiveness to pheromone under light (Cardé et al., 1975) and to simulate typical night-time summer temperatures in Michigan. Light intensity was 1800-2000 lux inside of the tunnel and was generated by 8 fluorescent bulbs (Philips model F96T12, 95 Watt) mounted 22 cm above the top of the wind tunnel. A variable speed, blower-type fan (Dayton 5C090C, Northbrook, IL) pushed air through the tunnel at 0.3 m/s. The pheromone plume emerging from the tunnel was expelled from the building through a roof-mounted stack. The upwind fan pushed air first through both a Vari-flow II filter for fine, particulate matter and a Vari-Pure high capacity, activated carbon filter; both filters were obtained from Airguard (Louisville, KY). Finally, air was pushed through a 1.3 x 0.8 x 0.2-m hardwood frame attached tightly to the upwind end of the tunnel and enclosed with cloth dampening screens (20 mesh/cm) stretched tightly across each opening. The down-wind end of the tunnel was enclosed with wire-mesh screen.

A variable-speed continuous belt painted with alternating orange and white 0.15 m-wide transverse stripes was installed below the tunnel floor. The belt was driven in the same direction as the wind by an electric motor in experiments requiring sustained flights. Belt speed could be regulated by a rheostat that modulated voltage input into the

motor. During sustained flights (see below), belt speed was adjusted in order to maintain moths near the tunnel's mid-point.

Wind Tunnel Assay Procedures

Male moths, 1-4 days old, were collected during the last 0.5 h of the photophase and placed into cylindrical (17 cm long x 8 cm diam.) wire-mesh release cages. The cages containing 1 or 5 moths (depending on experiment) were placed into the wind tunnel for 1 h of acclimation prior to assays. Subsequently, bioassays ran for a maximum of 1.5 h terminating at 2 h into the moths' normal scotophase. At the upwind end of the tunnel, pheromone dispensers (lures or ropes) were placed 1 cm above a horizontal 7.5 x 12.5 cm yellow card attached to a horizontally-clamped 9 cm glass rod attached to a steel ring-stand. Pheromone was released 25 cm above the tunnel floor in stationary-floor experiments and 10 cm above the floor in moving-floor experiments. Wire-mesh release cages holding 5 male moths of a given species were placed at the down-wind end of the tunnel at a height matching that of the pheromone dispenser. In sustained-flight experiments, release cages contained only one male moth.

Males were allowed 3 min to respond to an inserted pheromone dispenser in assays where the floor was held stationary and 2 min in sustained-flight assays employing the moving floor. The behaviors recorded were: wing-fanning; non-anemotactic flight from the release cage; upwind anemotactic flight without touching the release device; upwind anemotactic flight followed by landing on the platform and touching the release device. Also, the numbers of individuals exhibiting no detectable behavioral change were recorded.

Release cages, ring-stands, and glass rods were thoroughly washed with acetone after daily use. The interior of the wind tunnel was also briefly scrubbed with an acetone-soaked rag and immediately rinsed with water so as not to damage the plexiglas. The exhaust fan ran for at least 4 h after assays were completed.

Experiment 1.

This experiment tested the effect of brief exposures of *A. velutinana* and *C. rosaceana* to pheromone plumes generated either by lures or rope dispensers on subsequent responsiveness of male moths to these pheromone sources either 15 min or 24 h after the initial exposure treatment. We predicted that *A. velutinana* would be more adversely affected by the exposure than *C. rosaceana* given previously published reports that mating disruption is easier to achieve for *A. velutinana* than *C. rosaceana* (Stelinski et al., 2003 a and references within). Groups of 5 male moths of each species were released in plumes generated by: 1) a rubber septum described above (standard lure used for monitoring each species); 2) a rope (standard pheromone dispenser used in mating disruption); and 3) a combination of a species-specific lure with a rope placed 5 cm directly above it. Moths were allowed 3 min to respond during all pre-exposure treatments. Moths reaching or orienting to the pheromone source but not contacting it were segregated from those that either did nothing, wing-fanned, or flew out of release cages without orienting. Recaptured moths (both 'orienters' and 'non-orienters') were assayed in the wind tunnel again to each pheromone source either 15 min or 24 h after the initial pheromone pre-exposure. Moths tested 24 h after the pre-exposure treatment were kept in an environmental chamber under the temperature and light-cycle conditions described above for the interval prior to testing. The treatments were: 1) pre-exposure to

a lure followed by wind tunnel assay using a lure; 2) pre-exposure to a rope followed by wind tunnel assay using a lure; 3) pre-exposure to lure and rope combination followed by wind tunnel assay using a lure; 4) pre-exposure to a rope followed by wind tunnel assay using a rope; 5) pre-exposure to clean air followed by wind tunnel assay using a lure. 'Naïve' will refer to moths having no prior exposure to pheromone or the wind tunnel prior to assay. 'Control' will refer to moths pre-exposed to moving air, but no pheromone in the wind tunnel. 'Pre-exposed' will refer to moths pre-exposed to pheromone in the wind tunnel. This pheromone may have emanated from a lure, a rope, or a lure and rope combination.

To avoid bias due to possible slight variations between days, all treatment combinations were tested each day of testing. Treatment order was also randomized daily to equalize any effect of time after what would have been the onset of normal scotophase.

Experiment 2.

This experiment tested the effect of brief exposures of *A. velutinana* and *C. rosaceana* to pheromone plumes generated by high-release rope dispensers on the duration of sustained flights of male moths 24 h after initial exposure. Individual male moths of each species were released in plumes generated by a rope dispenser. As in Experiment 1, moths reaching the pheromone source or orienting to the source but not contacting it were segregated from those that either did nothing, wing-fanned only, or flew out of release cages without orienting to the pheromone. Recaptured moths were then placed in plumes generated by lures 24 h after the initial pheromone exposure. After moths locked onto the pheromone plume generated by a lure, the moving floor was

activated to prolong flights. We recorded both the time it took moths to exit cages and to lock onto the plume, as well as the duration of sustained flight in the plume.

Electroantennogram Assays (Experiment 3)

This experiment tested the hypothesis that briefly exposing *A. velutinana* and *C. rosaceana* to pheromone plumes generated by high-release rope dispensers affected EAG responses of moths 15 min or 24 h after initial exposure. The EAG system and test protocols were detailed by Stelinski et al. (2003 a). EAG cartridges were made by pipetting various concentrations (2 μg – 2 mg) of pheromone (96.1 % (*Z*)-11-tetradecenyl acetate and 3.9% (*E*)-11-tetradecenyl acetate as determined by gas chomatography) in hexane (20 μL total solution) onto 1.4 x 0.5 cm strips of Whatman No. 1 filter paper. After 5 min in a fume hood for solvent evaporation, treated strips were inserted into disposable glass Pasteur pipettes, sealed with Parafilm, and allowed to equilibrate for 24 h prior to use. Pheromone dosages were delivered alternately to both naïve and pheromone pre-exposed moths (15 min or 24 h after initial exposure) in ascending order of dosage ($N = 25$ per treatment). Four 1-ml puffs spaced 12 s apart were administered to each antenna at each dosage.

Statistical Analyses

For experiments 1 and 2, a logistic model was used to measure the probability that a combination of the thee factors: pheromone delivery device (rubber septum or rope) x moth type (naïve, pre-exposed orienter, or pre-exposed non-orienter) x species (*C. rosaceana* or *A. velutinana*) would result in a particular behavioral category as defined previously using the PROC GENMOD procedure in SAS (SAS Institute, 2000). Subsequently, analyses of numbers of male moths responding were carried out using the

G statistic (Sokal and Rolf, 1981). Proportions of moths responding within each behavioral category were compared both within each individual species under study (Tables 1 and 2) and between species (Table 3). In addition, for Experiment 2, data for sustained-flight duration and elapsed time to leave release cages were transformed to ln (x + 1) (which normalized the distributions) and then subjected to ANOVA; differences in pairs of means were separated using Tukey's multiple comparisons test (SAS Institute, 2000). For Experiment 3, data were subjected to ANOVA and differences in pairs of means were separated using Tukey's multiple comparisons test (SAS Institute, 2000). In all cases, the significance level was $\alpha < 0.05$.

RESULTS

Experiment 1. Responses of C. rosaceana.

Compared to naïve or control (Air then Lure) males of the same age, significantly more *C. rosaceana* males contacted their respective lure 24 h after brief pre-exposure to pheromone plumes generated by a rope dispenser (Table 2). This result occurred irrespective of whether or not male *C. rosaceana* oriented to the rope during the pre-exposure treatment. Compared to naïve or control males, significantly more male *C. rosaceana* oriented to lures without contacting the source 24 h after brief pre-exposure to a lure, a rope, or a lure-rope combination (Table 2). Again, this result generally held for both males that oriented during the pre-exposure procedure and those that did not. The only exception was for pre-exposed non-orienters after brief exposure to a rope. Nearly 100 % of pre-exposed *C. rosaceana* locked onto plumes (oriented with or without source contact) generated by lures 24 h after pre-exposure compared to 58 or 60 % for control or naïve moth, respectively. Significantly fewer male *C. rosaceana* oriented (with or

without source contact) to ropes compared to lures before and 24 h after pre-exposure to such ropes (Table 2). If they did not orient to those dispensers during the pre-exposure treatment, significantly fewer male *C. rosaceana* contacted lures 15 min after pre-exposure to a lure, rope, or the lure-rope combination (Table 2). Statistically equal numbers of naïve and control male *C. rosaceana* contacted or oriented to lures (Table 2).

Responses of A. velutinana.

Of male *A. velutinana* orienting to the pheromone sources during pre-exposure treatments, significantly fewer contacted the lure compared to naïve moths 15 min after pre-exposure to the lure-rope combination and 24 h after pre-exposure to the rope and lure-rope combination (Table 3). Of those male *A. velutinana* not orienting during the pre-exposure treatment, significantly fewer contacted the lure compared to naïve moths after pre-exposure to the lure, rope, and lure-rope combination 15 min and 24 h after pre-exposure (Table 3). A significantly greater proportion of male *A. velutinana* contacted ropes 24 h after orienting to ropes compared to the proportion of naïve moth orienting to a lure, rope, or lure-rope combination (Table 3).

Table 2. Response of male *Choristoneura rosaceana* to lures or ropes 15 min or 24 hr after pre-exposure to either clean air, a lure, rope, or lure-rope combination.

Moth type and Pheromone dispenser	*N*	Proportion of males exhibiting the indicated response[a]		
		No behavioral change	Orientation without source contact	Source contact
Naïve males				
Lure	107	0.14bc[b]	0.30c	0.30bc
Rope	110	0.23bc	0.25cd	0.04d
L + R[c]	123	0.13bcd	0.42b	0.04d
Pre-exposed orienters (15 min)				
Lure then Lure	45	0.10cd	0.38bc	0.18c
Rope then Lure	25	0.33a	0.25cd	0.25c
L + R then Lure	39	0.19bc	0.38bc	0.00d
Pre-exposed non-orienters (15 min)				
Lure then Lure	25	0.26ab	0.39bc	0.00d
Rope then Lure	48	0.28ab	0.26c	0.09d
L + R then Lure	44	0.33a	0.31c	0.10d
Pre-exposed orienters (24 hr)				
Lure then Lure	32	0.00d	0.52a	0.48ab
Rope then Lure	25	0.00d	0.46ab	0.54a
L + R then lure	38	0.00d	0.57a	0.43abc
Rope then Rope	27	0.37a	0.22cd	0.00d
Pre-exposed non-orienters (24 hr)				
Lure then Lure	29	0.00d	0.55a	0.31bc
Rope then Lure	41	0.00d	0.37bc	0.59a
L + R then Lure	31	0.03d	0.42b	0.45ab
Rope then Rope	38	0.39a	0.13d	0.00d
Air then Lure[c]	61	0.15bc	0.25cd	0.33bc

[a]Proportions of moths wing-fanning only or flying out without anemotactic orientation not shown.

[b]Numbers in the same column followed by the same letter are not significantly different (G^2 test of homogeneity, $P = 0.05$). [c]'L + R' stands for 'Lure + Rope.' [d]Refers to control treatment.

However, a statistically equal proportion of male *A. velutinana* contacted ropes 24 h after pre-exposure if they did not orient during the pre-exposure treatment compared to naïve moths (Table 3). Of the male *A. velutinana* orienting to pheromone sources during pre-exposure, statistically equal proportions oriented (without source contact) to lures 15 min and 24 h after orienting to a lure, rope, or lure-rope combination compared with naïve moths. Of those male *A. velutinana* not orienting during the pre-exposure treatment to pheromone plumes, significantly fewer oriented (without source contact) to lures 15 min after pre-exposure to a rope or lure-rope combination and significantly fewer oriented to lures 24 h after pre-exposure to the lure-rope combination compared to naïve moths (Table 3). Statistically equal numbers of naïve and control male *A. velutinana* contacted or oriented to lures (Table 3).

Comparison of responses between species.

When comparing responses between species, statistically equal proportions of naïve males of both species either contacted or oriented without source contact to their respective lures (Table 4). Compared to naïve *A. velutinana*, significantly fewer naïve *C. rosaceana* contacted ropes; however, statistically equal proportions of both species oriented to ropes without source contact. Compared to pre-exposed *A. velutinana*, a significantly greater proportion of *C. rosaceana* contacted lures 24 h after pre-exposure to a lure, rope or lure-rope combination, irrespective of whether or not they oriented during pre-exposure (Table 4). Compared to pre-exposed *C. rosaceana,* significantly more *A. velutinana* contacted ropes or oriented to ropes without source contact 24 h after orienting to ropes; no *C. rosaceana* contacted ropes 24 h after pre-exposure to ropes.

Table 3. Response of male *Argyrotaenia velutinana* to lures or ropes 15 min or 24 hr after pre-exposure to either clean air, a lure, rope, or lure-rope combination.

Moth type and Pheromone dispenser	*N*	Proportion of males exhibiting the indicated response[a]		
		No behavioral change	Orientation without source contact	Source contact
Naïve males				
Lure	127	0.16cd[b]	0.35ab	0.24b
Rope	135	0.19c	0.42a	0.14cd
L + R[c]	123	0.10d	0.44a	0.23b
Pre-exposed orienters (15 min)				
Lure then Lure	38	0.05d	0.42a	0.16bcd
Rope then Lure	29	0.31b	0.38ab	0.17bc
L + R then Lure	28	0.21bc	0.50a	0.04de
Pre-exposed non-orienters (15 min)				
Lure then Lure	25	0.47ab	0.26bc	0.00e
Rope then Lure	25	0.63a	0.16c	0.11d
L + R then Lure	28	0.55ab	0.09d	0.05de
Pre-exposed orienters (24 hr)				
Lure then Lure	42	0.07d	0.45a	0.17bc
Rope then Lure	40	0.18c	0.43a	0.13cd
L + R then lure	47	0.32b	0.49a	0.11d
Rope then Rope	35	0.03d	0.46a	0.40a
Pre-exposed non-orienters (24 hr)				
Lure then Lure	34	0.24bc	0.38ab	0.03e
Rope then Lure	34	0.26bc	0.47a	0.03e
L + R then Lure	24	0.79a	0.07d	0.00e
Rope then Rope	29	0.52ab	0.10d	0.07de
Air then Lure[d]	53	0.15cd	0.28bc	0.34ab

[a]Proportions of moths wing-fanning only or flying out without anemotactic orientation not shown.

[b]Numbers in the same column followed by the same letter are not significantly different (G^2 test of homogeneity, $P = 0.05$). [c]'L + R' stands for 'Lure + Rope.' [d]Refers to control treatment.

Table 4. Comparison of the response of *Choristoneura rosaceana* vs. *Argyrotaenia velutinana* males to lures or ropes 24 hr after pre-exposure to either clean air, a lure, rope, or lure-rope combination.

Moth type and Pheromone dispenser	*N*	Proportion of males exhibiting the indicated response[a]		
		No behavioral change	Orientation without source contact	Source contact
C. rosaceana				
Naïve males				
Lure	107	0.14de[b]	0.30c	0.30c
Rope	110	0.23cd	0.25cd	0.04e
L + R[c]	123	0.13de	0.42bc	0.04e
Pre-exposed orienters (24 hr)				
Lure then Lure	32	0.00e	0.52a	0.48a
Rope then Lure	25	0.00e	0.46b	0.54a
L + R then lure	38	0.00e	0.57a	0.43ab
Rope then Rope	27	0.37bc	0.22d	0.00e
Pre-exposed non-orienters (24 hr)				
Lure then Lure	29	0.00e	0.55a	0.31c
Rope then Lure	41	0.00e	0.37c	0.59a
L + R then Lure	31	0.03e	0.42bc	0.45ab
Rope then Rope	38	0.39b	0.13de	0.00e
Air then Lure	61	0.15bc	0.25cd	0.33bc
A. velutinana				
Naïve males				
Lure	127	0.16d	0.35c	0.24cd
Rope	135	0.19d	0.42bc	0.14d
L + R[a]	123	0.10e	0.44b	0.23cd
Pre-exposed orienters (24 hr)				
Lure then Lure	42	0.07e	0.45b	0.17d
Rope then Lure	40	0.18d	0.43b	0.13de
L + R then Lure	47	0.32bc	0.49ab	0.11e
Rope then Rope	35	0.03e	0.46b	0.40b
Pre-exposed non-orienters (24 hr)				
Lure then Lure	34	0.24c	0.38bc	0.03e
Rope then Lure	34	0.26c	0.47b	0.03e
L + R then Lure	24	0.79a	0.07e	0.00e
Rope then Rope	29	0.52b	0.10e	0.07e
Air then Lure[c]	53	0.15cd	0.28c	0.34abc

[a]Proportions of moths wing-fanning only or flying out without anemotactic orientation not shown. [b]Numbers in the same column followed by the same letter are not significantly different (G^2 test of homogeneity, $P = 0.05$). [c]'L + R' stands for 'Lure + Rope.' [d]Refers to control treatment.

Statistically equal proportions of clean-air pre-exposed (control) moths of both species contacted lures or oriented to lures without making contact 24 h after pre-exposure (Table 4).

Experiment 2

Provided they had been pre-exposed to ropes 24 h earlier, a significantly greater proportion of male *C. rosaceana* oriented to plumes generated by lures, irrespective of whether or not they oriented during pre-exposure compared to naïve males of the same age (Fig. 19 A). Male *C. rosaceana* pre-exposed to ropes and not orienting during pre-exposure flew to lures for a significantly longer period than male *C. rosaceana* having oriented to ropes during pre-exposure 24 h earlier (Fig. 19 B). All rope pre-exposed male *C. rosaceana* (whether they oriented or not 24 h earlier) sustained flights to lures significantly longer than did naïve moths of the same age (Fig. 19 B). There were no significant differences in the time required by pre-exposed and naïve male *C. rosaceana* to leave the release cages (Fig 19 B).

A significantly greater proportion of naïve male *A. velutinana* oriented upwind toward plumes generated by lures compared to those pre-exposed to ropes 24 h earlier (Fig. 19 C). Naïve male *A. velutinana* sustained flights to lures significantly longer than did moths of the same age that had been pre-exposed to rope plumes 24 h earlier (Fig. 19 D). There were no significant differences in the time required to leave the release cage between pre-exposed and naïve male *A. velutinana* (Fig 19 D).

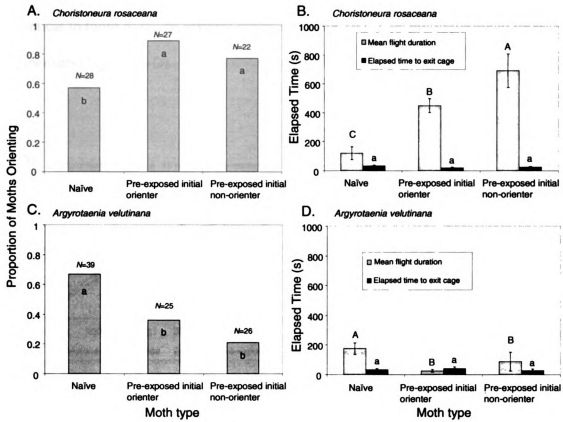


Figure. 19. A. Proportion of naïve and pheromone-rope pre-exposed *Choristoneura rosaceana* males that locked onto and oriented to lures in sustained-flight wind tunnel with moving floor 24 h after pre-exposure. B. Duration of sustained-flights of naïve and pheromone-rope pre-exposed *C. rosaceana* 24 h after pre-exposure. C. Proportion of naïve and pheromone-rope pre-exposed *Argyrotaenia velutinana* males that locked onto and oriented to lures in sustained-flight wind tunnel with moving floor 24 h after pre-exposure. D. Duration of sustained-flights of naïve and pheromone-rope pre-exposed *A. velutinana* 24 h after pre-exposure. Means within a panel followed by the same letter and case of letter are not significantly different at $\alpha < 0.05$.

Experiment 3

Mean EAG responses of naïve male *C. rosaceana* and *A. velutinana* were identical to those of moths assayed either 15 min or 24 h after pre-exposure to rope dispensers (Fig. 20 A-D).

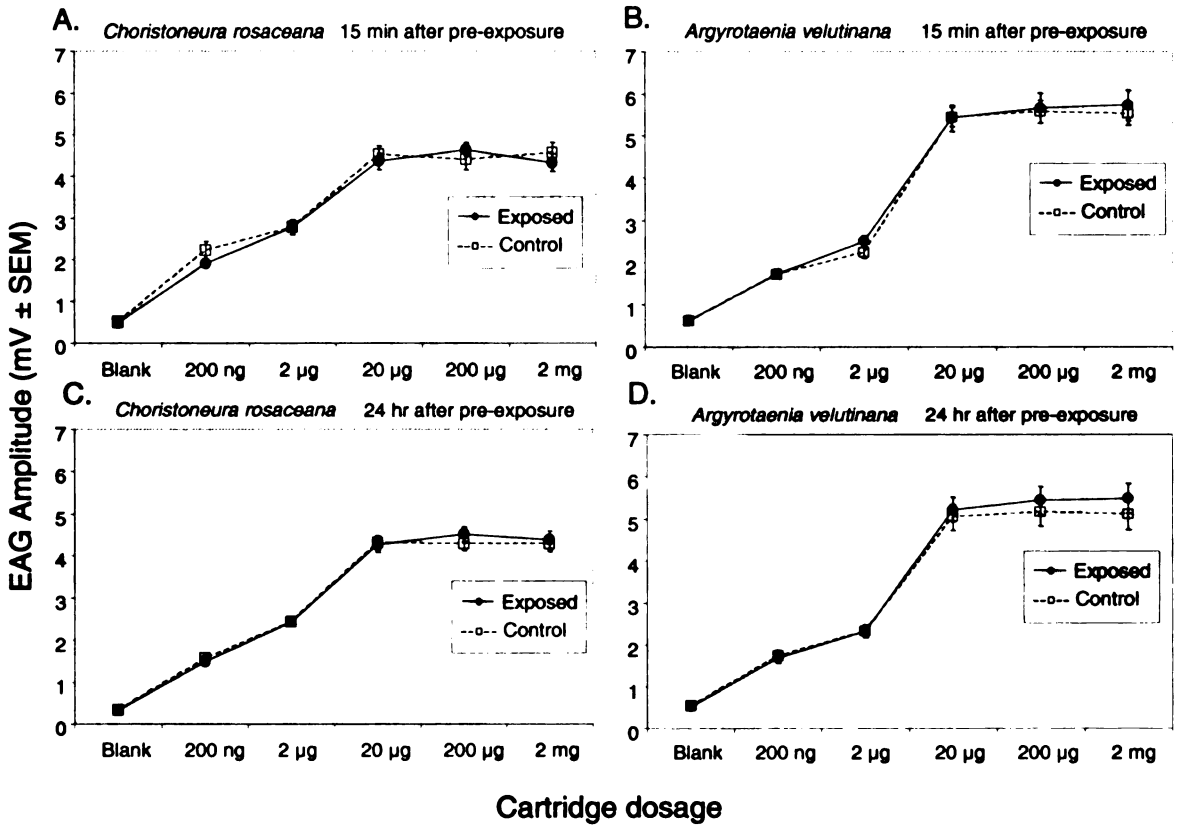


Figure 20. EAG dosage-response relationships for naïve and pheromone-rope pre-exposed *Choristoneura rosaceana* (A. 15 min, C. 24 h after pre-exposure) and *Argyrotaenia velutinana* (B. 15 min, D. 24 h after pre-exposure) using live-insect antennal preparations.

DISCUSSION

Brief pre-exposures of male *C. rosaceana* and *A. velutinana* to plumes arising from rubber septa releasing pheromone blends specifically tuned for each species or by a commercial rope dispenser targeting both species induced markedly different effects on subsequent behavioral responses to pheromone. The clearest behavioral differences between the two species came 24 h after the pre-exposure when *C. rosaceana*'s propensity for orienting toward lures containing a 3-component pheromone blend tuned for that species was heightened after brief pre-exposure to a lure, a rope dispenser, or a combination of a lure and rope. Under these conditions a greater proportion of *C. rosaceana* males successfully locked onto and progressed toward lures. Moreover, durations of sustained flights to lures were substantially prolonged relative to those of naïve, unexposed moths of the same age. But, such pre-exposed male *C. rosaceana* were not more likely to fly toward ropes 24 h after pre-exposure to ropes (Table 1). By contrast, pre-exposed *A. velutinana*'s propensity for locking onto pheromone plumes generated by lures emitting a 3-component pheromone blend tuned for that species was greatly reduced 24 h after pre-exposure to a lure, a rope, or the lure-rope combination. Duration of anemotactic flights was also much reduced. This decreased propensity for orientation to lures was most dramatic in those individuals not orienting during the pre-exposure treatment. However, the proportion of male *A. velutinana* that contacted ropes 24 h after initially orienting to ropes was larger than that for naïve moths (Table 2). Overall, nearly identical proportions of naïve male *C. rosaceana* and *A. velutinana* locked onto and progressed toward their respective lures; mean durations of sustained flights of naïve moths of the two species toward their respective lures were also nearly

identical (Fig. 19 B, D). Thus, naïve moths of both species behaved similarly in plumes of their respective lures; but, their behaviors diverged considerably 24 h after pheromone pre-exposure.

Numerous studies have investigated the effects of short and prolonged pre-exposures of moths to their species-specific synthetic pheromones and geometric isomers (Bartell and Roelofs, 1973; Bartell and Lawrence, 1976 a, b, c; Linn and Roelofs, 1981; Sanders, 1985; Evenden et. al., 2000); however, few have focused on long-term effects of pheromone pre-exposure on subsequent behavioral responses (Figuerdo and Baker, 1992; Anderson et., al. 2003). Figuerdo and Baker (1992) observed significantly decreased behavioral responses of male *G. molesta* to rubber septa releasing specifically tuned, multi-component pheromone blends day(s) after pre-exposure to such septa. In contrast, Anderson et al. (2003) documented increased responsiveness of *S. littoralis* to female pheromone gland extracts and synthetically prepared lures 27 h after brief pre-exposure to female-produced plumes. A similar response, although to off-blends, occurred in *C. fumiferana* after pheromone pre-exposure (Sanders, 1984; 1995).

Other recent investigations have focused on short-term intervals after pre-exposure of moths to pheromone. For example, Evenden et al. (2000) found no change in the ability for female plume-following by Western *C. rosaceana* males pre-exposed to pheromone for 1 h and then assayed in a wind tunnel 10-30 min after exposure. This short interval between exposure and assay chosen by Evenden et al. (2000) may not have permitted detection of the neurophysiological effects of pheromone pre-exposure uncovered by the current study, demonstrated by waiting 24 h to assay effects of

pheromone pre-exposure, as well as using a sustained-flight tunnel to quantify durations of anemotactic flights.

The neurophysiological changes induced in both leafroller species by pheromone pre-exposure likely occurred in the CNS. No changes were detected at the periphery at both 15 min and 24 h after pre-exposure as evidenced by EAGs (Fig. 20 A-D). For *A. velutinana*, the long-lasting effect of decreased attractiveness of a lure (lower-release and more complete blend pheromone source) and increased attractiveness of a rope (higher-release and less complete blend pheromone source) is consistent with an increase in response threshold, requiring a higher concentration of stimulus to elicit normal responses.

For *C. rosaceana*, these data suggest a decreased response threshold perhaps combined with an increased ability for pheromone blend discrimination given the greater attractiveness of a lower-release, natural blend lure. Serotonin and octopamine enhance male responsiveness to pheromone and modulate the activity of neurons both in the CNS (Linn et al., 1992; Linn, 1997) and the peripheral nervous system (Pophof, 2000; 2002; Stelinski et al., 2003 c). Alternatively, the behavioral changes documented herein may have resulted from some type of learning, similar to associative or aversive learning of *Drosophila* traceable to the mushroom bodies (Bell and Heisenberg, 1994; Yin et al., 1994).

Our wind-tunnel data suggest that the induced behavioral modifications may have involved more than just changes in response thresholds. The propensity of pre-exposed *C. rosaceana* to contact lures had also increased as evidenced by the longer sustained-flight durations relative to naïve individuals. In addition, those male *C. rosaceana* not orienting

during pre-exposure flew significantly longer than did those that oriented during pre-exposure (Fig. 19 C). Perhaps initial non-orienters were exposed to more total pheromone than were their associated orienters. During pre-exposure, orienters usually left the release cage within 30 s and spent only *ca.* 10-30 s orienting to the source in a stationary-floor tunnel before landing on the tunnel floor or walls. In contrast, the majority of non-orienters spent a full 3 min in the release cages placed directly within the plume of pheromone. For *C. rosaceana*, longer duration of pre-exposure was positively correlated with longer sustained flights to lures 24 h after pre-exposure to ropes.

It is doubtful that the previously documented difference between *C. rosaceana* and *A. velutinana* in the duration of peripheral adaptation after prolonged exposure to pheromone (Stelinski et al., 2003 a) influenced the behaviors documented in this study. We had postulated that long-lasting peripheral adaptation of *C. rosaceana* might shield the CNS in this species during prolonged exposures to pheromone, while the short-lived peripheral adaptation of *A. velutinana* might permit marked impact of pheromone exposure on the CNS (Stelinski et al., 2003 a, b). The current study did not support such a difference. Rather, both species revealed effects at the level of the CNS, albeit differently. The relatively brief and discontinuous pheromone pre-exposure in the current study did not induce long-lasting adaptation in *C. rosaceana* as measured by EAGs (methods as above; $N = 10$ moths; data not shown).

Could this finding that a pre-exposure to pheromone can in some cases reduce and in other cases enhance subsequent responses to pheromone plumes explain some of the variation observed across moth species in their susceptibility to mating disruption? Western *C. rosaceana* are reported to be successfully controlled by mating disruption

(Evenden et al., 1999 a, b). In contrast, eastern and midwestern populations of this pest are thought to be relatively difficult to control via mating disruption, perhaps requiring near-true blend formulations of pheromone for successful disruption (Novak et al., 1978; Novak and Roelofs, 1985; Lawson et al., 1996; Miller et. al., unpublished). It is intriguing that pheromone pre-exposure caused Michigan *C. rosaceana* to improve at locking onto and progressing toward a lower release-rate and more complete blend pheromone emitter. The converse result was documented for *A. velutinana*, a species easily disrupted with only the main component of its pheromone blend (Novak et al., 1978; Reissig et al., 1978; Novak and Roelofs, 1985). Thus, the behavioral evidence uncovered in the current experiments seems sufficient to explain why *C. rosaceana* is more difficult to disrupt than *A. velutinana* and why a more complete blend of disruptant may be necessary to achieve success for the former species but not the latter. One day after pheromone pre-exposure, a lower-release and more complete blend lure was more attractive to *C. rosaceana*, while the converse was true for *A. velutinana*.

After having completed this study, we learned that the Isomate OBLR/PLR Plus rope dispensers contained a small, undeclared amount (1.5 %) of (*Z*)-9-tetradecenyl acetate. Evenden et al. (1999 c) showed that addition of > than 1.0 % of (*Z*)-9-tetradecenyl acetate to 100 μg of the 4-component blend (Vakenti et al., 1988), formulated in a rubber septum lures, reduced the responsiveness of *C. rosaceana* compared with lures containing the pheromone alone. Therefore, the existence of this so-called "antagonist" in Isomate OBLR/PLR Plus rope dispensers may explain why responsiveness of naïve and pheromone-exposed *C. rosaceana* was lower than that of *A. velutinana*. If false-plume-following is an important mechanism mediating mating

disruption of *C. rosaceana*, then perhaps the presence of this antagonist renders these dispensers less attractive to *C. rosaceana* and therefore less effective. Further work is needed to determine whether this component alone played a significant role in increasing responsiveness of *C. rosaceana* after pre-exposure.

Of the numerous published studies on the behavioral responses of moths to pheromone after pheromone pre-exposure, only a few have specifically focused on high-dosage dispenser technologies like ropes (Cardé et al., 1997; 1998), currently the predominant dispenser for mating disruption. The explanations arising from most laboratory investigations of mating disruption are extrapolations from tests using low-release pheromone dispensers. Also, many have focused only on effects of minutes-long pheromone pre-exposure. Our results suggest that long-term effects of pheromone pre-exposure may be important and that commercially available pheromone ropes induce such changes. A critical question is whether leafrollers actually approach such rope dispensers under mating disruption in the field and remain in their vicinities sufficiently long to induce such behavioral modifications. We have gathered extensive field data that both *C. rosaceana* and *A. velutinana* do approach rope dispensers identical to those used in the current laboratory study, under standard application densities, and that behavioral responses to ropes in the field are very similar to those seen in the wind tunnel (Stelinski et al., 2004a). More attention needs to be focused on the sequence of events as these moths interact with pheromone dispensers in the field. The finding that appreciable numbers of moths can be observed approaching rope dispensers both in the laboratory and the field (Stelinski et al., 2004a,b) suggests that these experimental approaches are tractable. Moreover, these findings add to the growing body of evidence that false-plume-

following is likely to be a major explanatory mechanism of pheromone disruption using rope dispensers.

Finally, the current data suggest that using a common rope dispenser to disrupt communication of both of these leafrollers may not be the optimal control tactic. The Isomate OBLR/PLR Plus pheromone rope dispenser appears to be well-suited to disrupting sexual communication of *A. velutinana* but not for *C. rosaceana*. Our data suggest a better tactic for *C. rosaceana* would be to use a dispenser releasing a lowered rate of a blend closely matching the natural pheromone of this species. We are hopeful that efficacy for *C. rosaceana* can be optimized while maintaining acceptable cost.

CHAPTER FIVE

Captures of two leafroller moth species in traps baited with varying dosages of pheromone lures or commercial mating-disruption dispensers in untreated and pheromone-treated orchard plots

Also published as:

Stelinski, L. L., J. R. Miller, and L. J. Gut. 2005. Captures of two leafroller moth species in traps baited with varying dosages of pheromone lures or commercial mating-disruption dispensers in untreated and pheromone-treated orchard plots. *The Canadian Entomologist.* Accepted for publication 14, November, 2004.

ABSTRACT

A 2-yr study conducted in 0.6 ha apple blocks examined the effects of treatment with pheromone rope dispensers on catches of the obliquebanded leafroller, *Choristoneura rosaceana* (Harris), and the redbanded leafroller, *Argyrotaenia velutinana* (Walker), in traps baited with varying dosages of pheromone lures or Isomate OBLR/PLR Plus pheromone rope dispensers. In untreated blocks, captures of male *A. velutinana* were high and did not differ among: 1) traps baited with a standard lure loading used to monitor this pest, 2) lure loadings 10 and 100 times the standard loading, and 3) traps baited with an Isomate OBLR/PLR Plus pheromone rope dispenser. In pheromone-treated blocks, captures of *A. velutinana* in traps were reduced 94 - 99 % for all loading dosages tested (up to 1000 times the standard loading). The results for *C. rosaceana* were different. In untreated blocks, traps baited with 10 or 30 standard lures captured significantly more *C. rosaceana* in 2002 than traps baited with a single standard lure; but, traps baited with the standard lure loading captured significantly more moths than traps baited with 100 and 1000 times the standard loading in 2003. Also, traps baited with Isomate OBLR/PLR Plus pheromone dispensers captured significantly fewer *C. rosaceana* than traps with standard lures in untreated blocks. In pheromone-treated blocks, traps baited with standard monitoring lures and lures with higher loadings (10 and 1000 times the standard) captured equivalent numbers of *C. rosaceana*; the capture of moths was reduced only between 50 and 71 %. We conclude that Isomate OBLR/PLR Plus pheromone ropes deployed in Michigan, USA are effective in disrupting orientation of *A. velutinana*; however, they are not very effective for *C. rosaceana*. In addition, increasing lure loading dosage above that of 1x monitoring lures (rubber septa or

membrane-type) does not appear to reliably increase monitoring effectiveness of males of either leafroller species in orchards where pheromone ropes are deployed at recommended densities.

INTRODUCTION

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is native to North America and is widely distributed from British Columbia to Nova Scotia and south to Florida (Chapman et al., 1968). *C. rosaceana* has an extremely wide host range; however, the preferred hosts are woody plants including Rosaceae. *C. rosaceana* is an important pest of pome fruits in North America. The redbanded leafroller, *Argyrotaenia velutinana* (Walker) (Lepidoptera: Tortricidae), is sympatric with *C. rosaceana* and native to temperate Eastern North America (Chapman, 1973). This species also has a broad host range. It feeds on leaves of diverse plant species except conifers. The larvae feed on many unrelated plants, including most common fruits, vegetables, weeds, flowers, ornamentals and shrubs (Hull et al., 1995). Among the fruits, *A. velutinana* prefers apples and commonly occurs in the apple-growing areas of the Midwestern and Eastern United States and Eastern and Western Canada (Howitt, 1993; Hull et al., 1995).

C. rosaceana and *A. velutinana* share the major components of their pheromone blends; (*Z*)11-14:Ac and (*E*)11-14:Ac in a 98:2 ratio for *C. rosaceana* and 93:7 ratio for *A. velutinana* (Roelofs and Arn, 1968; Roelofs and Tette, 1970; Roelofs et al., 1975; Cardé and Roelofs, 1977; Hill and Roelofs, 1979). Efforts to disrupt mating of *A. velutinana* using synthetic pheromones to achieve crop protection have been judged successful (Novak et al., 1978; Roelofs and Novak, 1981; Cardé and Minks, 1995). In

contrast, lower success of mating disruption has been documented for *C. rosaceana* in New York using single-pheromone-component dispensers (Reissig et al., 1978) as well as more complex 3-component blends (Agnello et al., 1996; Lawson et al., 1996).

We continue to use these two sympatric leafroller species as a study system to investigate the reasons underlying differences in susceptibility to mating disruption between these moth species. Recent studies with leafroller moths revealed species-specific expression and duration of long-lasting peripheral adaptation following exposure to pheromone dispensers used in mating disruption (Stelinski et al., 2003a, b). This adaptation was expressed in *C. rosaceana* caged within a few cm of pheromone dispensers but was not present in *A. velutinana.* Furthermore, in behavioural studies in a flight tunnel, *C. rosaceana* became more responsive to lower-release lures containing an attractive synthetic blend of pheromone components 24 hr after brief exposure to pheromone, while *A. velutinana* became more responsive to high-release, off-blend rope dispensers 24 hr after brief exposure to pheromone (Stelinski et al., 2004a).

The objectives of the current study were to: 1) determine the effectiveness of Isomate OBLR/PLR rope dispensers for disrupting orientation of *A. velutinana* and *C. rosaceana* to synthetically-baited pheromone traps in Michigan, 2) compare lure-dosage versus moth-capture relationships for both species in pheromone-treated and untreated orchards to identify possible lure loadings that may permit monitoring of male activity of both species in pheromone-treated orchards, and 3) investigate the attractiveness of Isomate OBLR/PLR pheromone rope dispensers deployed in delta-style monitoring traps relative to monitoring lures releasing highly attractive synthetic blends of pheromone.

MATERIALS AND METHODS

General methods for field study

This experiment was carried out at the Trevor Nichols Research Complex (TNRC) of Michigan State University in Fennville, MI during the summers of 2002 and 2003. Experiments were conducted within plots of 12 and 18 year old Rome and Delicious apple trees, respectively, both with *ca.* 2-3 m canopy heights. Trees were planted on a 3 m within- and 6 m between-row spacing. Pheromone plots were established at the beginning of the season using Isomate OBLR/PLR Plus pheromone rope dispensers (Pacific Biocontrol Co., Vancouver, WA) containing 274 mg of 93.4 % (*Z*)-11-tetradecenyl acetate, 5.1 % (*E*)-11-tetradecenyl acetate, and 1.5 % (*Z*)-9-tetradecenyl acetate. Ropes were applied at 500 dispensers per ha.

In 2002, the monitoring lures (1x) used for *A. velutinana* were red rubber septa (The West Company, Linville, PA, USA) loaded with 0.93 mg (*Z*)- and 0.07 mg (*E*)-11-tetradecenyl acetates and 2.0 mg dodecyl acetate (Roelofs et al., 1975; Cardé and Roelofs, 1977). For *C. rosaceana*, identical rubber septa were loaded with 0.485 mg of (*Z*)- and 0.015mg (*E*)-11-tetradecenyl acetates and 0.026 mg of (*Z*)-11-tetradecenol (Hill and Roelofs, 1979). Pheromone blend solutions were prepared in HPLC grade hexane and stored at -18 ° C. All pheromone lures were deployed in Scenturion Guardpost LPD traps (delta-style trap with 20 x 19 cm sticky trapping surface, Suterra, Bend, OR).

2002 tests

The experimental design was a split-plot. Orientational disruption using rope dispensers at the rate described above or no disruption was applied to whole-plots (0.6 ha) at random. Each whole-plot contained five levels of the subplot factor. These were

different loading rates of the optimally attractive blends of each species' pheromone achieved by varying the number of rubber septum lures placed inside traps. There were three replicates of the two whole-plot factors applied to 0.6 ha apple blocks (three treated with pheromone and three not treated). Four sub-samples (traps) containing each of the five subplot levels (lure loadings) were placed within each whole-plot. The subplot factor levels were: 1) unbaited traps (control), 2) traps baited with 1 rubber septum containing 1/10 of a standard (1x) loading dosage described above, 3) traps baited with 1 standard (1x) rubber septum as described above, 4) traps baited with 10 standard (1x) rubber septa, 5) traps baited with 30 standard (1x) rubber septa. Rubber septa were affixed to the roofs of traps by pinning them to the traps' plastic surface. In cases where multiple septa were affixed in traps, septa were tightly clustered (in direct contact) in rows of ten. Neither the area of the sticky trapping surface nor the trap openings were affected by the presence of rubber septa. Although pheromone dispenser surface area differed between traps containing one, ten, and thirty rubber septa, the large-scale structure of pheromones plumes emanating from such traps was likely similar. This is because the surface area of the traps' openings rather than that of the pheromone dispensers within the traps likely determined the large-scale structure of plumes released from traps (Murlis et al. 1992).

All traps were hung in trees *ca.* 1.5-2 m above ground level in the upper 1/3rd of the tree canopy. Species-specific traps within whole-plots were spaced 20 m apart. Five traps were placed per 120 m long row of trees with 3 m tree spacing. Trap placement for each species was alternated between rows resulting in at least 9 m spacing between traps for *C. rosaceana* and *A. velutinana*. There were five rows used per species within each plot. Pairs of pheromone-treated and untreated whole-plots were separated by at least 85

m. Moths captured in traps were counted and removed twice weekly. Lures were replaced at the onset of each moth generation.

2003 tests

As in 2002, the experiment was arranged in a split-plot design with presence or absence of pheromone disruption using rope dispensers as the whole-plot factor and lure loading dosage in pheromone traps as the subplot factor. Pheromone treatment was randomly applied to whole-plots. Each whole-plot contained six levels of the subplot factor. The subplot factor levels included four load rates of the optimally-attractive pheromone blends of each species, as described previously, in decade steps ranging from 1x to 1000x. The final two subplot levels were unbaited traps (control) and traps baited with a single Isomate OBLR/PLR Plus rope. There were four replicates of the two whole-plot factors applied to 0.6 ha apple blocks (four treated with pheromone and four not treated). One set of the six subplot levels was placed in each whole-plot.

We elected to test higher loading dosages in 2003 because it appeared maximal captures were not reached with the highest dosages tested in 2002 (see Fig. 1). The 1x monitoring lures for *A. velutinana* were plastic membrane lures loaded with 0.93 mg (*Z*)- and 0.07 mg (*E*)-11-tetradecenyl acetates and 2.0 mg dodecyl acetate (BioLure, Suterra, Bend, OR). The 1x membrane lures for *C. rosaceana* were loaded with 0.485 mg of (*Z*)- and 0.015mg (*E*)-11-tetradecenyl acetates and 0.026 mg of (*Z*)-11-tetradecenol. For the 10x and 100x treatments, the mg amounts of the various pheromone components were appropriately multiplied and loaded into lures. These lures were custom-manufactured by Suterra (Bend, OR). All pheromone lures were deployed in the traps described above; the 1000x treatment was achieved by placing ten 100x lures in a single trap. All lures were

affixed to the roofs of traps with adhesive tape. We elected to use membrane lures in 2003 rather than varying the numbers of rubber septa in traps as was done in 2002 in order to achieve higher loading dosages and to minimize the effects of volume changes of the pheromone-release apparatus on the dispersion pattern of the pheromone plume.

All traps were hung in trees *ca.* 1.5-2 m above ground level in the upper 1/3rd of the tree canopy. Traps within whole-plots were spaced at least 20 m apart and placed within plots as described above. Traps were checked twice per week at which point all moths were counted and removed. Lures were replaced at the onset of each moth generation.

Data analysis

Comparisons of mean moth catches in traps per loading dosage of pheromone in lures were made with a split-plot analysis of variance (ANOVA) and Fisher's protected least significant difference (LSD) multiple comparison procedure (SAS Institute, 2000). Because no males were attracted to unbaited (control) traps, these data were excluded from the analysis. Data were square-root transformed $[(x + 0.5)^{1/2}]$ before analysis. In all cases, significance level was $\alpha < 0.05$.

RESULTS

2002 field trials for C. rosaceana *using rubber septum lures*

There was a significant effect of both the whole-plot factor (application of ropes) ($F = 10.1$, $df = 1, 29$ $P < 0.05$) and the subplot factor (lure dosage) ($F = 58.1$, $df = 4, 29$, $P < 0.001$) on captures of *C. rosaceana*. In addition, there was a significant interaction between the application of pheromone disruption ropes and lure dosage in traps on captures of *C. rosaceana* ($F = 4.6$, $df = 4, 29$, $P < 0.001$).

Within the subplot factor, significantly more male *C. rosaceana* were captured in traps baited with 1/10x, 1x, 10x and 30x lure dosages under no pheromone disruption than under disruption (Fig. 21 A).

Within the whole-plot factor, significantly more male *C. rosaceana* were captured in traps baited with 10x and 30x lure dosages than were captured in traps baited with a 1/10x or 1x dosages under both no pheromone disruption and disruption (Fig. 21 A). Significantly more male *C. rosaceana* were captured in traps baited with 1x lures than in traps baited with 1/10x lures in both untreated and pheromone-treated plots (Fig. 21 A).

2002 field trials for A. velutinana *using rubber septum lures*

There was a significant effect of both the whole-plot factor (application of ropes) ($F = 82.7$, $df = 1, 23$, $P < 0.001$) and the subplot factor (lure dosage) ($F = 5.9$, $df = 3, 23$, $P < 0.001$) on captures of *A. velutinana*. In addition, there was a significant interaction between application of pheromone disruption ropes and lure dosage in traps on captures of *A. velutinana* ($F = 4.4$, $df = 3, 23$, $P < 0.01$).

Within the subplot factor, significantly more male *A. velutinana* were captured in traps baited with 1/10x, 1x, 10x and 30x lure dosages under no pheromone disruption than under disruption (Fig. 21 B).

Within the whole-plot factor, there was no difference in the number of male *A. velutinana* captured in traps baited with 1x, 10x or 30x loading dosages under no pheromone disruption (Fig. 21 B). However, traps with 1x, 10x or 30x loading dosages captured significantly more *A. velutinana* than traps baited with the 1/10x dosage in the untreated plots (Fig. 21 B). Very few *A. velutinana* were captured with any of the lure

loadings under disruption treatment and lure dosage had no significant effect on captures of *A. velutinana* in pheromone-treated plots (Fig. 21 B).

2003 field trials for C. rosaceana using membrane lures

There was a significant effect of both the whole-plot factor (application of ropes) ($F = 20.0$, $df = 1, 39$, $P < 0.01$) and the subplot factor (lure dosage) ($F = 3.7$, $df = 4, 39$, $P < 0.05$) on captures of *C. rosaceana*. However, the interaction between the application of pheromone disruption ropes and lure dosage in traps on captures of *C. rosaceana* was not significant ($F = 1.47$, $df = 4, 39$, $P = 0.21$).

Within the subplot factor, significantly more male *C. rosaceana* were captured in traps baited with 1x, 10x, 100x, 1000x lure dosages, and ropes under no pheromone disruption than under disruption (Fig. 22 A).

Within the whole-plot factor, significantly more male *C. rosaceana* were captured in traps baited with 1x lures than in traps baited with 100x and 1000x lures, or ropes in plots not treated with pheromone (Fig. 22 A). Also, there were no differences in captures of *C. rosaceana* in traps baited with 10x, 100x, 1000x lure dosages and ropes in untreated plots (Fig. 22 A). Under pheromone disruption, significantly more *C. rosaceana* were captured in traps baited with 1x and 1000x lure dosages than in traps baited with ropes (Fig. 22 A). Also, there was no difference in captures of *C. rosaceana* in traps baited with 10x, 100x lure dosages, and ropes under pheromone disruption (Fig. 22 A).

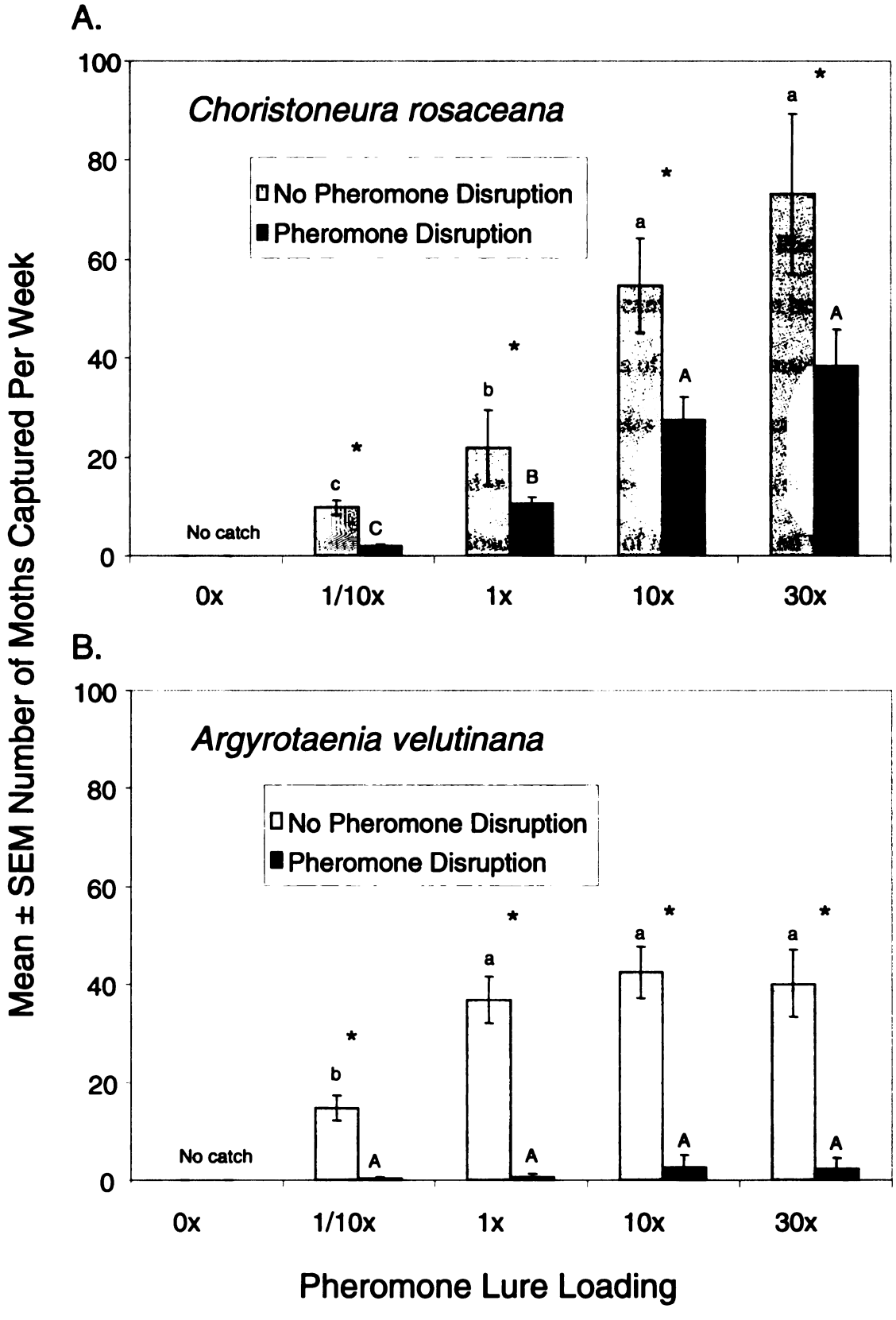

A.
Choristoneura rosaceana
No Pheromone Disruption
Pheromone Disruption
No catch
0x
1/10x
1x
10x
30x
B.
Argyrotaenia velutinana
No Pheromone Disruption
Pheromone Disruption
No catch
0x
1/10x
1x
10x
30x
Mean ± SEM Number of Moths Captured Per Week
Pheromone Lure Loading

Figure 21. A. Captures of male *Choristoneura rosaceana* in pheromone traps baited at various loading rates using rubber septum dispensers in untreated plots (light bars) and in pheromone-treated plots (dark bars). Within the subplot factor (pheromone lure loading), pairs of means marked with an asterisk above them are significantly different. Within the whole plot factor (pheromone disruption application), means not followed by a letter of the same case (small case for No Pheromone Disruption and capitals for Pheromone Disruption) are significantly different. There was a significant interaction between pheromone treatment and lure loading dosage. B. Captures of male *Argyrotaenia velutinana* in pheromone traps baited at various loading rates using rubber septum dispensers in untreated plots (light bars) and in pheromone-treated plots (dark bars). Within the subplot factor (pheromone lure loading), pairs of means marked with an asterisk above them are significantly different. Within the whole plot factor (pheromone disruption application), means not followed by a letter of the same case (small case for No Pheromone Disruption and capitals for Pheromone Disruption) are significantly different. There was a significant interaction between pheromone treatment and lure loading dosage.

2003 Field Trials for A. velutinana using membrane lures

There was a significant effect of the whole-plot factor (application of ropes) ($F = 17.7$, $df = 1, 39$, $P < 0.01$) on captures of *A. velutinana* but no significant effect of the subplot factor (lure dosage) ($F = 1.8$, $df = 4, 39$, $P = 0.12$) In addition, the interaction between the application of pheromone disruption ropes and lure dosage in traps on captures of *A. velutinana* was not significant ($F = 1.5$, $df = 4, 39$, $P = 0.19$).

Within the subplot factor, significantly more male *A. velutinana* were captured in traps baited with 1x, 10x, 100x, 1000x lure dosages, and ropes under no pheromone disruption than under disruption (Fig. 22 B).

Within the whole-plot factor, significantly more *A. velutinana* were captured in traps baited with 1x lure dosages than in traps baited with 1000x lure dosages in plots not treated with pheromone (Fig. 22 B). There was no difference in captures of *A. velutinana* in traps baited with 1x, 10x, 100x lures, or ropes in plots with no pheromone disruption (Fig. 22 B). Very few *A. velutinana* were captured with any of the lure loadings under pheromone disruption and lure dosage had no significant effect on captures of *A. velutinana* in pheromone-treated plots (Fig. 22 B).

DISCUSSION

Application of pheromone dispensers (Isomate OBLR/PLR Plus) at a rate of 500 per ha releasing primarily (*Z*)-11-tetradecenyl acetate and containing a small amount (*ca.* 1.5 %) of a pheromone antagonist of *C. rosaceana*, (*Z*)-9-tetradecenyl acetate, (Evenden *et al* 1999*c*) significantly decreased captures of both *C. rosaceana* and *A. velutinana* in pheromone traps relative to those in untreated plots. However, this pheromone treatment caused greater disruption of orientation to pheromone-baited traps for *A. velutinana* than for *C. rosaceana* as measured over a wide range of lure loading dosages. For *C. rosaceana*, orientation to traps baited with 1x rubber septum and membrane lures containing an optimally-attractive blend was disrupted only *ca.* 52 and 71 % for the 2002 and 2003 seasons, respectively. In contrast, disruption of orientation for *A. velutinana* to attractive 1x rubber septum and membrane lures was 98 and 94 % for the 2002 and 2003 seasons, respectively.

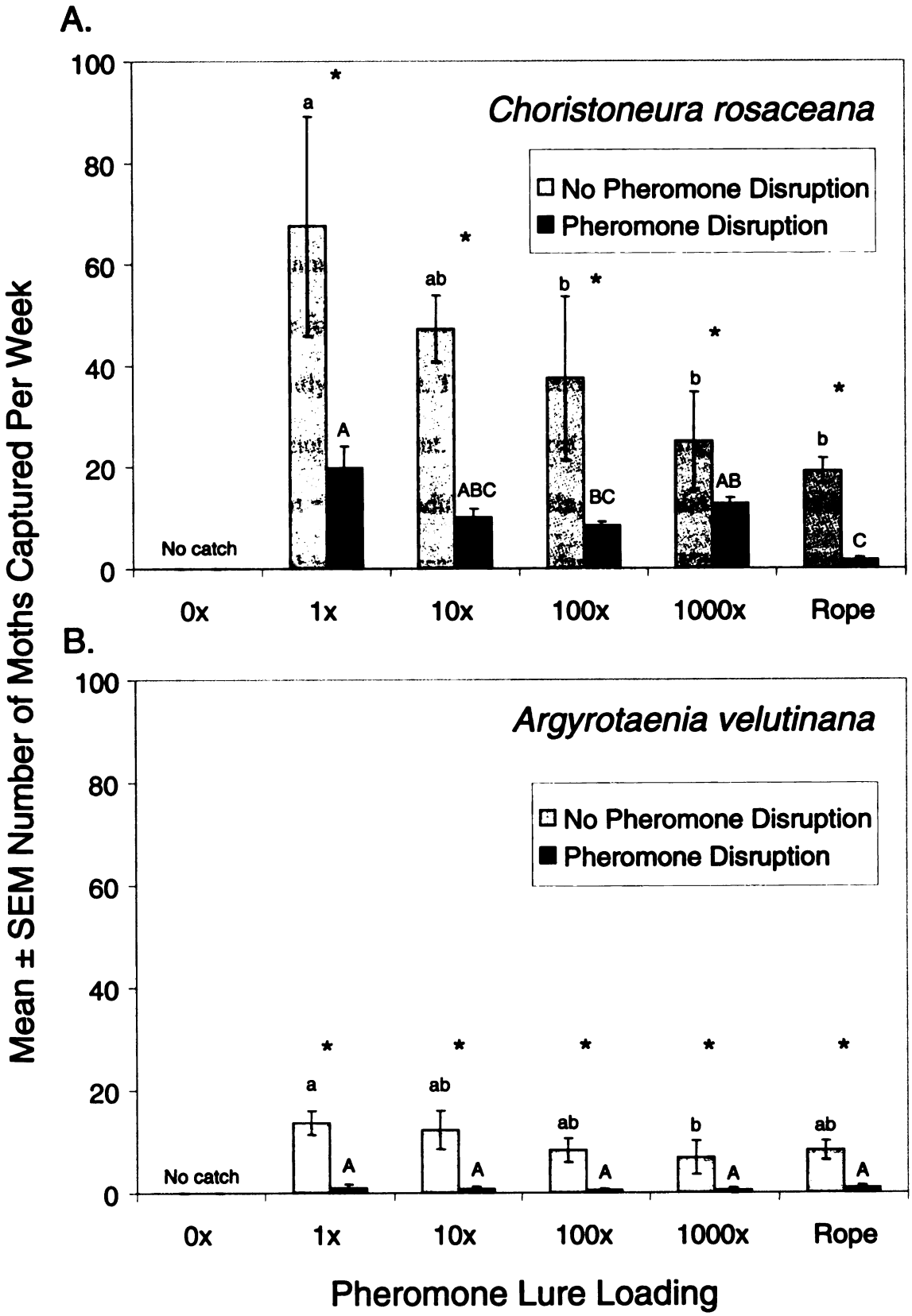

A.
Choristoneura rosaceana
No Pheromone Disruption
Pheromone Disruption
Mean ± SEM Number of Moths Captured Per Week
100
80
60
40
20
0
No catch
a
A
ab
ABC
b
BC
b
AB
b
C
*
0x
1x
10x
100x
1000x
Rope
B.
Argyrotaenia velutinana
No Pheromone Disruption
Pheromone Disruption
100
80
60
40
20
0
No catch
a
A
ab
A
ab
A
b
A
ab
A
*
0x
1x
10x
100x
1000x
Rope
Pheromone Lure Loading

Figure 22. A. Captures of male *Choristoneura rosaceana* in pheromone traps baited at various loading rates using membrane dispensers in untreated plots (light bars) and in pheromone-treated plots (dark bars). Within the subplot factor (pheromone lure loading), pairs of means marked with an asterisk above them are significantly different. Within the whole plot factor (pheromone disruption application), means not followed by a letter of the same case (small case for No Pheromone Disruption and capitals for Pheromone Disruption) are significantly different. The interaction between pheromone treatment and lure loading dosage was not significant. B. Captures of male *Argyrotaenia velutinana* in pheromone traps baited at various loading rates using membrane dispensers in untreated plots (light bars) and in pheromone-treated plots (dark bars). Within the subplot factor (pheromone lure loading), pairs of means marked with an asterisk above them are significantly different. Within the whole plot factor (pheromone disruption application), means not followed by a letter of the same case (small case for No Pheromone Disruption and capitals for Pheromone Disruption) are significantly different. The interaction between pheromone treatment and lure loading dosage was not significant.

These results agree with Roelofs and Novak (1981), who also found that *A. velutinana* was easier to disrupt than *C. rosaceana* when using only the main component of their pheromone blends ((*Z*)-11-tetradecenyl acetate). However, greater levels of disruption (88-96 %) have been achieved for *C. rosaceana* using dispensers containing a 93:7 ratio of (*Z*)- and (*E*)-11-tetradecenyl acetates and without traces of the antagonist, (*Z*)-9-tetradecenyl acetate (Deland et al., 1994). Addition of this antagonist to currently marketed Isomate rope dispensers for leafrollers may explain the reduced level of

disruption reported in our current study for *C. rosaceana* relative to Deland et al. (1994), particularly if false-plume-following to ropes was an important operating mechanism. Although reduction in the numbers of males trapped at synthetic pheromone sources cannot be reliably used as the sole indicator that pheromone treatment prevents males from finding virgin females (Agnello et al., 1996), a substantial difference in percent disruption of pheromone-baited traps between these two species suggests that *A. velutinana* should be easier to control than *C. rosaceana* with Isomate OBLR/PLR dispensers.

A. velutinana responded to very high loadings (up to 1000x in membrane lures) of its 3-component pheromone blend. In addition, it is notable that captures of *A. velutinana* in traps baited with high-release pheromone ropes equaled captures in traps baited with the 3-component lures shown to be highly attractive to *A. velutinana* (Cardé and Roelofs, 1977). In associated studies, we also found that *ca.* 50 % of unexposed *A. velutinana* and > 80 % of pheromone pre-exposed *A. velutinana* oriented to such ropes in a wind tunnel (Stelinski et al., 2004a) and observed numerous feral *A. velutinana* orienting to these ropes in the field (Stelinski et al., 2004b). Linn et al. (1985) showed that responses of *A. velutinana* in a wind tunnel are highest (75-100 % response) to the full seven-component blend of this species compared to less complete blends, especially at the lowest loadings tested (3 and 10 μg / septum), which still elicited *ca.* 75 % response. However, significant responses (*ca.* 75 - >80 %) were also elicited by the incomplete 92:8 Z / E mixture when presented at higher loadings of 100 and 300 μg / septum. Thus, Isomate OBLR/PLR Plus ropes attract *A. velutinana* males despite releasing a sub-optimally attractive Z / E ratio that also lacks other minor components of the pheromone. Perhaps the high pheromone

loading and corresponding release-rate from such dispensers accounts for their marked attractiveness to *A. velutinana*.

In contrast to the results for *A. velutinana*, significantly more *C. rosaceana* were captured in traps baited with lures containing 0.526 mg (1x in rubber septum or membrane lure) of a blend optimally-attractive for this species than traps baited with single Isomate OBLR/PLR Plus pheromone ropes or with lure loadings 100 times above the 1x load. In addition, significantly fewer *C. rosaceana* responded to ropes (ca. 25 %) compared with *A. velutinana* in a wind tunnel (Stelinski et al., 2004a). Although we have observed numerous feral *C. rosaceana* closely approaching (within 100 cm) Isomate OBLR/PLR ropes in orchard plots (Stelinski et al., 2004b), it is possible that the presence of (*Z*)-9-tetradecenyl acetate, a pheromone antagonist, in these ropes resulted in fewer captures of *C. rosaceana* in rope-baited traps relative to lure-baited traps. However, increasing the lure loading dosage of an optimally-attractive blend lacking the antagonist also decreased captures of *C. rosaceana* in the field (Fig. 2 A), while the responses of *A. velutinana* remained equivalent over a wide range of high loadings (Fig. 2 b). Overall, our results are similar to earlier work by Klun and Robinson (1972) showing *A. velutinana* was equally responsive to traps baited with its major pheromone component alone ((*Z*)-11-tetradecenyl acetate) over a wide range of loadings in olive oil (3.5 – 3000 μg). Those authors also found that *C. rosaceana*'s responsiveness to lures reached an upper limit at a loading of 896 μg of (*Z*)-11-tetradecenyl acetate and captures in traps significantly decreased as loading dosages were increased up to 3000 μg. Our data obtained with increasing doses of 3-component blends corroborate the same dose-response trends observed for *A. velutinana* and *C. rosaceana* by Klun and Robinson

(1972), who varied only the major pheromone component of each species, (Z)-11-tetradecenyl acetate.

We observed contrasting lure-dosage versus moth-catch relationships for *C. rosaceana* under no pheromone disruption in 2002 versus 2003 (Fig. 1 A and Fig. 2 A). It is likely that we documented the upward trend of the dose-versus-catch relationship in 2002 at the lower lure dosages tested, while the downward trend was documented in 2003 when much higher lure dosages were tested. Both upward and downward trends of such a histogram can be found in Fig. 1 of Klun and Robinson (1972), where captures of *C. rosaceana* in traps increased up to a maximum at loadings of 896 μg of (Z)-11-tetradecenyl acetate / lure and then decreased beyond that loading. As mentioned previously, there appears to be an upper lure-loading limit for *C. rosaceana* above which captures of moths in traps are reduced. The methodological switch from varying numbers of equally-loaded rubber septa in 2002 to varying loading by increasing release rate of pheromone in single membrane-type lures in 2003 also possibly accounts for the discrepancy in captures between years. However, our results suggest that maximum captures of *C. rosaceana* using a 3-component blend occur between 1x (membrane lure) and 30x (rubber septa) of the standard 0.526 mg loading of the attractive, 3-component blend.

Collectively, these results indicate that *A. velutinana* and *C. rosaceana* respond differently to varying quantities of their pheromone emitted from lures. *A. velutinana* appears to have a higher response threshold (less sensitive) than *C. rosaceana*. Recent electrophysiological studies (Stelinski et al., 2003a) have shown that the peripheral receptors of *C. rosaceana* exhibit a long-lasting adaptation following prolonged exposure

to its pheromone components, while the same is not true for *A. velutinana*; however, it is unclear whether these species-specific patterns of long-lasting receptor adaptation contributed to the differing behavioral responses to lures in the field. As documented by Stelinski et al. (2004b) feral *C. rosaceana* and *A. velutinana* males are attracted to and closely approach (within 0 to 100 cm) Isomate OBLR/PLR dispensers. Furthermore, laboratory-reared males of both species approach such dispensers in a 2.4 m long wind tunnel (Stelinski *et al.* 2004*a*). However, feral males of both species do not remain in close proximity (within 10 cm) to Isomate OBLR/PLR dispensers in the field long enough (males leave within 10s) to receive the required pheromone pre-exposure dosage necessary to induce long-lasting adaptation as quantified by Stelinski et al. (2003b). These results suggest long-lasting adaptation may not be a contributing factor to the species-specific differences in susceptibility to mating disruption.

In addition to long-lasting adaptation, instantaneous reductions of peripheral sensitivity may influence moth behavior as they approach sources of concentrated pheromone. Keunen and Baker (1981) documented instantaneous antennal adaptation using EAGs for moths continuously exposed to pheromone in the laboratory. However, no long-term effects were found after removal from pheromone. Interestingly, tortricid moths are known to orient along the edges of concentrated pheromone plumes or walls of pheromone in wind tunnels (Kennedy et al., 1981; Willis and Baker, 1984). Such moths effectively modulate their exposure dosage while being attracted to the source of concentrated plumes. Therefore, the antennal exposure to pheromone of moths edge-following along concentrated pheromone plumes may be discontinuous and less severe than that achieved under constant laboratory exposures. Systematic studies of the

similarities or differences in short-term or instantaneous adaptation (Stelinski et al., 2003a) between *C. rosaceana* and *A. velutinana* have not yet been conducted and may contribute to our understanding of the behavioural differences documented in this study.

Significantly more codling moths, *Cydia pomonella* (L.), are captured in traps baited with 10-mg lures of codlemone (*E*8,*E*10-12OH) than traps baited with 1-mg lures of codlemone under mating disruption using pheromone ropes at recommended application rates for that species (Charmillot, 1990; Barrett, 1995; Judd et al., 1996). If a mating disruption treatment raises the response threshold of male moths via adaptation of peripheral receptors or habituation of the central nervous system (Vickers and Rothchild, 1991), then it is conceivable that higher-release lures should be more attractive than lower-release lures under the conditions of mating disruption. However, we did not obtain this outcome for either leafroller species in this study as has been documented for *C. pomonella* (Charmillot, 1990; Barrett, 1995). Ineffectiveness of high-load lures in trapping leafrollers under mating disruption was most dramatic for *A. velutinana*, for which captures in traps baited with every lure dosage tested were nearly completely shut down. For *C. rosaceana* in 2002, traps baited with 10 or 30 1x rubber septum lures did capture more moths than a trap baited with a single 1x rubber septum lure in plots under mating disruption. However, this outcome did not hold in 2003 when traps baited with 10 to 1000 times the 1x loading dosage in membrane lures captured significantly fewer moths than did a trap baited with single 1x membrane lure in plots under mating disruption. Thus, increasing lure loading dosage above that of 1x monitoring lures (rubber septa or membrane-type) does not appear to reliably increase monitoring

effectiveness of males of either leafroller species in orchards where pheromone ropes are deployed at recommended densities.

The results of this study indicate that Isomate OBLR/PLR ropes, currently marketed for mating disruption of *C. rosaceana*, provide lower levels of orientational disruption of this species than has been documented in previous studies that used similar dispensers lacking traces of the antagonist, (*Z*)-9-tetradecenyl acetate (Deland et al., 1994; Knight et al., 1998). In small plot trails, Evenden et al. (1999c) found that application of dispensers containing a 1:1 mixture (*Z*)-9-tetradecenyl acetate and the 4 component Western *C. rosaceana* pheromone (Vakenti et al., 1998) achieved levels of orientational disruption to virgin-females baited traps (>83 %) that were approximately equal to that achieved with dispensers containing the pheromone alone. Perhaps these differing responses of *C. rosaceana* in British Columbia and Michigan are due to 'racial' differences between these geographically separated populations.

Based on their results, Evenden et al. (1999a, b, c) concluded that mating disruption was unlikely to be mediated by false-plume-following and more likely to be mediated by camouflage or receptor adaptation, given that dispensers with incomplete blends or containing a behavioural antagonist were not less effective than those containing multi-component attractive blends. Furthermore, it was suggested that both *C. rosaseana* and *Pandemis limitata* could be controlled concurrently using dispensers releasing the pheromone components of both species, despite the presence of a behavioral antagonist for *C. rosaceana* within such a blend (Evenden et al., 1999a, c). Thus, the undeclared addition of (*Z*)-9-tetradecenyl acetate by Shin-Etsu Chemical Co. (Tokyo, Japan) to Isomate OBLR/PLR dispensers also containing the major components of both

C. rosaceana and *P. limitata* was likely made to improve the effectiveness of this dispenser for concurrent mating disruption of these two sympatric leafroller species. Our recent laboratory (Stelinski et al., 2004a) and field (Stelinski et al., 2004b) studies in Michigan suggest that false-plume-following is an important mechanism mediating mating disruption of *C. rosaceana* by polyethylene-tube dispensers such as Isomate OBLR/PLR Plus. Despite the presence of this antagonist, *C. rosaceana* are attracted to and closely approach or contact Isomate OBLR/PLR Plus dispensers in flight tunnel studies (Stelinski et al., 2004a) and under natural apple orchard conditions (Stelinski et al., 2004b). However, the addition of a behavioral antagonist of *C. rosaceana* ((Z)-9-tetradecenyl acetate) to such dispensers may have made them less effective by decreasing attractiveness relative to dispensers lacking this compound. Further work should be conducted to determine whether the presence of this antagonist in dispensers impacts mating disruption of *C. rosaceana* in Michigan and in the Eastern United States. In contrast, orientational disruption of *A. velutinana* was nearly complete using Isomate OBLR/PLR dispensers suggesting high effectiveness for control of this species.

CHAPTER SIX

Field observations quantifying attraction of four tortricid moth species to high-dosage, polyethylene-tube pheromone dispensers in untreated and pheromone-treated orchards.

Also published as:

Stelinski, L. L., L. J. Gut, A. V. Pierzchala, and J. R. Miller. 2004. Field observations quantifying attraction of four tortricid moth species to high-dosage pheromone rope dispensers in untreated and pheromone-treated apple orchards. *Entomologia Experimentalis et Applicata.* 113: 187-196.

ABSTRACT

Orientational responses of four species of feral tortricid moths to polyethylene-tube pheromone dispensers were observed in a 0.8 ha apple orchard treated with such pheromone dispensers and in an untreated 0.8 ha orchard. Male oblique-banded leafrollers, *Choristoneura rosaceana* (Walker) (mean 7.2 ± 0.4 over 21 nights), Oriental fruit moths, *Grapholita molesta* (Busck) (mean 10.5 ± 2.1 over 20 evenings), and the redbanded leafrollers, *Argyrotaenia velutinana* (Walker) (mean 2.0 ± 1.1 over 14 nights) were attracted within 100 cm of their respective polyethylene-tube pheromone dispensers in the untreated orchard. Furthermore, *C. rosaceana* (mean 1.95 ± 0.7 over 17 nights) and *G. molesta* (mean 1.53 ± 0.4 over 20 evenings) came within 100 cm of their respective polyethylene-tube pheromone dispensers in the pheromone-treated orchard. Most visits lasted less than 10 s, after which the majority of moths departed by flying upwind. In the untreated orchard, the number of *C. rosaceana* observed orienting to polyethylene-tube dispensers was greater than the number captured in optimized monitoring traps (1.9 ± 0.4) per night of observation. The numbers of *A. velutinana* (2.0 ± 1.1) or *G. molesta* (10.5 ± 2.1) attracted to polyethylene-tube dispensers in the untreated orchard did not differ statistically from the numbers captured in optimized monitoring traps per night of observation. In the pheromone-treated orchard, the number of *C. rosaceana* (2.0 ± 0.4) or *G. molesta* (1.2 ± 0.2) observed orienting to polyethylene-tube dispensers did not differ statistically from the numbers of male moths of these species captured in optimized monitoring traps per night of observation. No codling moth, *Cydia pomonella*, were observed orienting to or landing near their respective polyethylene-tube dispensers in either the untreated or pheromone-treated orchards, despite the fact that substantial numbers were

captured in monitoring traps per night of observation (6.0 ± 1.7) in the untreated orchard. Attraction of male moths to polyethylene-tube dispensers occurred in three of the four species observed. These results provide support for the idea that false-plume-following is an important component of the mechanisms mediating communicational disruption in moths by polyethylene-tube dispensers.

INTRODUCTION

Pheromone-based mating disruption is an important biorational pest-management tactic for insects relying on long-distance pheromones for mate finding (Cardé and Minks, 1995). However, the effectiveness of the approach has been variable (Reissig et al., 1978; Sanders, 1982; Audemard, 1988). Factors influencing the efficacy of mating disruption treatments may include: completeness of pheromone blend in a disruption formulation (Minks and Cardé, 1988; Evenden et al., 1999a), high population densities, which increase competition between calling females and pheromone dispensers (Schmitz et al., 1995; Weissling and Knight, 1996; Suckling and Angerelli, 1996; Knight and Turner, 1999), variability in canopy structure and wind direction, which affects pheromone plume structure (Cardé and Minks, 1995), and varying durations of antennal adaptation across species (Stelinski et al., 2003 a, b).

Several investigations have attempted to uncover why mating disruption sometimes succeeds and at other times fails (Sanders, 1985; Sanders, 1996; Valeur and Löfstedt, 1996; Cardé et al., 1997; Cardé et al., 1998; Sanders, 1998; Eveden et al., 2000, Stelinski et al., 2004 a, b). Such investigations have focused on the behavioral and physiological mechanisms underlying mating disruption. The mechanisms postulated to underlie mating disruption have been reviewed by Rothschild (1981), Bartell (1982), and

Cardé (1990). They include: camouflage, imbalance of sensory input, false-plume-following, and adaptation and/or habituation.

Our recent research interests have included the physiological (Stelinski et al., 2003a,b,c) and behavioral (Stelinski et al., 2004a) differences among certain leafroller moth species, which may contribute to differences in the effectiveness of mating disruption as a control tactic for these species (Gut et al., 2004). Efforts to disrupt the redbanded leafroller, *Argyrotaenia velutinana* (Walker), using synthetic pheromones have been judged successful (Novak et al., 1978; Roelofs and Novak, 1981; Cardé and Minks, 1995), while lower success has been documented for the oblique-banded leafroller, *Choristoneura rosaceana* (Harris) (Reissig et al., 1978; Agnello et al., 1996; Lawson et al., 1996). Studies comparing these two species have revealed species-specific expression and duration of long-lasting peripheral adaptation following exposure to polyethylene-tube pheromone dispensers used in mating disruption (Stelinski et al, 2003 a; b). Specifically, exposure to pheromone induces minutes-long effects on peripheral reception in *C. rosaceana* but not *A. velutinana* (Stelinski et al., 2003a,b) and day-long effects on behavioral responsiveness to pheromone in both *C. rosaceana* and *A. velutinana* (Stelinski et al., 2004a).

The next step in this comparative study was to describe the behavioral interactions of these two species with commercially available mating disruption dispensers in orchards. This chapter describes behavioral observations of four tortricid moth species (*C. rosaceana*, *A. velutinana*, Oriental fruit moth, *Grapholita molesta* (Busck), and codling moth, *Cydia pomonella* (L.)) in close proximity to their mating disruption dispensers in orchards.

MATERIALS AND METHODS

Field Observations.

This study was conducted May-September of 2003 at the Trevor Nichols Research Complex (TNRC) of Michigan State University in Fennville, MI. Visual observations were conducted on sunny and calm evenings starting at 16:30 and lasting through 24:30 in two 0.8 ha orchards of 18 year old Delicious apple trees with *ca.* 2-3 m canopy heights. Trees were planted on a 3 m within- and 6 m between-row spacing. The orchards were spaced *ca.* 85 m apart. One orchard was left untreated while the second received treatments of three types of polyethylene-tube pheromone dispensers at recommended label rates (see description below). The pheromone-treated orchard was located downwind of the untreated orchard based on the prevailing wind direction at the TNRC. The pheromone treatments targeted four species of tortricids known to infest these orchards: *G. molesta*, *C. pomonella*, *A. velutinana*, and *C. rosaceana*.

Pheromone Dispensers. The polyethylene-tube pheromone dispensers used for observations and applied as mating disruption treatments were: 1) Isomate-M Rosso containing 250 mg of 88.5 % (*Z*)-8-dodecen-1-yl-acetate, 5.7 % (*E*)-8-dodecen-1-yl-acetate, 1.0 % Z-8-dodecen-1-ol, and 4.8 % inert ingredients (500 dispensers per ha) targeting *G. molesta*; 2) Isomate-C Plus containing 205 mg of 53.0 % (*E,E*)-8,10-dodecadien-1-ol, 29.7 % dodecanol, 6.0 % tetradecanol, and 11.3 % inert ingredients (1000 dispensers per ha) targeting *C. pomonella*; and 3) Isomate OBLR/PLR Plus containing 274 mg of 93.4 % (*Z*)-11-tetradecenyl acetate, 5.1 % (*E*)-11-tetradecenyl acetate, and 1.5 % (*Z*)-9-tetradecenyl acetate (500 dispensers per ha) targeting both *C. rosaceana* and *A. velutinana*. All dispensers were hung in trees *ca.* 1.5 - 2 m above the

ground and in the upper third of the tree canopy. Isomate-M Rosso, Isomate-C Plus, and Isomate-OBLR/PLR dispensers were applied in the orchard on 1 May, 27 May, and 12 June, respectively. After 12 June, all three dispenser types were present concurrently. Dispensers used in field observations were field-aged to match the age of those used for mating disruption.

Observational Arena.

The observational arena was a 71 cm high, vinyl-covered table measuring 86 x 86 cm. The table top was demarcated with tape in a 10 x 10 cm grid to aid estimation of the proximity of observed moths to a polyethylene-tube pheromone dispenser affixed above the table's center. An individual pheromone dispenser was twisted onto an apple branch (*ca.* 50 cm long, 1.5 cm diameter) removed from a tree in the experimental plot. The apple branch was then affixed horizontally and approximately 32 cm above the tabletop to a steel ring-stand. The stand was positioned in the center of the observational table. Wind direction at the observational arena was concurrently monitored using a 20 cm piece of flagging tape hanging from a second ring-stand placed adjacent to the one holding the apple branch with the pheromone dispenser. Detailed weather data including wind speed and direction were recorded by a weather station on the TNRC *ca.* 80 m from the observational arena (Michigan Automated Weather Network, http://www.agweather.geo.msu.edu/mawn). Wind speeds during observations varied from *ca.* 0.13 to 2.2 m/s. The observational arena was set up in openings created by missing trees within tree rows at 2, 4, or 6 rows interior from the border row of each plot; the specific site of the observational arena was randomly rotated among 5 locations nightly. The tree spacing and size described above was that of tightly planted, small trees

such that gaps created by missing trees were less than 3 m^3. Our aim was to insert the arenas into such small gaps, surrounded by foliage of neighboring trees, so as to mimic the position of a dispenser within an actual tree rather than conducting observations within the corridor between tree rows. The design of our arena allowed for conducting detailed quantifications of moth behaviors that may have been difficult to observe directly within trees.

Observed events were spoken into a hand-held microcassette audio recorder by an investigator sitting 0.75 m from the observational arena. Data recorded included: anemotactic orientations to the dispenser, closest approach to the dispenser, landings at the observation arena, time during the diel period, and duration of visits. Observations after dusk were assisted by night-vision goggles (Rigel 3100, DeWitt, IA) with a 40° field of view, 0.5 – 200 m viewing distance and resolution of 28 lines / mm. In rare cases with little sky light (*ca.* below 0.01 lux), an infrared illuminator mounted on the goggles was activated to improve resolution. In preliminary laboratory tests, this illuminator did not appear to affect moth behavior and no differences were noted in moth behavior in the field whether it was on or off. At peak moth activity during the diel cycle (see results), multiple moth visits occasionally occurred simultaneously; however, we estimate that more than 90 % of all moth visits were documented.

Concurrent with the observations, attraction of male moths to sex pheromone was monitored using pheromone traps (LPD Scenturian Guardpost, Suterra, Bend, OR) placed in both the untreated and pheromone-treated orchards. Traps for each species were baited with monitoring lures containing pheromone blends attractive to each species. For *A. velutinana*, rubber septa were loaded with 0.93 mg (*Z*)- and 0.07 mg (*E*)-11-tetradecenyl

acetates (93 : 7 ratio of *Z* : *E*) and 2.0 mg dodecyl acetate (Roelofs et al., 1975). For *C. rosaceana*, rubber septa were loaded with 0.485 mg of (*Z*)- and 0.015 mg (*E*)-11-tetradecenyl acetates (92.2 : 3.0 ratio of *Z* : *E*) and 0.026 mg of (*Z*)-11-tetradecenol (Hill and Roelofs 1979). For *C. pomonella*, septa were loaded with 1 mg of (*E*,*E*)-8,10-dodecadien-1-ol. Finally, for *G. molesta*, septa were loaded with 3 μg of (*Z*)-8-dodecenyl acetate : (*E*)-8-dodecenyl acetate : Z-8-dodecen-1-ol in a 100 : 6 : 10 blend. Pheromone blend solutions used to load rubber septa were prepared in HPLC grade hexane and stored at -18 ° C. Three traps were deployed in each plot per species; traps were maintained *ca.* 50 m from the observation arena. They were hung *ca.* 1.5 - 2 m above ground level in the upper third of the tree canopy. New pheromone lures were deployed every two weeks for each trap. Moths captured in traps were counted and removed following each observational period. Moth captures in pheromone traps were monitored only on nights during which direct observations were conducted.

For each species, direct observations were carried out between two and five times per week during their respective adult generations. The first and second generations of *G. molesta* were observed 5 May – 9 June and 7 June – 2 September, respectively. For *G. molesta*, observations were made on 20 evenings in the untreated orchard and on 20 evenings in the pheromone-treated orchard. The first and second generations of *C. rosaceana* were observed 17 June – 2 September and the second and third generations of *A. velutinana* were observed 30 June – 8 September. For *C. rosaceana* and *A. velutinana*, observations were made on 21 and 14 evenings, respectively, in the untreated orchard and on 17 and 14 separate evenings, respectively, in the pheromone-treated orchard. The first and second generations of *C. pomonella* were captured in traps 3 June –1 September. For

C. pomonella, observations were made on 15 evenings in the untreated orchard and on 15 separate evenings in the pheromone-treated orchard.

Statistical Analyses.

Comparisons of mean moth catches in traps and mean numbers of moths observed throughout the entire season at the observational arena in untreated versus pheromone-treated orchards were accomplished with analysis of variance (ANOVA) and Fisher protected least significant difference (LSD) multiple comparison procedure (SAS Institute, 2000). Comparisons of mean moth catches in traps versus numbers of moths observed at the observational arena per night were accomplished by two-sided (tailed) *t* tests. Because no *C. pomonella* were observed in either pheromone-treated or untreated orchards and no *A. velutinana* were observed in the pheromone-treated orchard, these data were excluded from the analysis. All data were square-root transformed $[(x + 0.5)^{1/2}]$ before analysis. In all cases, significance level was $\alpha < 0.05$.

RESULTS

Number of moths observed at polyethylene-tube dispensers and in monitoring traps.

Over the course of their respective flight periods, *C. rosaceana*, *G. molesta*, and *A. velutinana* were consistently attracted within 100 cm of their respective polyethylene-tube pheromone dispensers in the untreated orchard (Table 5). Likewise, *C. rosaceana* and *G. molesta* came within 100 cm of their respective dispensers in the pheromone-treated orchard (Table 5).

Significantly more *C. rosaceana* and *G. molesta* approached their respective pheromone dispensers in the untreated orchard than in the pheromone-treated orchard (Table 5). In addition, *A. velutinana* approached their pheromone dispensers only in the

untreated orchard (Table 5). In contrast, *C. pomonella* were not observed approaching their dispensers in either the untreated or pheromone-treated orchards (Table 5). However, *C. pomonella* were captured in optimized monitoring traps in both orchards (Table 5).

The mean number of *C. rosaceana* approaching pheromone dispensers per night in the untreated orchard was significantly ($P < 0.05$) greater than the mean number captured in monitoring traps (Table 5). However, the mean number of *A. velutinana* approaching pheromone dispensers per night in the untreated orchard was not statistically ($P > 0.05$) different from the mean number captured in monitoring traps (Table 5). Likewise, the mean number of *G. molesta* approaching pheromone dispensers in the untreated orchard was not statistically ($P > 0.05$) different from the mean number captured in monitoring traps (Table 5).

In the pheromone-treated orchard, the mean number of *C. rosaceana* approaching pheromone dispensers was not statistically ($P > 0.05$) different from the mean number captured in monitoring traps (Table 5). Similarly, the mean number of *G. molesta* approaching pheromone dispensers in the pheromone-treated orchard was not statistically ($P > 0.05$) different from the mean number captured in monitoring traps (Table 5).

Duration of stay and proximity to ropes.

Nearly all *C. rosaceana* observed were attracted within 100 cm of their polyethylene-tube dispenser in both untreated and pheromone-treated orchards and a third of those observed approached within 10 cm of the dispenser (Fig. 23 A). However, no individuals of this species were observed landing at the observational arena. The majority

of *C. rosaceana* left the observational arena within 10 s of initial sighting in both untreated and pheromone-treated orchards (Fig. 23 B).

All of the *G. molesta* observed approached within 100 cm of their dispenser (Fig. 23 C). Of the four moth species observed, *G. molesta* was the only one to occasionally land at the observational arena. Specifically, 95 (out of 220 total) and 3 (out of 22 total) *G. molesta* landed at the observational arena in the untreated and pheromone-treated orchards, respectively. The *G. molesta* moths that landed at the arena wing-fanned vigorously and walked rapidly, remaining in motion for the duration of their stay. A total of 14 *G. molesta* landed on the branch upon which a polyethylene-tube dispenser was hung and four directly contacted a dispenser. The majority of *G. molesta* observed were attracted within 20-60 cm of their dispenser (Fig 23 C) and left within 10 s in both untreated and pheromone-treated orchards (Fig. 23 D). The three *G. molesta* that landed at the observational arena in the pheromone-treated orchard left within 20 s.

All *A. velutinana* approached within 70 cm of the dispenser in the untreated orchard (Fig. 23 E). The majority of *A. velutinana* observed in the untreated orchard left the observational arena within 10 s of initial sighting (Fig. 23 F). No *A. velutinana* were observed approaching their dispensers in the pheromone-treated orchard. Also, no individuals of this species were observed landing at the observational arena in the untreated orchard.

G. molesta oriented toward their respective polyethylene-tube pheromone dispensers placed at the observational arena from heights (*ca.* 0.5 – 0.8 m above ground level) below that of the table.

Table 5. Mean ± SEM numbers of moths captured in traps and observed visiting polyethylene-tube dispensers per night.

Species followed by Treatment Pairs	Mean number ± SEM of moths captured in traps per night		Mean number ± SEM of observed visits to dispensers per night
Choristoneura rosaceana (21 nights of observation)			
Untreated plot	1.9 ± 0.4a[a]	*	7.2 ± 0.4a
Pheromone-treated plot	1.2 ± 0.2a	NS	1.95 ± 0.7b
Argyrotaenia velutinana (14 nights of observation)			
Untreated plot	6.3 ± 1.0	NS	2.0 ± 1.1
Pheromone-treated plot	0.0 ± 0.0		0.0 ± 0.0
Grapholita molesta (20 evenings of observation)			
Untreated plot	8.5 ± 1.0a	NS	10.5 ± 2.1a
Pheromone-treated plot	0.3 ± 0.1b	NS	1.53 ± 0.4b
Cydia pomonella (15 evenings of observation)			
Untreated plot	6.0 ± 1.7a		0.0 ± 0.0
Pheromone-treated plot	0.09 ± 0.01b		0.0 ± 0.0

[a]Pairs of means in the same column for each species followed by the same letter are not significantly different ($P < 0.05$, ANOVA followed by LSD test). Paired values within rows marked with an asterisk are significantly different ($P < 0.05$, t test) and NS indicates lack of significance.

In contrast, *C. rosaceana* and *A. velutinana* oriented toward their respective polyethylene-tube dispensers from heights (*ca.* 2 - 3 m above ground level) above the table. Upon leaving the arena, the majority of moths from all species flew upwind.

Activity period.

C. rosaceana (17 June – 2 September) and *A. velutinana* (30 June – 8 September) were observed at the observational arena and captured in traps after sunset between 21:45 and 00:15. During their first generation of adult flight (5 May – 9 June), *G. molesta* were observed at the observational arena and captured in traps before sunset between 16:40 and 20:15. Activity of subsequent generations occurred later, between 19:30 and 21:30 (before sunset) July through September. The above described diel rhythms of responsiveness of *C. rosaceana* and *G. molesta* to polyethylene-tube pheromone dispensers were identical in both the untreated and pheromone-treated orchards. *C. pomonella* were captured in traps after sunset between 21:30 and 23:30 June through late August.

DISCUSSION

Throughout their respective adult generations, *C. rosaceana, G. molesta*, and *A. velutinana* were consistently attracted within 100 cm of their respective high-release, polyethylene-tube pheromone dispensers in an untreated apple orchard. *C. rosaceana* and *G. molesta* were also frequently observed orienting to their respective polyethylene-tube dispensers in a comparison orchard treated with standard densities (*ca.* 1 per tree) of such dispensers for mating disruption. Using field wind tunnels, Cardé et al. (1997; 1998) observed the behavioral interactions of laboratory-reared pink bollworm moths,

Pectinophora gossypiella (Saunders), with high-release pheromone disruption dispensers targeting this species (PBW-Ropes, Shin-Etsu, Tokyo, Japan).

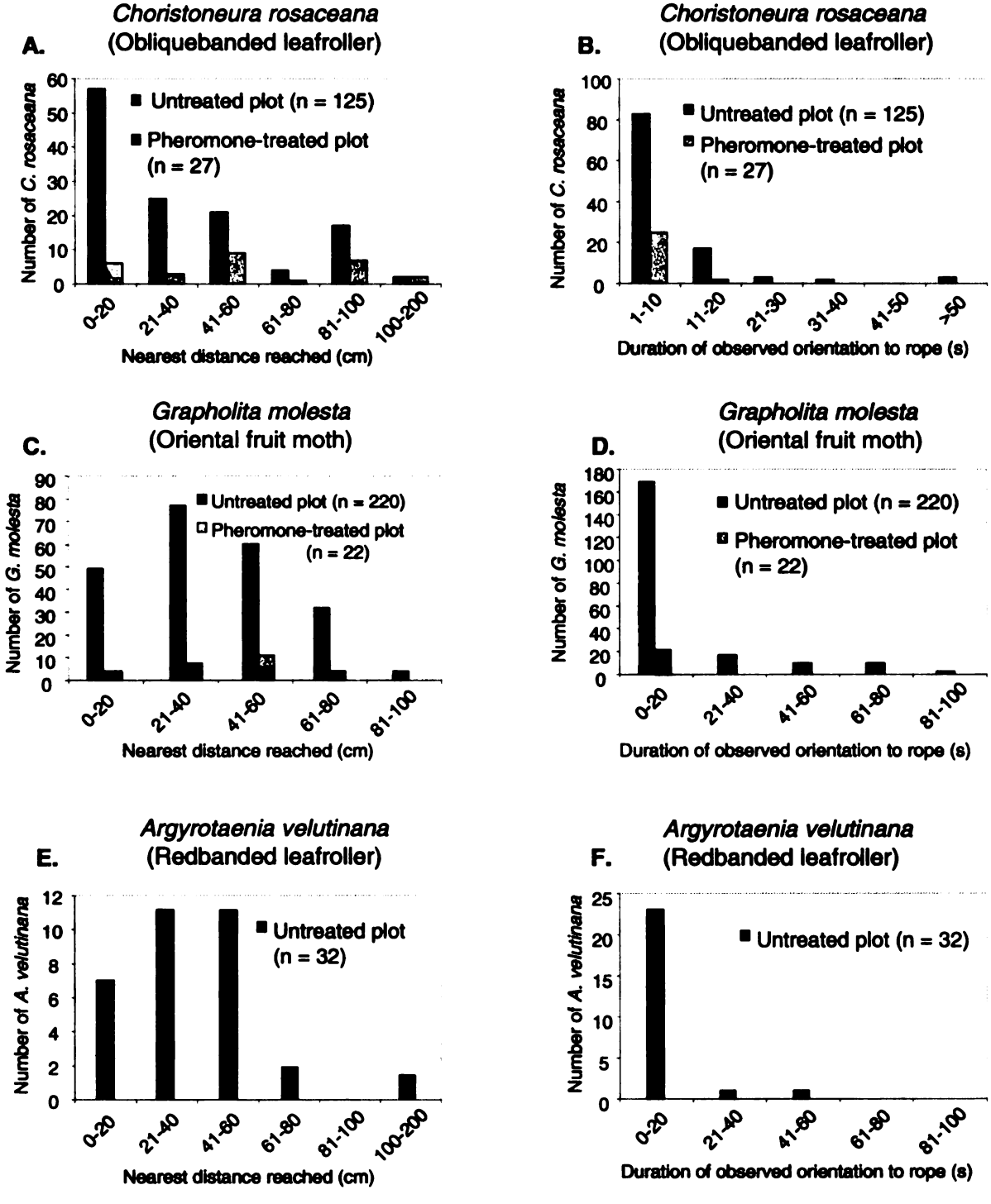


Figure 23 A. Nearest distance reached by feral oblique-banded leafrollers, *Choristoneura rosaceana*, attracted to Isomate OBLR/PLR dispensers in an untreated or pheromone-treated orchard. B. Duration of stay of *C. rosaceana* observed in close proximity to Isomate OBLR/PLR dispensers. C. Nearest distance reached by feral Oriental fruit moths, *Grapholita molesta*, attracted to Isomate-M Rosso dispensers in an untreated or pheromone-treated orchard. D. Duration of stay of *G. molesta* observed in close proximity to Isomate-M Rosso dispensers. E. Nearest distance reached by feral redbanded leafrollers, *Argyrotaenia velutinana*, attracted to Isomate OBLR/PLR dispensers in an untreated or pheromone-treated orchard. F. Duration of stay of *A. velutinana* observed in close proximity to Isomate OBLR/PLR dispensers. No *A. velutinana* were observed approaching Isomate OBLR/PLR dispensers in the pheromone-treated orchard.

In that study, released *P. gossypiella* also approached and often contacted dispensers. The majority of these males left the dispensers within a few minutes and many walked while wing-fanning on cotton foliage nearby. Our data corroborate and extend the Cardé et al. (1998) findings documenting that moths approach and interact with high-release polyethylene-tube dispensers.Attraction of male moths to polyethylene-tube dispensers occurred in three of the four species observed in this study. This finding suggests that false-plume-following may play an important role in mating disruption using such dispensers. Polyethylene-tube dispensers attracted approximately equal numbers of *A. velutinana*, *G. molesta*, and *C. rosaceana* as were captured in traps with optimally tuned monitoring lures in an untreated orchard. If the nightly observations made at a single

polyethylene-tube dispenser in this study reflect what is taking place at the majority of dispensers in a treated plot, then direct competition by sources of sex-attractant would decrease the time available for males to find females. However, under high population densities of calling females, such competition may be insufficient to prevent males from eventually finding and mating with a female after orientations to synthetic pheromone dispensers. Therefore, efficacy of mating disruption under conditions of high populations may necessitate that the management strategy invoke other mechanisms such as camouflage of female plumes.

An additional effect of false-plume-following to dispensers may, in some cases, be the neurophysiological modifications induced by exposure to high dosages of pheromone (Cardé et al., 1997; 1998). Consequential neurophysiologically-mediated modifications of normal responsiveness to pheromone occur in moths following laboratory exposures to pheromone (Bartell and Lawrence, 1977; Linn and Roelofs, 1981; Sanders, 1985; 1996; Figuerdo and Baker, 1992; Anderson et. al., 2003; Stelinski et al., 2004a) at dosages similar to those occurring adjacent to polyethylene-tube dispensers. The most recent measures of average air-borne pheromone concentrations achieved at sites roughly equidistant from polyethylene-tube dispensers deployed at recommended densities were 1.7 ± 15 ng/m^3 of *P. gossypiella* pheromone and 1.9 ± 0.4 ng/m^3 of *C. pomonella* pheromone (Koch et al., 1997, Koch et al., 2002). However, studies with various moth species reveal that the concentrations of pheromone necessary to reduce positive behavioral responsiveness or peripheral sensitivity are substantially greater than those that can be achieved in an average volume of air in a crop under mating disruption. For example, confinement of *Lobesia botrana* Den and Schiff., in

vineyard plots treated with polyethylene-tube dispensers (1 dispenser/5 m^2, each dispenser containing 500 mg of (*E*)-7,(*Z*)-9-dodecadienyl acetate) for 8 hr did not affect the moths' ability to subsequently find pheromone point-sources in untreated plots and reduction of behavioral responsiveness to pheromone was only induced when exposure concentrations in the laboratory reached 4 µg/l of pheromone (Schmitz et al., 1997). Furthermore, reduction of behavioral responsiveness to pheromone for male *G. molesta* occurred only after one h of laboratory exposure to its pheromone at a concentration of 65 $\mu g/m^3$ (3200 female equivalents) (Rumbo and Vickers, 1997). In addition, EAG measurements revealed that long-term effects on peripheral sensitivity in *C. rosaceana* occurred only after minutes-long confinement in the laboratory at concentrations of pheromone of at least 1 ng/ml or in the field only when confined for 24 hr within a few cm of Isomate OBLR/PLR dispensers (Stelinski et al., 2003b). Collectively, current data suggest that air-borne concentrations of pheromone in field plots treated with mating disruption dispensers are insufficient to reduce positive behavioral responses to pheromone. Therefore, we suggest that neurophysiological modifications, thought to be important contributors to mating disruption, are induced only when moths come close to high-release devices. Thus, it may be critically important that mating disruption formulations/dispensers attract moths within cm of the source. The data presented here establish that at least three species of tortricids do come within a few cm of their respective polyethylene-tube dispensers.

In addition to long-term neurophysiological modifications, real-time effects on peripheral sensitivity may influence moth behavior as they approach polyethylene-tube dispensers in the field. Kuenen and Baker (1981) documented that moths, challenged by

continuous exposure to pheromone in the laboratory, experienced instantaneous antennal adaptation as measured by EAGs. However, no long-term effects were found after removal of moths from pheromone. Therefore, it is possible that peripheral adaptation may be induced in moths orienting along plumes generated by polyethylene-tube dispensers. Interestingly, tortricid moths, such as *G. molesta*, are capable of orienting along the edges of concentrated pheromone plumes or walls of pheromone in wind tunnels (Kennedy et al., 1981, Willis and Baker, 1984). Such documented behaviors may partially explain how tortricids were able to orient along plumes from high-dosage polyethylene-tube dispensers in this study.

The exception to the general pattern of male moth attraction to polyethylene-tube dispensers was that no *C. pomonella* were observed orienting to or landing near their dispensers throughout the entire season in either the untreated or pheromone-treated orchards, despite being present at the research site (Table 5). However, *C. pomonella* has been documented to closely approach an earlier version of commercial polyethylene-tube dispensers (Isomate-C, containing 180 mg of 51.8 % of (*E*,*E*)-8,10-dodecadien-1-ol, 29.1 % dodecanol, 6.0 % tetradecanol, and 13.1 % inert ingredients) (Barrett, 1995) that contained 25 mg less total pheromone than the dispensers used in the current study. The finding that *C. pomonella* did not approach Isomate-C Plus dispensers in this study was not due to low moth population densities, given high captures of male *C. pomonella* in monitoring traps (Table 1) and in orchards directly surrounding ours (data available online: http://www.maes.msu.edu/tnrc/). Despite the lack of observed visits to Isomate-C Plus dispensers by *C. pomonella* in either the untreated or pheromone-treated orchard, *C.*

pomonella captures in monitoring traps were nearly completely inhibited by the pheromone treatment (Table 5).

We can only speculate why the Isomate-C Plus dispensers used in the current study did not elicit close visits. It is possible that *C. pomonella* locked onto plumes from these high-release pheromone dispensers but became arrested (Baker and Cardé, 1979) and exited those plumes at a distance downwind of the observational arena before such behavior could be noted. Whether lower-release, point-source dispensers could attract *C. pomonella* closer than high release counterparts should be investigated further (Charmillot, 1990). Alternatively, it is plausible that disruption of *C. pomonella* orientation to traps occurred through a different mechanism, e.g., plume camouflage.

It is also possible that we did not see *C. pomonella* approach dispensers at the observational arena because they may have been positioned too low with respect to this species' normal residence within the tree canopy. Dispensers at the observational arena were positioned *ca.* 1.0 m above ground level amongst trees averaging 2 m canopy heights. *C. pomonella* have been reported to occur primarily in the upper third of the tree canopy (Rothschild, 1982) and recommendations for placement of monitoring traps have also been set within the upper third of the canopy (Reidl et al., 1986) or at "mid-canopy" height (Gut and Brunner, 1994). Thus, further observations of field-deployed Isomate-C Plus dispensers at varying heights above 1.0 m must be conducted before it can be concluded that *C. pomonella* never approach them closely.

Witzgall et al. (1999) also observed behaviors of *C. pomonella* in orchards treated with pheromone. The dispensers used were either resin-treated cellulose flakes or polyethylene-tubes (similar to the ones in the current study) containing codlemone

(*E*8,*E*10-12OH; OH), codlemone acetate (*E*8,*E*10-12Ac; Ac) or a blend of these two components. Overall, more male *C. pomonella* were observed flying in pheromone-treated orchards compared with untreated controls, implying that male *C. pomonella* were attracted to the treated orchards. However, close-range attraction to dispensers, as observed for *C. rosaceana*, *G. molesta* or *A. velutinana* in this study, was rarely observed for *C. pomonella*. Also, Witzgall et al. (1999) observed approximately equal attraction of male *C. pomonella* to dispensers releasing codlemone as to dispensers releasing both codlemone and the behavioral antagonist, codlemone acetate (Hathaway et al., 1974, Witzgall et al., 1997). Furthermore, they observed increased attraction to dispensers releasing codlemone in the presence of dispensers containing codlemone acetate. These authors postulated that adaptation or habituation might explain the observed attractiveness of dispensers releasing the behavioral antagonist, codlemone acetate. In addition, they concluded that using blends of pheromonal attractants and antagonists may improve disruption of *C. pomonella*, as such blends may reduce the chance of long-range attraction of male *C. pomonella* to treated orchards and concurrently induce increased close-range responsiveness to pheromone dispensers.

Interestingly, we also observed significant attraction of male *C. rosaceana* to Isomate OBLR/PLR dispensers in this study and in a recent wind tunnel investigation (Stelinski et al., 2004) even though they contained the behavioral antagonist, (*Z*)-9-tetradecenyl acetate (Evenden et al., 1999 c). Evenden et al. (1999 c) found that application of dispensers containing a 1:1 mixture of (*Z*)-9-tetradecenyl acetate and the 4 component Western *C. rosaceana* pheromone (Vakenti et al., 1998) achieved levels of orientational disruption to virgin-females (>83 %), in small-plot trials, that were

approximately equal to that achieved with dispensers containing the pheromone alone. Evenden et al. (1999 a, b, c) concluded that mating disruption was unlikely to be mediated by false-plume-following and more likely to be mediated by camouflage or receptor adaptation, given that dispensers with incomplete blends or containing a behavioral antagonist were not less effective than those containing multi-component, attractive blends. The results of the current study caution that direct behavioral observations are required before inferentially ruling out the importance of false-plume-following because of the presence of a compound that in different circumstances performed as an antagonist. If false-plume-following is an important mechanism mediating mating disruption of *C. rosaceana,* then perhaps the addition of this antagonist to currently marketed dispensers for mating disruption has made them less effective. More work needs to be conducted examining whether the addition of this antagonist to dispensers influences the level of mating disruption for *C. rosaceana.*

Improved understanding of the general mechanisms underlying mating disruption among pest species should help pest managers improve the efficacy and practicality of this management tactic. The comparative approach taken here, where multiple species are studied in a common habitat, was valuable given the behavioral differences uncovered between species. Although some investigators have stressed the difficulty in conducting direct visual observations of male moths responding to pheromone sources under authentic field conditions, our experience is that this is indeed possible. Moreover, the data produced are highly useful in elucidating the mechanisms of pheromone disruption.

CHAPTER SEVEN

Effects of seconds-long pre-exposures to rubber septum or polyethelene-tube pheromone dispensers on subsequent behavioral responses of male *Grapholita molesta* (Lepidoptera: Tortricidae) in a sustained-flight tunnel.

Also submitted for pupilication in:

Stelinski, L. L., K. J. Vogel, J. R. Miller, and L. J. Gut. Effects of seconds-long pre-exposures to rubber septum or polyethelene-tube pheromone dispensers on subsequent behavioral responses of male *Grapholita molesta* (Lepidoptera: Tortricidae) in a sustained-flight tunnel. *Environmental Entomology*. Submitted November, 2004.

ABSTRACT

Male Oriental fruit moths, *Grapholita molesta* (Busck), were briefly pre-exposed in a wind tunnel to plumes from a rubber septum lure releasing a 3-component, optimally-attractive pheromone blend for this species or to plumes generated by Isomate-M Rosso pheromone dispensers. A greater proportion of *G. molesta* males took flight and successfully oriented toward a lure 15 min and 24 h after briefly orienting in plumes generated by an identical lure compared with unexposed, naïve moths or control moths pre-exposed to clean air. The mean duration of sustained flights of lure-pre-exposed male *G. molesta* in plumes generated by a lure was significantly shorter 15 min and 24 h after pre-exposure compared with that of naïve moths. Statistically equal proportions of lure-pre-exposed *G. molesta* not orienting during the pre-exposure treatment contacted the lure 15 min after exposure compared with naïve or control moths. In addition, statistically equal proportions of lure-pre-exposed *G. molesta* not orienting during the pre-exposure treatment contacted the lure or oriented without source contact 24 h after exposure. The mean duration of sustained flights to lures by *G. molesta* not orienting during the pre-exposure treatment was significantly shorter than that for naïve moths. Only 9 % of naïve *G. molesta* oriented when placed into plumes generated by Isomate-M Rosso dispensers during and after pre-exposure treatments and none contacted this type of dispenser. The proportion of male *G. molesta* contacting lures 15 min and 24 h after pre-exposure to ropes was not statistically different from the proportions of naïve or control moths contacting the lure or orienting without source contact. However, as observed with moths pre-exposed to a lure, the mean duration of sustained flights of male *G. molesta* pre-exposed to an Isomate-M Rosso dispenser was significantly shorter than that of naïve

moths 15 min and 24 h after pre-exposure. Mean durations of sustained flights of male *G. molesta* pre-exposed to a lure or rope were significantly longer after 24 h compared with 15 min after the exposure treatment, indicating that the effect of pheromone pre-exposure decayed over time. Electroantennograms recorded 15 min and 24 h after pre-exposures to lures or Isomate-M Rosso dispensers in the flight tunnel were indistinguishable from those recorded from unexposed moths. We suggest that false-plume-following by naïve male *G. molesta* combined with decreases in duration of subsequent anemotactic orientations following previous bouts of false-plume-following may explain why Isomate-M Rosso dispensers are effective in mating disruption experiments with *G. molesta.*

INTRODUCTION

The Oriental fruit moth, *Grapholita molesta* (Busck), is a major pest of stone fruit trees (Rosaceae) worldwide; it attacks shoots and feeds internally within fruits (Rothschild and Vickers 1991). Efforts to develop mating disruption as a means of controlling this pest began decades ago (Gentry et al. 1974, 1975, Rothchild 1975, Cardé et al. 1977, 1979). Promising results from some of these early studies led to the development of a commercial polyethylene dispenser (Isomate-M, Shin-Etsu Chemical Co., Tokyo, Japan) for releasing *G. molesta* pheromone into orchards (Vickers 1990). These and subsequent versions (Isomate-M 100 and M Rosso) of dispensers proved effective in numerous field trials (Pfeiffer and Killian 1988, Audemard et al. 1989, Rice and Kirsh 1990, Pree et al. 1994, Trimble et al. 2001, Atanassov et al. 2002, Trimble et al. 2004). Thus, *G. molesta* is a good subject for studying the factors underlying effective mating disruption.

Moth exposures to pheromone induce a range of responses. Various investigations have documented peripheral adaptation or sensory fatigue in moths after intense exposure to their species-specific pheromones (Bartell and Roelofs 1973, Bartell and Lawrence 1976a, 1976b, 1976c, Linn and Roelofs 1981, Sanders 1985, Stelinski et al. 2003 a, b). Such studies have shown that prolonged exposure to pheromone decreases stereotyped behavioral responses by males, including wing fanning and rapid walking (Bartell and Roelofs 1973), captures in pheromone-baited traps in the field, and orientation to optimized pheromone dispensers in wind tunnels (Rumbo and Vickers 1997, Daly and Figueredo 2000). In addition, peripheral adaptation of moth olfactory receptor neurons has been quantified electrophysiologically (e.g., Baker et al. 1989, Kuenen and Baker 1981, Marion-Poll and Tobin 1992, Schmitz et al. 1997, Stelinski et al. 2003a); major outcomes are decreased electroantennogram (EAG) responses or decreased spike frequencies from single sensillae during and after prolonged pheromonal stimulation.

In contrast to decreases in behavioral or antennal responsiveness after pheromone exposure, other studies report increases in behavioral response in moths briefly pre-exposed to pheromone. For example, Sanders (1984, 1995) found that the percentage of male *Choristoneura fumiferana* successfully locking onto and flying to sources of pheromone off-blends dramatically increased following brief pre-exposures to calling females or a 95:5 blend of *E*:*Z*-11-tetradecenal. This effect was termed "priming" and thought to possibly increase false-plume-following of males in mating disruption regimes using pheromone off-blends. Similarly, responses of *Grapholita molesta* (Busck) to off-blends of pheromone (blends containing high % *E*) that normally elicited few completed

flights from naïve moths, increased after minutes-long pre-exposures to *E*-8-dodecenyl acetate (Linn and Roelofs 1981).

Heightened behavioral responses to female-produced or optimally-tuned synthetic sources of pheromone following pre-exposure to such chemicals are also known to be markedly long-lasting. Specifically, Anderson et al. (2003) described a pronounced increase in behavioral response to presentations of the female sex-pheromone in male *Spodoptera littoralis* having been briefly pre-exposed to a female-produced plume. This abnormally high responsiveness in exposed moths lasted up to 27 h and was not associated with a change in peripheral sensitivity. More recently, Stelinski et al. (2004a) have described enhanced behavioral responses of *Choristoneura rosaceana* to pheromone sources in wind tunnel studies following seconds- and minutes-long pre-exposures to pheromone dispensers, including Isomate OBLR/PLR polyethylene tubes intended for mating disruption. Not only did the proportion of male moths locking onto sources of pheromone increase following pre-exposure, but also the duration of sustained flights increased by up to 4-fold. Interestingly, the same study revealed that the behavioral responses of *Argyrotaenia velutinana*, after identical exposures to those imposed on *C. rosaceana*, decreased for up to 24 after the pre-exposure treatment and the study found no evidence of "behavioral priming" for this species following pheromonal pre-exposure (Stelinski et al. 2004a). These authors suggested that the difference in behavioral responses between *C. rosaceana* and *A. velutinana* following brief pre-exposure to pheromone may explain why successful mating disruption has been more difficult to achieve in the former species compared with the latter as determined by field studies (Novak et al. 1978, Reissig et al. 1978, Novak and Roelofs 1985).

The current study focused on describing the effects of brief pre-exposure to the currently marketed Isomate-M Rosso dispensers and optimally-tuned, rubber septum lures, which approximate females, on the subsequent behavior of *G. molesta* in an effort to gain insights into why mating disruption has proven so successful for this species. The specific objectives were to determine whether/how brief pre-exposures to low- and high-dose pheromone dispensers affects: 1) initiation of anemotaxis, 2) duration of sustained anemotactic flight, and 3) peripheral sensitivity 15 min and 24 h after exposure.

MATERIALS AND METHODS

Insects.

G. molesta were drawn from a two-year-old laboratory colony at Michigan State University (East Lansing, MI, USA) originally collected as larvae from apple orchards in Southwest Michigan. Moths were reared at 24 °C and 60 % RH on pinto bean-based diet (Shorey and Hale, 1965) under a 16:8 (L:D) photoperiod. Pupae were sorted by sex and emerged in 1 L plastic cages containing 5 % sucrose in plastic cups with cotton dental wick protruding from their lids.

Chemicals and Release Devices.

The behavioral responses of *G. molesta* were quantified in a sustained-flight tunnel using two types of pheromone dispensers. First, a pheromone blend attractive to this species was formulated in red rubber septa. The rubber septa were loaded with 3 µg of (*Z*)-8-dodecen-1-yl-acetate : (*E*)-8-dodecen-1-yl-acetate : (*Z*)-8-dodecen-1-ol in a 100 : 6 : 10 blend (Willis and Baker, 1984). Pheromone blend solutions used to load rubber septa were prepared in HPLC grade hexane and stored at -18 ° C. Henceforth, such rubber septum dispensers formulated specifically for this species will be referred to as

'lures'. The polyethylene-tube pheromone dispensers used were Isomate-M Rosso containing 250 mg of 88.5 % (*Z*)-8-dodecen-1-yl-acetate, 5.7 % (*E*)-8-dodecen-1-yl-acetate, 1.0 % (*Z*)-8-dodecen-1-ol, and 4.8 % inert ingredients. These polyethylene tube dispensers will be referred to as 'ropes'. All rope dispensers were aged for 2 wk in a laboratory fume hood prior to use in behavioral assays to allow dissipation of pheromone that might have built up on rope surfaces during shipping and freezer storage.

Wind tunnel.

Behavioral assays were conducted in the Plexiglas sustained-flight tunnel detailed by Stelinski et al. (2004a). Briefly, the working section of this wind tunnel measured 1.3 x 0.8 m in cross section and 2.4 m long. It was housed in a temperature-controlled chamber maintained at 23 °C and 50-70 % RH. Light intensity was 700 lux inside the tunnel and was generated by 2 fluorescent bulbs (Philips model F96T12, 95 Watt) mounted 22 cm above the top of the wind tunnel. A variable speed, blower-type fan (Dayton 5C090C, Northbrook, IL) pushed air through the tunnel at 0.3 m/s. The pheromone plume emerging from the tunnel was expelled from the building through a roof-mounted stack.

Wind Tunnel Assays.

The wind tunnel assay procedures were a slight modification of those described by Stelinski et al. (2004). Male *G. molesta*, 4-6 days old, were placed into cylindrical (17 cm long x 8 cm diam.) wire-mesh release cages 3 h prior of the end of a 16 h photophase. Each cage, containing 1 or 5 moths (depending on experiment), was placed into the wind tunnel for 1 h of acclimation prior to assays. Subsequently, bioassays ran for a maximum of 1.5 h terminating at 0.5 h prior to the end of the moths' normal photophase. At the

upwind end of the tunnel, pheromone dispensers (lures or ropes) were placed 1 cm above a horizontal 7.5 x 12.5 cm yellow card attached to a horizontally-clamped 9 cm glass rod attached to a steel ring-stand. Pheromone was released 25 cm above the tunnel floor in stationary-floor experiments and 10 cm above the floor in moving-floor experiments. Wire-mesh release cages holding 5 male moths were placed at the down-wind end of the tunnel at a height matching that of the pheromone dispenser. In sustained-flight experiments, release cages were 10 cm long x 8 cm diam. and contained only one male moth.

Males were allowed 5 min to respond to an inserted pheromone dispenser. The behaviors recorded were: wing-fanning only; non-anemotactic flight from the release cage; upwind anemotactic flight without touching the release device; upwind anemotactic flight followed by landing on the platform and touching the release device. Also, the numbers of individuals with no detectable behavioral change were recorded. Across all pheromonal treatments tested, there were few instances of significant differences between mean numbers of moths wing fanning only or exhibiting non-anemotactic flights out of release cages between treatments tested. Thus, for clarity, in table 6 I only present the proportions of moths that oriented with and without source contact versus those exhibiting no behavioral change.

Release cages, ring-stands, and glass rods were thoroughly washed with acetone after daily use. The interior of the wind tunnel was also briefly scrubbed with an acetone-soaked rag and immediately rinsed with water so as not to damage the plexiglas. The exhaust fan ran for at least 4 h after assays were completed.

Experiment 1.

This experiment tested the effect of brief exposures of *G. molesta* to pheromone plumes generated either by lures or rope dispensers on subsequent responsiveness of male moths to these pheromone sources either 15 min or 24 h after the initial exposure treatment. Groups of 5 male moths were released in plumes generated by: 1) a rubber septum described above; or 2) a rope (standard pheromone dispenser used in mating disruption). Moths were allowed 5 min to respond during all pre-exposure treatments. Moths reaching or orienting to the pheromone source but not contacting it were segregated from those that either did nothing, wing-fanned, or flew out of release cages without orienting. Mean duration of moth orientations during the pre-exposure treatment was *ca.* 15 s. Recaptured moths (both 'orienters' and 'non-orienters') were assayed in the wind tunnel again to each pheromone source either 15 min or 24 h after the initial pheromone pre-exposure. Moths tested 24 h after the pre-exposure treatment were kept in an environmental chamber under the temperature and light-cycle conditions described above for the interval prior to testing. The combined initial pre-exposure treatments and subsequent assays were: 1) pre-exposure to a lure followed by wind-tunnel assay using a lure; 2) pre-exposure to a rope followed by wind tunnel assay using a lure; 3) pre-exposure to a rope followed by wind-tunnel assay using a rope; 4) pre-exposure to clean air followed by wind-tunnel assay using a lure. 'Naïve' will refer to moths having no prior exposure to pheromone or the wind tunnel prior to assay. 'Control' will refer to moths pre-exposed to moving air, but no pheromone in the wind tunnel. 'Pre-exposed' will refer to moths pre-exposed to pheromone in the wind tunnel. This pheromone may have emanated from a lure or rope.

To avoid bias due to possible slight variations between days, all treatment combinations were tested each day of testing. Treatment order was also randomized daily to equalize any effect of time before what would have been the onset of scotophase.

Experiment 2.

This experiment tested the effect of brief exposures of *G. molesta* to pheromone plumes generated by lures or rope dispensers on the duration of sustained flights of male moths 15 min and 24 h after initial exposure. As in Experiment 1, moths reaching the pheromone source or orienting to the source but not contacting it during pre-exposure were segregated from those either doing nothing, wing-fanning only, or flying out of release cages without orienting to the source. The response of recaptured moths to plumes generated by lures was assayed 15 min and 24 h after the initial pheromone exposure. After moths locked onto the pheromone plume generated by a lure, the moving floor was activated to prolong flights. We recorded the duration of sustained flight along the pheromone plume. For pre-exposed male *G. molesta*, mean durations of sustained flights following the pre-exposure treatment did not differ statistically between those moths that oriented during pre-exposure versus those that did not (see results). Therefore, for comparison between treated and control moths mean flight durations of pre-exposed moths were combined whether or not they oriented during pre-exposure (see Fig. 24).

Experiment 3.

This experiment also tested the effect of brief pre-exposures of male *G. molesta* to pheromone plumes generated by 3 μg lures as above on the duration of sustained flights of male moths 15 min after initial exposure. However, moths were assayed using lures of various loading dosages 15 min after the pre-exposure treatment. Our hypotheses were

that if pre-exposure treatments increased moth sensitivity 15 min later, then durations of sustained flights should increase as lure loading dosage decreased. Alternatively, if pre-exposure treatments decreased moth sensitivity, then durations of sustained flights should increase as lure dosage increased. The combined initial pre-exposure treatments and subsequent assays were: 1) pre-exposure to a 3 μg lure followed by wind-tunnel assay using a 9 μg lure; 2) pre-exposure to a 3 μg followed by wind tunnel assay using a 3 μg lure; 3) pre-exposure to a 3 μg lure followed by wind-tunnel assay using a 1 μg lure; 4) pre-exposure to a 3 μg lure followed by wind-tunnel assay using a 0.5 μg lure; 5) pre-exposure to clean air followed by wind-tunnel assay using a 3 μg lure; 6) pre-exposure to clean air followed by wind-tunnel assay using a 0.5 μg lure. All lures were loaded with the pheromone blend described under *Chemicals and Release Devices* and durations of sustained flights were measured as described under *Experiment 2*. All treatment combinations were tested each day of testing and treatment order was randomized daily to equalize any effect of time before what would have been the onset of scotophase.

Electroantennogram Assays (Experiment 4).

This experiment tested the hypothesis that briefly exposing *G. molesta* to pheromone plumes generated by lures or high-release rope dispensers affected EAG responses of moths 15 min or 24 h after initial exposure. The EAG system and test protocols were detailed by Stelinski et al. (2003a, 2004a). EAG cartridges were made by pipetting various concentrations (2 μg – 20 mg) of pheromone in hexane (20 μL total solution) onto 1.4 x 0.5 cm strips of Whatman No. 1 filter paper. After 5 min in a fume hood for solvent evaporation, treated strips were inserted into disposable glass Pasteur pipettes. Pre-exposed *G. molesta* were mounted for EAG analysis either 15 min or 24 hr

following pre-exposure. Pheromone dosages were delivered alternately to both naïve and pheromone pre-exposed moths in ascending order of dosage ($N = 12$ per treatment). Four 1-ml puffs spaced 12 s apart were administered to each antenna at each dosage.

Statistical Analyses.

For Experiment 1, a logistic model was used to measure the probability that a combination of the two factors: pheromone delivery device (rubber septum or rope) x moth type (naïve, pre-exposed orienter, or pre-exposed non-orienter) would result in a particular behavioral category as defined above using the PROC GENMOD procedure in SAS (SAS Institute, 2000). Subsequently, analyses of numbers of male moths responding were carried out using the G statistic (Sokal and Rolf, 1981). For Experiments 2 and 3, data for sustained-flight duration were transformed to ln (x + 1) (which normalized the distributions) and then subjected to ANOVA; differences in pairs of means were separated using Tukey's multiple comparisons test (SAS Institute, 2000). For Experiment 3, data were subjected to ANOVA (SAS Institute, 2000). In all cases, the significance level was $\alpha < 0.05$.

RESULTS

Experiment 1.

Compared with naïve or control males of the same age, a significantly greater proportion of male *G. molesta* contacted their respective lure 15 min and 24 h after brief pre-exposure to pheromone plumes generated by such a lure (Table 6). This result occurred only for those *G. molesta* orienting to the lure during the pre-exposure treatment. Compared with naïve or control males, statistically equal proportions of males oriented to lures without contacting the source 15 min and 24 h after brief pre-exposure

to a lure (Table 6). Compared with naïve or control moths, statistically equal proportions of males not orienting during the pre-exposure treatment to lures contacted the lure 15 min after exposure and contacted the lure or oriented without source contact 24 h after exposure.

Only 9 % of naïve *G. molesta* oriented when placed into plumes generated by ropes and none contacted this dispenser (Table 6). The proportion of male *G. molesta* contacting or orienting to lures without source contact 15 min and 24 h after pre-exposure to ropes was not significantly different from the proportions of naïve or control moths exhibiting these behaviors (Table 6). No *G. molesta* oriented to ropes 15 min or 24 h after pre-exposure to ropes.

Experiment 2.

Naïve male *G. molesta* sustained flights to lures significantly longer than did moths of the same age but pre-exposed to a lure or rope plume 15 min or 24 h earlier (Fig. 24). Furthermore, mean durations of sustained flights of male *G. molesta* pre-exposed to a lure or rope were significantly longer 24 h compared with 15 min after exposure treatment indicating that the effect of exposure decayed over time (Fig. 24). Mean durations of sustained flights 15 min after pre-exposure were nearly identical irrespective of pheromone dispenser type (Fig. 24). Likewise, mean durations of sustained flights 24 h after pre-exposure were similar between those moths pre-exposed to lures or ropes (Fig. 24).

Experiment 3.

Fifteen minutes after pre-exposure, male *G. molesta* exposed to clean air devoid of pheromone sustained flights to 3 and 0.5 μg lures significantly longer than did males

pre-exposed to 3 μg lures irrespective of the loading dosage of lures used during the assay after pre-exposure (Fig. 25). There were no significant differences in mean durations of sustained flights to the four assay dosages tested 15 min after pre-exposure to 3 μg lures, but the mean duration of sustained flights to 0.5 μg lures was slightly shorter compared with the other dosages tested (Fig. 25). However, moths pre-exposed to clean air (control) sustained significantly shorter flights to 0.5 μg lures compared with 3 μg lures (Fig. 25).

Table 6. Response of male *Grapholita molesta* to lures or Isomate rope dispensers 15 min or 24 hr after pre-exposure to either clean air, a lure or a rope dispenser.

Moth type and Pheromone source	*N*	Proportion of males exhibiting the indicated response[a]		
		No behavioral change	Orientation without source contact	Source contact
Naïve males				
Lure	105	0.21c[b]	0.18a	0.34b
Rope	96	0.72a	0.09b	0.00c
Pre-exposed orienters (15 min)				
Lure then Lure	73	0.08c	0.19a	0.66a
Rope then Lure	20	0.60ab	0.15a	0.20b
Rope then Rope	9	0.78a	0.00b	0.00c
Pre-exposed non-orienters (15 min)				
Lure then Lure	76	0.55b	0.05b	0.20b
Rope then Lure	75	0.33bc	0.12ab	0.28b
Rope then Rope	74	0.80a	0.00b	0.00c
Air then Lure[c]	70	0.21c	0.13ab	0.37b
Pre-exposed orienters (24 hr)				
Lure then Lure	72	0.27c	0.21a	0.50a
Rope then Lure	14	0.57b	0.29a	0.14bc
Rope then Rope	10	0.90a	0.00b	0.00c
Pre-exposed non-orienters (24 hr)				
Lure then Lure	67	0.20c	0.20a	0.39b
Rope then Lure	68	0.43b	0.17a	0.30b
Rope then Rope	67	0.82a	0.00b	0.00c
Air then Lure[c]	90	0.22c	0.14ab	0.46b

[a]Proportions of moths wing-fanning only or flying out without anemotactic orientation not shown.

[b]Numbers in the same column followed by the same letter are not significantly different (G^2 test of homogeneity, $P = 0.05$).

[c]Refers to control treatment.

Experiment 4.

Mean EAG responses of naïve male *G. molesta* were indistinguishable from those of moths assayed either 15 min or 24 h after pre-exposure to lures or rope dispensers (Fig. 26 A, B).

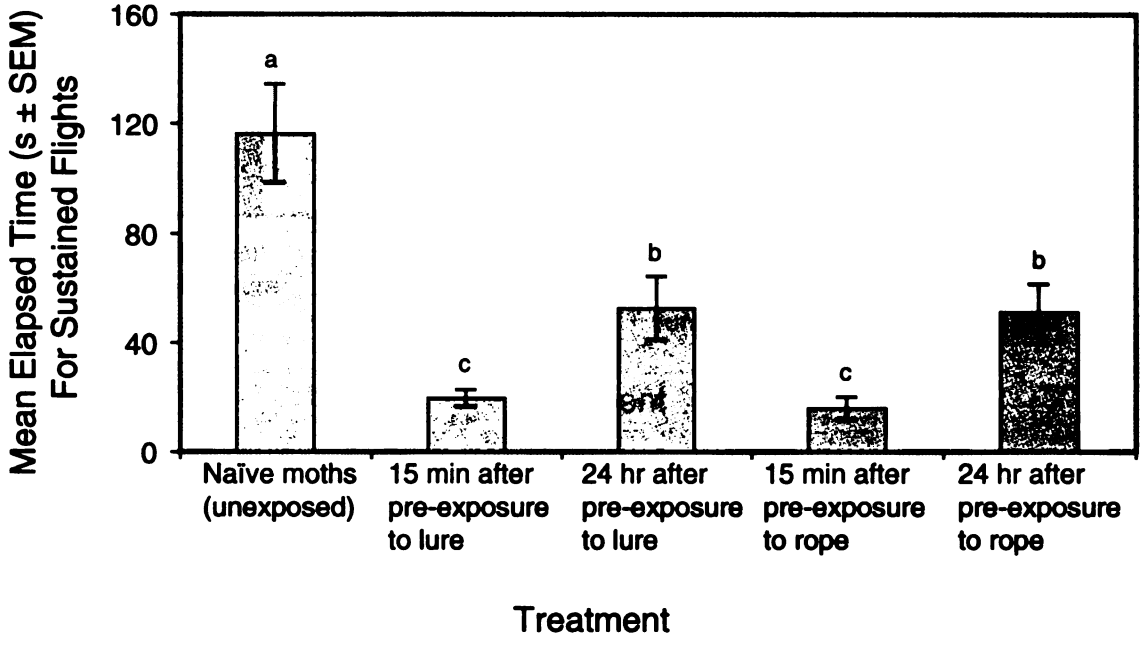


Figure 24. Duration of sustained-flights of naïve and pheromone pre-exposed *Grapolita molesta* 15 min and 24 h after pre-exposure to a lure or rope. $N = 41$ per treatment. Means followed by the same letter are not significantly different at $\alpha < 0.05$.

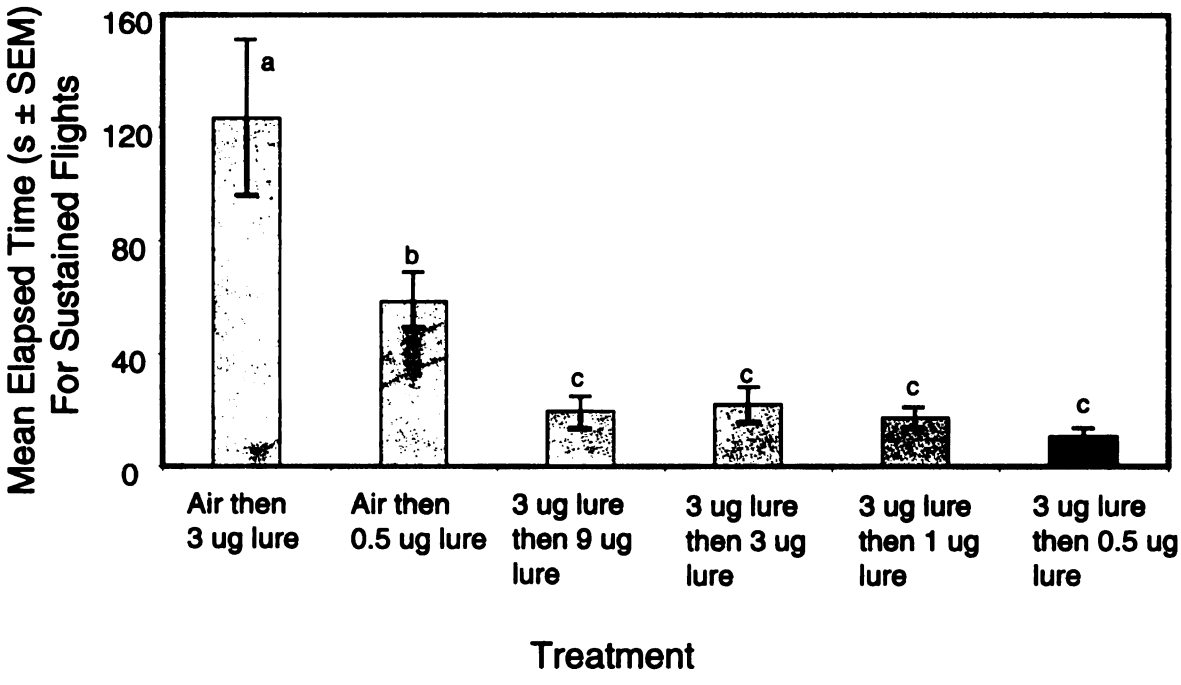


Fig. 25. Durations of sustained-flights to lures of various loading dosages recorded from male *Grapolita molesta* pre-exposed to clean-air or a 3 μg pheromone lure 15 min prior. $N = 30$ per treatment. Means followed by the same letter are not significantly different at $\alpha < 0.05$.

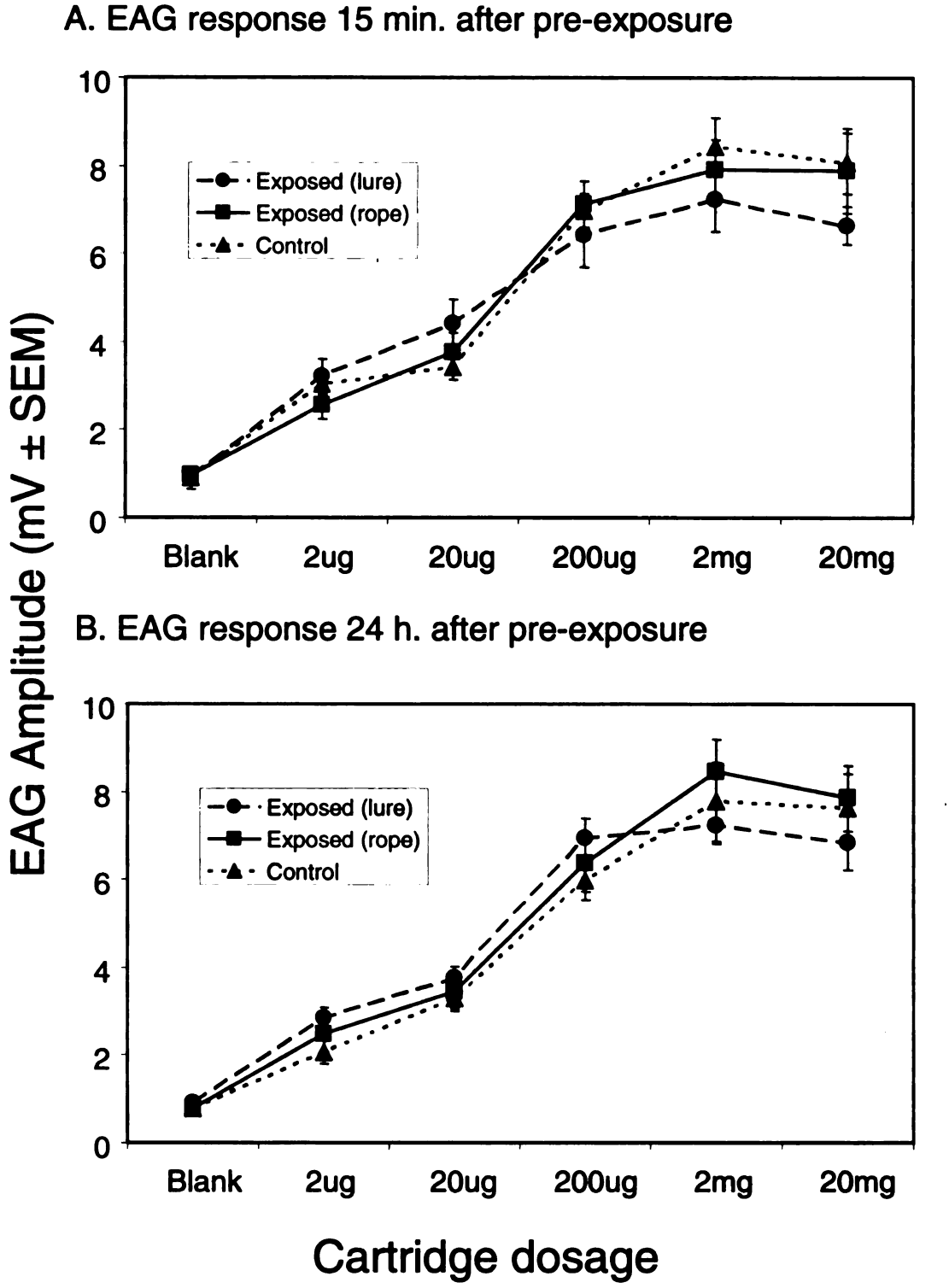


Figure 26. EAG dosage-response relationships for naïve and pheromone lure or rope pre-exposed *Grapholita molesta* (A. 15 min, B. 24 h after pre-exposure) using live-insect antennal preparations.

DISCUSSION

The proportion of completed anemotactic flights to a dispenser loaded with 3 μg of pheromone (3-componet tuned blend) increased only in those *G. molesta* orienting along a plume produced by such a dispenser either 15 min or 24 h prior. Such increased responsiveness to pheromone following pheromone pre-exposure has been documented in flight tunnel studies for *C. fumiferana* (Sanders 1984), *C. rosaceana* (Stelinski et al. 2004a), and *S. littoralis* (Anderson et al. 2003). Furthermore, increased responsiveness to pheromone has been observed in *G. molesta* following pre-exposure. Specifically, Linn and Roelofs (1981) found enhanced subsequent responses to off-blends of pheromone containing high % *E* following minutes-long pre-exposures of male *G. molesta* to *E*-8-dodecenyl acetate. In addition, Rumbo and Vickers (1997) found slightly increased responses of *G. molesta* to pheromone sources in a flight tunnel following 10 and 60 min (20 and 30 min of recovery, respectively) of pre-exposure in sealed containers with pheromone dispensers at release rates up to 320 times that of a single female moth ("320 female equivalents"). Diminution of responsiveness in the flight tunnel occurred for *G. molesta* only after the release rate of pheromone during pre-exposure was increased to 3200 female equivalents (Rumbo and Vickers 1997).

In the various flight tunnel studies discussed above, the enhanced behavioral responses of moths following pre-exposure to their pheromone were detected as increases in the occurrence of various behaviors including: wing fanning, take off, and the more complex anemotactic orientation with or without source contact. However, even the most complex overt behavior analyzed in the above studies, i.e. source contact, was very brief in space and time, occurring in flight tunnels no longer than 2 m. The activation of a

moving floor in our 2.4 m-long, sustained-flight tunnel revealed that although a higher proportion of certain pre-exposed *G. molesta* responded and maintained relatively brief anemotactic flights, the duration of orientation of all pheromone pre-exposed moths was considerably shorter compared with that of naïve, unexposed counterparts.

Pre-exposed male *G. molesta* were equally or in certain cases more apt to initiate anemotaxis, however only for short periods (< 15 s). Varying the loading dosage of lures in the assay following pre-exposure to 3 μg lures did not change the duration of sustained flights as we had postulated (Fig. 2). Our results suggest that pre-exposed *G. molesta* were not more sensitive to pheromone given that durations of sustained flights to 1 and 0.5 μg lures, which likely released pheromone below that of calling females (Figuerdo and Baker 1992, Baker et al. 1980), did not increase. Perhaps pre-exposed moths were more susceptible to desensitization (peripheral or central) following pre-exposure, which did not influence initiation of anemotaxis but prevented extended flights within the plume. The data suggest this change in sensitivity took place in the central nervous system (CNS) as no changes were detected at the periphery both 15 min and 24 h after pre-exposure (Fig. 26).

In addition to quantifying the effects of pheromone pre-exposure to plumes at concentrations approximating females, we also compared the effects of pre-exposures within plumes generated by a current mating disruption dispenser for *G. molesta*, i.e. Isomate-M Rosso 'ropes' (Trimble et al. 2004), which on average release pheromone at 29 mg/ha/hr (Sexton and Il'ichev 2001). We observed relatively few (9 %) successful orientations to such high-release dispensers in our flight tunnel. Moreover, the proportions of male *G. molesta* exhibiting no detectable behavioral change when placed

in plumes generated by Isomate-M Rosso dispensers were high, ranging from 72 to 90 % (Table 6). However, in a companion study, where direct observations of Isomate-M Rosso dispensers were conducted in the field, the mean number of feral *G. molesta* approaching such dispensers per day was substantial and statistically equal to the mean number approaching optimally tuned lures such as those used herein (Stelinski et al. 2004b). Willis and Baker (1984) found that *G. molesta* failed to progress upwind in flight tunnels when placed within a continuous and homogeneous cloud of pheromone. These authors suggested that arrestment of response in this situation may have been due to the lack of "a necessary phasic stimulation to maintain state-switching" in the CNS rather than to receptor adaptation. In our study, it is conceivable that the high pheromone release rate from Isomate-M Rosso dispensers generated sufficiently large plumes to envelope moths within release cages so as to produce an effect similar to that described by Willis and Baker (1984). Alternatively, the general lack of behavioral response may have been due to a rapid receptor adaptation given the overwhelmingly high release rate generated by these dispensers.

It is important to note, however, that tortricids including *G. molesta* will orient along the edges of concentrated pheromone plumes or along clean air-pheromone boundaries generated within flight tunnels despite the fact that orientation is inhibited directly within such homogeneous clouds (Kennedy et al. 1981, Willis and Baker 1984). This may explain why numerous *G. molesta* were observed orienting to and closely approaching Isomate-M Rosso dispensers in the field (Stelinski et al. 2004b) but not in the current flight tunnel assays. Thus, direct field observations should be conducted

before the conclusion is reached that moths do not approach high-release dispensers of pheromone based solely on flight-tunnel investigations.

Brief pre-exposures of *G. molesta* to Isomate-M Rosso dispensers did not decrease their ability to subsequently initiate anemotaxis to lures 15 min and 24 h later. However, pre-exposure to Isomate-M Rosso dispensers did reduce the durations of sustained flights to the same level measured after exposure to lures. Furthermore, durations of sustained flights increased over of time following pre-exposure to either a lure or rope (Fig. 24). These results suggest that orientations to Isomate-M Rosso dispensers by *G. molesta* might not reduce the propensity of such moths to initiate subsequent anemotactic orientations to other pheromone sources, including calling females. But, the distance and duration of anemotactic flight within pheromone plumes following previous exposure to an Isomate-M Rosso dispenser may be substantially reduced for at least 24 h. Thus, false-plume-following by naïve male *G. molesta* combined with decreases in duration of subsequent anemotactic orientations following previous bouts of false-plume-following may explain why Isomate-M Rosso dispensers have been so successful in disrupting orientation of *G. molesta* to traps in the field as observed by Trimble et al. (2004) and Stelinski et al. (2004b).

Figueredo and Baker (1992) suggested that lowered behavioral responsiveness of male *G. molesta* due to habituation to the pheromone of conspecific females may be advantageous from an evolutionary perspective. It should prevent males from responding to plumes from distant females when closer females may be nearby. Thus, enhanced male orientation to closer rather than more distant calling females within high population densities could increase mating frequency. We extend this evolutionary hypothesis and

propose that male *G. molesta* having previously oriented along plumes of pheromone from females or mating disruption dispensers do not lose their ability for initiating anemotaxis to subsequent identical presentations of pheromone. However, the durations of sustained flights in such plumes are substantially reduced following pre-exposure. Our data support the above hypothesis in that the distance of male orientations following an initial experience with a female's plume may decrease and thus restrict the male's searching range. However, our findings also indicate that the ability of males to initiate anemotaxis within a putative 'restricted searching range' may increase or remain unchanged. The overall effect would be to intensify local or patch search (Bell and Tobin 1982) at the expense of wide foraging. This phenomenon is common when foragers encounter highly rewarding resources or the cues closely associated with them (Bell and Tobin 1982, White et al. 1984). However, in the context of mating disruption using pheromones, males might get duped into restricted patch search when it is inappropriate for the actual distribution of authentic females.

SUMMARY AND CONCLUSIONS

Attraction of male moths to polyethylene-tube dispensers occurred in three of the four species observed in the above-described field study in Chapter 6. Furthermore, in more recent studies with *C. pomonella* in which dispensers were observed directly within tree canopies, males of this species were also observed approaching rope dispensers. Polyethylene-tube dispensers attracted approximately equal numbers of *A. velutinana*, *G. molesta*, and *C. rosaceana* as were captured in traps with optimally tuned monitoring lures in an untreated orchard. These results suggest that false-plume-following is an important mechanism mediating mating disruption using polyethylene-tube "rope" dispensers.

An additional effect of false-plume-following to dispensers may, in some cases, be the neurophysiological modifications induced by exposure to high dosages of pheromone (Cardé et al., 1998). Indeed, the flight tunnel investigations described in Chapters 4 and 7 revealed long-lasting behavioral modifications in each of the three species investigated following seconds-long, close-proximity encounters with their respective rope dispensers. Specifically, the proportion of male *C. rosaceana* locking onto sources of pheromone and the duration of sustained flights increased by up to 4-fold following seconds-long pre-exposure to Isomate OBLR/PLR Plus dispensers. Furthermore, the same study revealed that the behavioral responses of *A. velutinana*, after identical exposures to those imposed on *C. rosaceana*, decreased for up to 24 h after the pre-exposure treatment. Finally, seconds-long pre-exposures of *G. molesta* to Isomate M-Rosso dispensers increased in the proportion of males locking onto pheromones plumes but decreases in the duration of sustained flights along such plumes.

The above-discussed behavioral modifications may be consequestial for achieving effective mating disruption. Specifically, perhaps the behavioral differences between *C. rosaceana* and *A. velutinana* documented above may explain why the former species is easier to disrupt compared with the latter. However, with respect to the impact of peripheral adaptation on mating disruption, the long-lasting effects documented above for *C. rosaceana* are not likely an important contributor. The concentration of pheromone required to induce long-lasting adaptation is well above that which could be achieved in the field. Also, as directly observed in the field, feral *C. rosaceana* do not remain near rope dispensers long enough to induce this physiological phenomenon.

Although the electrophysiological differences between *A. velutinana* and *C. rosaceana* are interesting and potentially important from a basic perspective, it is unlikely that they contribute to the differences in succeptability to mating disruption between these two species. As documented in Chapter 6, neither *C. rosaceana* nor *A. velutinana* remain in close proximity to ropes in the field long enough to receive the required pheromone pre-exposure to induce long-lasting adaptation documented in Chapter 2. However, it is possible that higher-release devices such as Microsprayers, Puffers, or MSTRS could induced LLA.

Pre-exposure to pheromone similar to that observed in the field resulted in increases or decreases in subsequent behavioral responses in all three species investigated above. However, it is important to consider that a high proportion of moths of each species retained the capability to initiate anemotaxis and plume follow after pheromone pre-exposure. This suggests that repeated visits to pheromone dispensers may occur for those moths, which will be most difficult to disrupt. Also, it implies that competitive

attraction between synthetic point sources of pheromone and authentic females may be the most important contributor to mating disruption. Under a regime in which competitive attraction is the dominant mechanism, the following consequences should be expected: 1) Completeness of pheromone blend should mater for those species requiring it for anemotaxis; 2) More low-release point sources rather than fewer high-release point sources should produce better disruption; 3) Higher moth population densities should result in decreased levels of mating disruption.

Previous investigators have stressed the difficulty in conducting direct visual observations of male moths responding to pheromone sources under authentic field conditions. The above investigation suggests that such an approach is indeed possible and valuable. Furthermore, the comparative approach taken here, where the behaviors of multiple species were compared both in the field and in the laboratory was valuable given the differences uncovered between species. The data suggest that a potentially important first step in mating disruption by polyethylene-tube "rope" dispensers is attraction of male moths within close proximity (150 cm or less). Subsequent modifications due to pheromone exposure following bouts of false-plume-following may result in adaptation, habituation, or sensitization depending on the moth species in question. Further work should focus on how such post-exposure behavioural modifications influence the level of mating disruption in the field. Improved understanding of the general mechanisms underlying mating disruption among multiple pest species should help pest managers improve the efficacy and practicality of this management tactic.

APPENDIX

Appendix 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2004-10

Title of thesis or dissertation (or other research projects):

Comparison of Monitoring Strategies and Evaluation of Spinosad Formulations for Management of Key *Rhagoletis* Species

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)

Lukasz L. Stelinski

Date _____

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America.
Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Choristoneura rosaceana (Harris)	Michigan Allegan Co. Fennville 15 July 2004 Host: on apple tree					10	10		MSU
Argyrotaenia velutinana (Walker)	Michigan Allegan Co. Fennville 24 July 2004 Host: on apple tree					10	10		MSU
Grapholita molesta (Busck)	Michigan Allegan Co. Fennville 25 June 2004 Host: on apple tree					10	10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Lukasz L. Stelinski

Date January 10, 2005

Voucher No. 2004-10

Received the above listed specimens for deposit in the Michigan State University Entomology Museum

Curator 11 Jan 2005

Date

LITERATURE CITED

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