Final Report: Development of pheromone-based mating disruption to control the red clover casebearer

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Project Team Leader: Dr. Maya Evenden

Project Team Members: Jennifer Otani, Calvin Yoder, Boyd Mori

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Table of Contents:

Chapter 1: Efficacy and mechanisms of communication disruption of the red clover casebearer moth (*Coleophora deauratella*) with attractive and unattractive pheromone formulations.

Abstract ........................................................................................................................................5

Introduction ..................................................................................................................................6

Materials and Methods ..............................................................................................................8

Results ........................................................................................................................................14

Discussion .................................................................................................................................16

Table 1 .........................................................................................................................................30

Figure 1 .......................................................................................................................................31

Figure 2 .......................................................................................................................................32

Figure 3 .......................................................................................................................................33

Figure 4 .......................................................................................................................................34

Figure 5 .......................................................................................................................................35

Figure 6 .......................................................................................................................................36

Chapter 2: Challenges of mating disruption using aerosol-emitting pheromone puffers in red clover seed production fields to control *Coleophora deauratella* (Lepidoptera: Coleophoridae)

Abstract .......................................................................................................................................38

Introduction ....................................................................................................................................39

Materials and Methods ..............................................................................................................41

Results ..........................................................................................................................................45

Discussion ....................................................................................................................................47

Figure 1 .........................................................................................................................................59

Figure 2 .........................................................................................................................................60
Chapter 3: Mating disruption of *Coleophora deauratella* (Lepidoptera: Coleophoridae) using laminate flakes in red clover seed production fields

Abstract...............................................................................................66

Introduction..........................................................................................67

Materials and Methods........................................................................69

Results..................................................................................................73

Discussion............................................................................................75

Table 1..................................................................................................85

Table 2..................................................................................................85

Table 3..................................................................................................85

Table 4..................................................................................................85

Figure 1..............................................................................................86

Figure 2..............................................................................................87

Figure 3..............................................................................................88

Figure 4..............................................................................................89

Figure 5..............................................................................................90
Efficacy and mechanisms of communication disruption of the red clover casebearer moth (*Coleophora deauratella*) with attractive and unattractive pheromone formulations

Boyd A. Mori* and Maya L. Evenden

Department of Biological Sciences, CW405 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

*Corresponding Author: Phone: 1-780-492-3080
Fax: 1-780-492-9234
Email: bmori@ualberta.ca

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Preface

In this chapter we evaluate hand-applied twist-tie pheromone dispensers for communication disruption of the red clover casebearer and determine the mechanisms by which mating disruption interferes with communication (Objective 1 and 2). We also assess the release rate and application density required for mating disruption (Objective 3).

Abstract

The red clover casebearer, Coleophora deauratella Leinig and Zeller (Lepidoptera: Coleophoridae) is a major pest of red clover (Trifolium pratense L.) grown for seed in Canada and parts of Europe. The efficacy and mechanisms of communication disruption were evaluated in small plot trials (0.25 ha) with twist-tie dispensers loaded with either the complete attractive pheromone blend (10:1 ratio of (Z)-7-dodecenyl acetate to (Z)-5-dodecenyl acetate) or the unattractive major component alone ((Z)-7-dodecenyl acetate). Both formulations reduced male C. deauratella orientation to pheromone traps (>99.6%). Interestingly, the unattractive major component reduced trap capture to a greater degree than the complete blend. In communication disruption-treated plots, males oriented to dispensers that release the complete pheromone blend, but not the major component. In the laboratory, male C. deauratella antennae became adapted as measured by electroantennograms conducted 5 minutes after pre-exposure to either the complete blend or the major component for 1 hour. Adaptation due to pre-exposure to either formulation resulted in a shift in the pheromone response threshold, antennae from pre-exposed moths responded to a greater degree to high pheromone stimuli dosages (5-50 μg) compared to untreated control moths. Antennae from moths held in clean air for 24 hours after pre exposure recovered and responded similarly to pheromone stimuli as antennae from control moths. These results suggest that both formulations have the potential to cause communication disruption of C. deauratella but the mechanisms of disruption differ between the two formulations.
**Introduction**

Mating disruption is an effective management tool for many lepidopteran pests (Cardé and Minks 1995; Stelinski et al. 2008; Witzgall et al. 2010). This technique interferes with mate-finding behaviour between male and female moths by treatment of the cropping area with high doses of synthetic sex pheromone (Howse et al. 1998). However, not all attempts to use mating disruption to control moth pests over the past several decades have met with success (Cardé and Minks 1995; Witzgall et al. 2010). The efficacy of mating disruption is dependent on factors of the target insect’s biology, the cropping environment, and the pheromone formulation. Insect population density influences the efficacy of mating disruption especially if pheromone dispensers are competing with calling females (Sanders 1981; Suckling and Angerelli 1996; De Lame et al. 2010). Migration of mated females into the treated area can decrease the effectiveness of pheromone treatment (Cardé and Minks, 1995). Crop and environmental factors that can affect pest control by mating disruption include variability in crop canopy, wind direction and wind speed which can affect pheromone plume structure (Cardé and Minks 1995), and pheromone adsorption and release by crop foliage (Suckling et al. 1996). Characteristics of the pheromone formulation affect the activity of mating disruption in different ways for different insect pests. The completeness of the pheromone formulation in dispensers (Minks and Cardé 1988; Evenden et al. 1999), the emission rate of pheromone (Reinke et al. 2014) and the number and distribution of pheromone point sources influence the effectiveness of mating disruption. Most often the development of successful mating disruption programs is hindered by the lack of knowledge on the mechanisms by which mate-finding behaviour is altered (Cardé et al. 1998). In many cases, the exact mechanisms of mating disruption are largely speculative, with mechanisms varying among even closely related species (Cardé and Minks 1995; Stelinski et al. 2004a,b; Reinke et al. 2014).

The mechanisms that mediate mating disruption can be classified into two categories: competitive, and non-competitive (Miller et al. 2006). Competitive mechanisms require an attractive pheromone blend and occur via false-trail following (competitive attraction) in which males orient to dispensers baited with synthetic pheromone rather than to calling females (Bartell 1982; Miller et al. 2006). Non-competitive mechanisms can be invoked by the complete attractive pheromone blend, off-ratio blends or the major component of the pheromone blend alone and include: sensory adaptation of the pheromone receptors on the antennae; habituation of
the central nervous system to process pheromone signals; sensory system imbalance in which males respond optimally to a pheromone blend not produced by the female; and camouflage of the natural pheromone plume by high amounts of synthetic background pheromone (Bartell 1982; Cardé and Minks 1995; Miller et al. 2006). In order to optimize mating disruption against various insect species, it is necessary to understand the mechanisms (competitive vs. non-competitive) by which it acts against the target species.

The red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae), is a severe pest of red clover (*Trifolium pratense* L.) grown for seed production in Canada (Ellis and Bjørnson 1996; Evenden et al. 2010). It is also an occasional pest in Europe (Markkula and Myllymäki 1960). Females lay eggs directly on the calyx of red clover inflorescences and immediately upon hatching, larvae burrow through the calyx and begin to feed on the developing seed (Landry 1991; Ellis and Bjørnson 1996). Larvae are capable of consuming 2-3 seeds/day which has led to ≥ 80% seed loss in red clover stands throughout Canada (Landry 1991; Ellis and Bjørnson 1996; Evenden et al. 2010). The internal feeding nature of larvae makes infestations difficult to control with insecticide and, currently, there are no insecticides registered for use against *C. deauratella* in Canada. Furthermore, any *C. deauratella* control strategy must be compatible with bee safety as red clover is highly dependent on both managed honey bees (*Apis mellifera* L.) and wild bumble bees (*Bombus* spp. Latreille) for pollination (Forester and Hadfield 1954; Holm 1966). As the need for judicious use of insecticides around bees is increasingly in the spotlight (Henry et al. 2012; Krupke et al. 2012; Cutler et al. 2013), alternative *C. deauratella* control strategies (i.e. mating disruption) that have minimal impact on non-target organisms need to be explored.

The recent identification of the sex pheromone of *C. deauratella* as a 10:1 ratio of (Z)-7-dodecenyl acetate (Z7-12:OAc) to (Z)-5-dodecenyl acetate (Z5-12:OAc) (Evenden et al. 2010) allows the potential for mating disruption to be explored. Evenden et al. (2010) found *C. deauratella* males were attracted to pheromone-baited traps with lures containing 100:10 μg of Z7-12:OAc to Z5-12:OAc (complete blend), whereas traps baited with 100 μg Z7-12:OAc (major component) were unattractive. The complete attractive pheromone blend may not be necessary for effective control with mating disruption as several pest species including the obliquebanded leaf roller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (Evenden et al. 1999), the mullein bug, *Campylomma verbasci* (Meyers) (Hemiptera: Miridae) (Judd et al. 1995), and
the Guatemalan potato moth, *Tecia solanivora* (Povolny) (Lepidoptera: Geliidae) (McCormick et al. 2012), do not need the complete pheromone blend to maintain effective disruption. As the major pheromone component alone is unattractive to *C. deauratella* males, we can examine the importance of competitive and non-competitive mating disruption mechanisms by comparison of disruption caused by the major component alone to the complete pheromone blend. Furthermore, the use of the major component in pheromone dispensers would be more cost effective than dispensers releasing the complete pheromone blend; however, the potential for resistance would need to be monitored (Mochizuki et al. 2002; Evenden and Haynes 2001).

Often the first step in determining if mating disruption has the capability to control insect pests is to conduct communication disruption studies. Communication disruption is a proxy for mating disruption that is measured using reduction of moth capture in pheromone-baited traps in treated areas as a measure of the efficacy of pheromone-treatment to interfere with pheromone-based communication, rather than mating *per se*. Here, we conduct initial proof-of-concept studies on *C. deauratella* to determine the potential for communication disruption in small plot-trials using the complete pheromone blend. We also compare the disruption capacity of the complete pheromone blend and the major component alone in the field to understand the mechanisms by which each pheromone formulation disrupts moth behavior. The density of dispensers necessary to successfully maintain disruption is also explored. Finally, we determine if pre-exposure of male moths to the complete pheromone blend and the major component alone can induce sensory adaptation to these compounds. The results of both the field and laboratory studies suggest that mating disruption is a viable control strategy to pursue for *C. deauratella* and adds to the growing body of literature on the possible mechanisms that are invoked by treatment with attractive and unattractive pheromone formulations.

**Materials and methods**

**Study Sites.** All field studies were conducted in 2010 and 2011 on red clover (*va. Altaswede*) fields in the Peace Region of Alberta, Canada. Field sites were predominately around the Town of Falher (55° 44’ 12.6” N, 117° 12’ 9.9” W) and at least 1 km separated individual sites. Experimental 0.25 ha plots (50 m x 50 m) were established in each field, 25 m from the field edge, and ≥ 100 m apart. In each experimental plot, four green unitraps (ConTech Enterprises, Delta, BC, Canada) placed 12.5 m from the centre along the cardinal directions and ~35 cm above the soil surface (Mori and Evenden 2013), were used to assess communication
disruption. Each unitrap was baited with a pre-extracted grey rubber septa loaded with 100:10 μg of Z7-12:OAc to Z5-12:OAc (ConTech Enterprises). Grey rubber septa were pre-extracted with a 5/95 solution of diethyl ether in methanol in a large capacity Soxhlet extractor for a minimum 24 hours (Contech Enterprises). A strip of Hercon Vaportape II (10% dichlorovos) (Hercon Environmental, Emigsville, PA, USA) was used as a killing agent in the unitraps. Wooden stakes (~122 cm x 3.81 cm x 1.27 cm), with a hole drilled 25.4 cm from the top, were used in all studies to support individual twist-tie dispensers in treated plots. In 2011, wooden stakes were placed in a uniform fashion at 500 stakes/ha in the treated plots only, as there was no effect of stakes in control plots in 2010.

**Pheromone Dispensers and Release Rates.** For the initial proof-of-concept experiment in 2010, twist-tie dispensers (Shin-Etsu Chemical Co., Tokyo, Japan) were formulated to release the complete pheromone blend (10:1 ratio of Z7-12:OAc to Z5-12:OAc). In 2011, twist-tie dispensers (Shin-Etsu Chemical Co.) were formulated to release the complete pheromone blend as in 2010, and a second dispenser was formulated to release the major component (Z7-12:OAc) alone. Both dispenser formulations contained 100 mg of the active pheromone ingredients (AI). Pheromone release rate was determined gravimetrically at 20.0 ± 0.1 °C and 30.0 ± 1.0 °C. Three lures were hung in a climate controlled chamber with continuous air flow (ConTech Enterprises). Periodically (every 1-4 days), lures were removed and weighed to four decimal places on a Mettler-Toledo AB204-S/FACT balance (Mettler-Toledo AG, Laboratory and Weighing Technologies, Greifensee, Switzerland) to determine the release rate of the chemicals.

**Experiment 1 - Proof-of-Concept.** In 2010, a small-plot proof-of-concept study was conducted to determine if treatment with the attractive pheromone blend disrupts communication between male and female *C. deauratella*. Four experimental plots were placed in a single field (~64.7 ha). The experiment was designed in a pair-wise fashion and replicated in time and space with the field containing two treated and two control plots. Wooden stakes were placed in a uniform fashion at 1000 stakes/ha, in both treatment and control plots in order to facilitate an even distribution of pheromone dispensers (1000 dispensers/ha) and account for any disturbance that setting up the stakes may have caused in control plots. In the treatment plots, twist-tie dispensers releasing the complete pheromone blend were twisted by hand around wooden stakes by looping the dispenser through the hole in the stake. Control plots did not receive dispensers. Plots were re-randomized after 14 days (28 June to 12 July) and the next trial ran for a
subsequent 14 days (12 July to 26 July) for a total of four replicates (two replicates per time period for two time periods, $N = 4$). At the end of the first trial all dispensers, wooden stakes, and pheromone-baited traps were removed from the plots and moths counted. Wooden stakes, dispensers, and pheromone-baited traps were replaced for the second trial and two of the four plots were randomly selected for dispenser treatment and the other two acted as controls. At the end of the second trial, trap contents were removed and moths were counted. Moths from all traps in each plot per trial were combined to give the total number of moths captured per plot.

Trap capture reduction was measured as the disruption index: $\% DI = \left(\frac{C-T}{C}\right) \times 100\%$, where $C =$ number of males captured in the control plot, and $T =$ number of males captured in the treated plot (Roelofs and Novak 1981; Rodriguez-Saona et al. 2009).

**Experiment 2 - Pheromone Components Necessary for Disruption.** In 2011, a small-plot communication disruption experiment was conducted (27 June to 12 July) to test the hypothesis that the major component alone causes a similar level of disruption as the complete pheromone blend as compared to an untreated control. Experiments were replicated in space with each field ($N = 3$) containing one plot for each treatment and an untreated control. Dispensers containing the complete attractive pheromone blend were applied to one plot in each field at 500 dispensers/ha. A second plot received dispensers loaded with the major component alone at 500 dispensers/ha and the final plot was left as an untreated control. To determine if *C. deauratella* males were attracted to dispensers releasing the complete blend or major component alone in communication disruption plots, yellow sticky cards (15 x 10 cm, ConTech Enterprises) were attached to the wooden stakes positioned 1 cm below the end of the pheromone dispenser. A yellow sticky card was attached to all wooden stakes positioned at the plot edge and diagonally from each corner through the plot interior. At the end of the experiment, all dispensers, yellow sticky cards, wooden stakes, and pheromone-baited traps were removed and moths captured were counted. All moths captured on yellow sticky cards around the plot edge and through the plot interior were combined to give a total number of moths caught at the edge and the interior of each plot. Moths captured in all four green unitraps were totaled for each plot.

**Experiment 3 - Pheromone Dispenser Density.** In 2011, a second small-plot communication disruption experiment was conducted (18 July to 1 August) to determine the density of dispensers needed for successful communication disruption. Experiments were replicated in space with each field ($N = 3$) containing four experimental plots. Plots were
randomly selected for the various treatments. One plot received a dispenser density of 1000 dispensers/ha on 500 point sources (effectively doubling the dose of pheromone) by placement of two dispensers on each wooden stake. Another plot received a dispenser density of 500 dispensers/ha on 500 point sources (one dispenser per stake), the last treated plot received 256 dispensers/ha (one dispenser per two stakes) and the final plot was left as an untreated control. At the end of the experiment, all wooden stakes, dispensers, and pheromone-baited traps were removed and moths were counted. The total number of moths captured in all four unitraps in each plot was combined to give the total number of moths captured per plot.

_Insect Collection for Electrophysiological Studies._ In early May 2013, field trash (stubble and leaf litter) was collected from a red clover field near Guy, Alberta (55° 31' 43"N, 117° 9' 44"W). The trash was collected in cotton bags and placed in refrigerated containers for transport to the laboratory at the University of Alberta. In the laboratory, trash was placed in plastic emergence bins (80 cm long by 40 cm wide by 45 cm high) equipped with two 500 ml emergence jars. The bins were placed on a laboratory bench at 22 ± 1°C under a 16:8 h light:dark cycle. Bins were misted with distilled water and checked daily for eclosed moths. Eclosed moths were separated by sex and placed in individual containers with access to 5 % sugar water _ad lib_. Male moths were placed in a growth chamber (Percival Intellus Environmental Controller, Model I30VL, Percival Scientific, Inc., Perry, IA, USA) on a 17:7 hr L:D cycle to closely follow the natural photoperiod experienced by moths at this time of year. Temperature was set at 21 °C, and 65% relative humidity. Additional moths were captured using sweep nets in red clover fields near Falher, Alberta several times throughout the summer and treated like the newly eclosed moths above. Electroantennogram (EAG) responses of control _C. deauratella_ males (those that were not pre-exposed to pheromone) found no effect of collection method or age on male moth response (data not shown). One to two-day-old lab-emerged _C. deauratella_ males were used in Experiment 4 and field collected males of unknown age were used in Experiment 5.

_Pre-exposure Apparatus for Electrophysiological Studies._ To test for adaptation of male _C. deauratella_ antennae, moths were pre-exposed for 1 hour to either a twist-tie dispenser containing the complete pheromone blend, the major component alone, or a blank (clean air) control. Twelve hours prior to the onset of the experiment, twist-ties were removed from the freezer and washed three times with HPLC grade hexane (EMD, Gibbstown, NJ, USA) to remove any residual pheromone on their surface. They were then left to equilibrate in a fume
hood until 1 hour prior to the beginning of the experiment when they were placed into the pheromone release chambers.

The pre-exposure apparatus consisted of three treatment arms (Fig. 1). Air was pumped through each treatment arm of the apparatus at 66 ml/min. Air was first filtered via an ARS charcoal filter (#ADS-STD-C2F, Analytical Research Systems Inc., Gainsville, Florida, USA) and then passed through distilled water in a 125 ml Büchner flask to humidify the air before being split into the three treatment arms. Subsequently, in each arm of the apparatus, air entered a 250 ml Büchner flask (pheromone release chamber) that contained one of three treatments: a twist-tie dispenser releasing the complete blend, a twist-tie dispenser releasing the major component, or a blank (clean air) control. From the pheromone release chamber, air traveled to a 500 ml glass jar (moth exposure chamber) with a metal lid equipped with two 0.6 cm (internal diameter) ports through which a LabPure Monobarb PTFE straight coupler (6 x 6 mm) (Thomas Scientific, Swedesboro, NJ, USA) was fixed to create an inlet for air. Air was vented from the moth exposure chamber via tubing to an exhaust port in a fume hood (Fig. 1). Moths were placed individually into the moth exposure chambers for 1 hour. Each moth exposure chamber was placed in an open top box made from white corrugated plastic to eliminate any visual influences and to maintain constant light (1066 lux). The entire three-arm pre-exposure apparatus was placed in a fume hood (temperature 21 ± 2 °C) in a laboratory adjacent to the laboratory containing the EAG to prevent any contamination of air in the EAG lab. At the end of each daily trial, all tubing was replaced and all glassware and metal lids were washed with soap and water and rinsed three times in acetone and hexane before being baked in an oven at 75 °C overnight.

Experiment 4 and 5 - Electrophysiological Studies after Pre-Exposure to Pheromone. The EAG system consisted of an IDAC-02 data acquisition controller system, a Syntech EAG probe (Type PRG-2, internal gain 10X), and EAG 2000 software (Syntech, Hilversum, The Netherlands). After the 1 hour pre-exposure period, moths were individually chilled at 4 °C for two minutes before the right antennae was excised and attached to a stainless steel antenna holder using a small quantity of Spectra 360 conductive gel (Parker Laboratories, Orange, NJ, USA). The stimuli consisted of the complete pheromone blend in a 10:1 ratio of Z7-12:OAc (96.6 % chemical purity) to Z5-12:OAc (98.6% chemical purity) (Bedoukian Research Inc., Danbury, CT, USA) serially diluted in HPLC grade hexane to obtain solutions between $5.0 \times 10^{-6} \mu g$ to $5 \mu g/ul$ hexane. Fifty μl of each solution was pipetted on to 0.2 cm by 7 cm strips of
Whatman No. 1 filter paper, placed into a disposable Pasteur pipette, and allowed to evaporate in the fume hood for 30 minutes. Fifty microliters of hexane and a common plant volatile, \((E)-2\)-hexenal (1 μg/μl), were also pipetted on to strips of filter paper and allowed to evaporate to act as a control and standard, respectively. Carbon-filtered and humidified air flowed constantly over each mounted antenna from a Syntech CS-55 stimulus controller at 50 ml/min. Stimulus puffs were triggered by hand via the stimulus controller with a pulse duration of 0.2 sec and flow of 10 ml/sec. Electroantennograms were measured as the maximum amplitude of depolarization by the applied stimulus. The stimuli were applied to each antenna once per minute in an ascending pheromone concentration order separated by the standard (hexane, 50 μg plant volatile, 5.0 x 10^{-5} μg pheromone, 50 μg plant volatile, 5.0 x 10^{-4} μg pheromone, 50 μg plant volatile, 5.0 x 10^{-3} μg pheromone, 50 μg plant volatile, 5.0 x 10^{-2} μg pheromone, 50 μg plant volatile, 0.5 μg pheromone, 50 μg plant volatile, 5 μg pheromone, 50 μg plant volatile, 50 μg pheromone, 50 μg plant volatile). Moth antennal response was normalized by dividing the mV response to each pheromone stimulus by the average mV response to plant volatile across the same antenna (Judd et al. 2005). Due to the length of time needed for EAGs, the experiments were conducted over several days. If experiments ran for longer than four hours, the stimuli were replaced. In Experiment 4, the first EAG stimulus was applied exactly 5 minutes after the moths were removed from the pre-exposure apparatus whereas in Experiment 5, we tested whether moths could recover normal antennal function 24 hours after pre-exposure. Thus, in Experiment 5, moths were removed from the pre-exposure apparatus, placed in individual cups with access to 5 % sugar water *ad lib*, and placed in clean air in a growth chamber (conditions as previously stated) until the following day. Electroantennograms were conducted exactly 24 hours after removal from the pre-exposure apparatus. We measured the response of antennae from ten males in each treatment group (complete pheromone blend, major component alone, or control) in both experiments.

Statistical Analyses. A general linear regression model was used to evaluate the release rate of dispensers containing the complete pheromone blend and the major component alone over time (60 days) at both 20.0 ± 0.1 °C and 30.0 ± 1.0 °C. Mean release rate of the three measured dispensers at each temperature and time period were transformed by the natural logarithm to give: \(\ln(\text{Release Rate}) = m \times \ln(\text{Day}) + b\), where \(m = \text{slope}\) and \(b = \text{y-intercept}\). The benefit of this equation is that the back-transformation equates to a power function \(y = ax^b\), where \(a =\)
Release Rate = e^b \times (\text{Day})^m \text{ (Crawley 2007).}

Male moth trap and yellow sticky card capture were analyzed to check for normality and heteroscedasticity using Shapiro-Wilks tests and visualization techniques (R Core Development Team 2013). Due to non-normal error distributions and overdispersion in the data a negative binomial error distribution was used in all models to evaluate Experiments 1-3 (package: glmmADMB) (Fournier et al. 2012). For Experiment 1, a repeated-measures mixed-effects model was used with time period specified as the repeated-measure and treatment as a fixed effect. For Experiments 2 and 3, a generalized linear mixed-effects model was used with site specified as a random effect and treatment as a fixed effect. Analysis of deviance tables and χ^2 goodness-of-fit statistics (analogous to F-values) were used to generate P-values for all fixed effects in generalized linear mixed-effects models. A post-hoc Tukey’s HSD test was used to determine significant differences between treatments (P < 0.05). For yellow sticky card data from Experiment 2, a generalized linear mixed-effects model was used specifying site as a random effect and treatment, yellow sticky card position, and a treatment X position interaction as fixed effects.

In Experiments 4 and 5, normalized antennal response data was transformed (ln(x + 1)) to meet the assumptions of normality. Stimulus dose and pheromone pre-exposure treatments, including a dose X treatment interaction, were specified as fixed effects and moth ID nested within day of the experiment was specified as a random effect in a general linear mixed-effects model. In Experiment 4, a significant dose X treatment interaction occurred, therefore at each stimulus dose tested, a generalized linear mixed-effects model was used to determine if differences in normalized mV antennal response existed between the different pheromone pre-exposure treatments. If treatment had a significant effect on normalized mV antennal response a post-hoc Tukey’s HSD test was used to determine significant differences between treatments (P < 0.05).

Results

Pheromone Dispenser Release Rates. The mean pheromone release rate from dispensers containing the complete blend and major component at 20 °C and 30 °C was fit to a power function curve (20 °C: complete blend dispenser F_{1,12} = 246.8, adj-\(r^2\) = 0.95, \(P < 0.001\); major component dispenser \(F_{1,12} = 387.4, \text{adj-}r^2 = 0.97, P < 0.001\); 30 °C: complete blend dispenser
The mean release rate of the complete pheromone blend dispensers declined from 4.19 ± 0.22 mg/day and 9.97 ± 0.16 mg/day on day 1 to 0.58 ± 0.01 mg/day and 0.38 ± 0.04 mg/day on day 60 at 20ºC and 30ºC, respectively. And the mean release rate of the major component alone dispensers declined from 3.53 ± 0.37 mg/day and 9.86 ± 0.54 mg/day on day 1 to 0.52 ± 0.02 mg/day and 0.34 ± 0.06 mg/day on day 60 at 20ºC and 30ºC, respectively.

Experiment 1 - Proof-of-Concept. Male *C. deauratella* trap capture in pheromone-treated plots was reduced by 99.6 ± 0.2 % compared to untreated controls ($\chi^2 = 113.7$, $df = 1$, $P < 0.001$) (Fig. 3). Trap capture in untreated control plots was two-fold higher in the second trial of the experiment compared to the first, but even with the higher population density, *C. deauratella* communication was disrupted. Male moth captures in control plots were ≥ 3 408 compared to ≤ 31 in the pheromone-treated plots over a 14-day period.

Experiment 2 – Components Necessary for Disruption. The number of male *C. deauratella* captured in pheromone-baited traps in plots treated with the complete blend and the major component were significantly lower than the untreated control ($\chi^2 = 7382.7$, $df = 2$, $P < 0.001$) (Fig. 4). Interestingly, pheromone-baited trap captures were lower in plots treated with the major component alone than with the complete blend (Fig. 4). Both treatments reduced pheromone-baited trap capture by > 99.9% (complete blend: 99.94 ± 0.06 %; major component: 99.98 ± 0.01 %). Due to wind damage of yellow sticky cards, only yellow sticky cards attached to the corner and middle wooden stakes at the plot edge ($N = 8$) and diagonally on every second stake from each corner through the plot interior ($N = 8$) were used in the analysis. More moths were captured on yellow sticky cards directly below dispensers releasing the complete blend compared to the major component ($\chi^2 = 44.2$, $df = 1$, $P < 0.001$). There was also a significant treatment X position interaction ($\chi^2 = 7.1$, $df = 1$, $P = 0.005$) with yellow sticky cards positioned below dispensers on the edge of the plots treated with the complete pheromone blend capturing numerically more moths than those in the interior of the same plot and at all locations in the plot treated with the major component (Fig. 5).

Experiment 3 - Pheromone Dispenser Density. All three pheromone treatments significantly reduced the number of male *C. deauratella* captured in pheromone-baited traps compared to untreated controls ($\chi^2 = 783.5$, $df = 3$, $P < 0.001$). No treatment effect was observed when the density of pheromone dispensers was doubled with the number of point sources
maintained constant (Fig. 5). Trap capture was reduced by $> 99.9 \pm 0.08\%$ in all treatments compared to untreated controls even at the lowest level of dispensers (256 dispensers/ha) tested in this study (Fig. 5).

Experiments 4 and 5 - Electrophysiological Effects after Pre-Exposure to Pheromone. After a 1 hour pre-exposure to either the complete pheromone blend or the major component alone followed by a 5 minute recovery period, there was a significant stimulus dose by pre-exposure treatment interaction ($F_{14,196} = 6.73$, $P < 0.001$) that affected electrophysiological response of male moth antennae. Therefore, at each stimulus dose tested, a separate model was used to determine the treatment effect. There was no significant effect of pre-exposure treatment on the normalized mV antennal response of moth antennae to hexane ($F_{2,25} = 1.83$, $P = 0.18$) (Table 1; Fig. 6). Pre-exposure to either the complete blend or the major component alone resulted in a significant reduction in the normalized mV antennal response at all stimulus doses from $5.0 \times 10^{-6}$ μg to 0.05 μg as compared to moths exposed to clean air prior to measurement. There were no significant differences between the two pheromone pre-exposure treatments on subsequent male moth antennal response (Table 1; Fig. 6). There was no significant difference between both pheromone pre-exposure treatments and the control at the 0.5 μg stimulus dose (Table 1; Fig. 6). At the high stimulus doses of 5.0 and 50 μg there was no significant difference in antennal response between moths that were pre-exposed to the major component alone or to the clean-air control. Interestingly, antennae pre-exposed to the complete blend had an elevated normalized mV response to the 5.0 and 50 μg stimulus doses (Table 1; Fig. 6).

After a 1 hour pre-exposure period followed by a 24 hour recovery period, there were no significant treatment or treatment X dose interaction effects on the normalized mV antennal response (treatment: $F_{2,26} = 1.0$, $P = 0.38$; treatment X dose: $F_{14,89} = 1.08$, $P = 0.38$). After the 24 hour recovery period, there was a slight elevation of response to the 0.5, 5.0, and 50 μg stimulus doses after pre-exposure to the complete pheromone blend, but this elevation was not significant.

Discussion

The present study demonstrates that orientation of male *C. deauratella* to pheromone traps in red clover seed production fields can be disrupted by treatment with pheromone formulations releasing either the full pheromone blend or the major component alone. Field trials combined with laboratory EAGs demonstrate that different mechanisms of disruption occur when *C. deauratella* are exposed to the complete blend or major component. False-trail
following and adaptation are mechanisms when the complete blend is used, whereas adaptation leading to sensory system imbalance appear to be the mechanisms when the major component is used.

In the initial proof-of-concept experiment, twist-tie dispensers releasing the complete pheromone blend significantly reduced the number of males captured in pheromone-baited traps (Fig. 3a). Although our results indicate successful communication disruption via a reduction in pheromone-baited trap captures, further studies are needed to determine if mating is actually disrupted by pheromone treatment, as trap capture reduction is not always indicative of successful mating disruption (Cardé and Minks 1995). Pea fields treated with the synthetic pheromone of *Cydia nigricana* (Fabricius) (Lepidoptera: Tortricidae) reduced male moth capture in pheromone-baited trap capture, but did not reduce damage in the field (Saucke et al. 2014). Ultimately, mating disruption of *C. deauratella* will only be successful if a reduction in female mating, oviposition and subsequent seed damage occurs in pheromone-treated plots.

This study shows that pheromone-based mating disruption has the potential to control *C. deauratella* because communication is disrupted even under high population densities. Thousands more male moths were captured in untreated control plots compared to pheromone-treated plots positioned in the same fields (Fig. 3a). Since male and female *C. deauratella* emerge in approximately equal numbers around peak flight (Mori, unpublished) the high male trap captures in control plots indicate high population densities of both sexes at our study sites. High pest populations generally cause mating disruption failures due to chance encounters between males and females without the need for long-range sex pheromone communication (Cardé and Minks 1995). Feldhege (1993) found mating disruption for *Lobesia botrana* (Dennis and Schiffermüller) (Lepidoptera: Tortricidae) in vineyards fails when moth density is greater than 4000 males and females/ha. In this study, trap capture over the 14-day period in the control plots equates to ≥ 13 632 males/ha. In the few instances when mating disruption is successful under high pest population pressures, non-competitive mechanisms are thought to drive treatment efficacy (Stelinski et al. 2008, Miller et al. 2006).

Mating disruption treatment with twist-tie pheromone dispensers releasing either the complete pheromone blend or the major component alone results in a reduction in the number of males captured in pheromone-baited traps compared to that in the untreated control plots (Fig. 4). These results correspond with previous studies on *Agrotis segetum* Dennis and Schiffermüller.
(Lepidoptera: Noctuidae) (Svensson et al. 1995), *C. rosacena* (Evenden et al. 1999), *Rhopobota naevana* (Hbn.) (Lepidoptera: Tortricidae) (Fitzpatrick et al. 2004), and *T. solanivora* (McCormick et al. 2012) which found that the major component alone or off-ratio pheromone blends disrupt pheromone-based communication to the same degree as more attractive complete pheromone blends. Interestingly, in this study, communication is disrupted to a greater degree in plots treated with dispensers releasing the major component than in those treated with the complete pheromone blend (Fig. 4). In other pest species, the major component or off-blend results in equal, but not better, disruption than the complete pheromone blend (Svensson et al. 1995; Evenden et al. 1999; Fitzpatrick et al. 2004; McCormick et al. 2012). However, the increased disruption of orientation to baited traps by male *C. deauratella* in plots treated with the major component may be an artifact of increased population density in plots treated with the attractive complete pheromone blend. In the current study, male *C. deauratella* are captured on yellow sticky cards positioned above dispensers releasing the complete pheromone blend in pheromone-treated plots and more males are captured on cards positioned at the edge of plots than the interior (Fig. 5). This suggests that male moths are attracted to pheromone released in plots treated with the attractive, complete pheromone blend which may have increased the male *C. deauratella* population density within the plots compared to plots treated with the unattractive major component. Future experiments should test for a reduction in the number of female matings and seed damage in large plots treated with the different pheromone formulations.

The successful communication disruption of *C. deauratella* males using the complete pheromone blend and major component allows for the examination of the mechanisms of mating disruption that are elicited by the different pheromone treatments. A larger number of male moths were captured on yellow sticky cards positioned below dispensers releasing the complete blend than those releasing only the major component in small pheromone-treated plots. Therefore, dispensers releasing the full pheromone blend elicit false trail following behavior by male *C. deauratella*. Several other moth species are also capable of orientation to and landing on or near high-release pheromone dispensers including *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Cardé et al. 1998), *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) (Reinke et al. 2014), *C. pomonella* (Barrett 1995) and *C. rosaceana* (Stelinski et al. 2004a). Although some moths are optimally responsive to pheromone over a narrow range of attractive pheromone doses (Willis and Baker 1984; Lofstedt et al. 1985; Evenden and Gries
C. deauratella males are capable of flight to the high-release dispensers in this study. This may be because the nervous system of the male moths is impacted by adaptation or habituation in the pheromone-treated environment that results in a shift in the threshold of response to pheromone which is further supported by the results of the electrophysiological study. In addition, C. deauratella is a moth species that does not exhibit a strong dose response to pheromone (Evenden et al. 2010) which may indicate that it is a species that is difficult to disrupt with pheromone-based mating disruption (Gut et al. 2004). The low numbers of C. deauratella males captured on yellow sticky cards positioned below dispensers releasing the major component is consistent with previous work that moths were not attracted to rubber septa lures baited with the major component alone (Evenden et al. 2010).

Male C. deauratella are more commonly captured on yellow sticky cards below dispensers releasing the full pheromone blend at dispensers positioned at the edge of treated plots compared to the interior. Overlapping pheromone plumes in the plot interior from multiple dispensers may invoke non-competitive mechanisms which prevent males from locating dispensers in the plot interior. This is similar to P. gossypiella in which males are more attracted to dispensers located in clean air than those surrounded by other dispensers (Cardé et al. 1998). Coleophora deauratella may orient along edges of concentrated pheromone plumes or at clean-air boundaries at the edge of plots, and then become disrupted within the more homogenous clouds of pheromone in the interior of the plots, as found with G. molesta and other tortricid species (Kennedy et al. 1981; Willis and Baker 1984). Based on our field studies with the complete pheromone blend, it appears false-trail following may be an initial mechanism when distinct pheromone plumes can be located, but upon subsequent exposure to pheromone other mechanisms such as adaptation/habituation or camouflage could occur. These results also provide support to the theory that non-competitive mechanisms are more conducive to effective control of high population densities as competitive mechanisms alone generally fail (Miller et al. 2006, Reinke et al. 2014, Stelinksi et al. 2008). As male moth capture on yellow sticky cards positioned below dispensers releasing the main pheromone component alone was low, false-trail following is not the main mechanism of communication disruption in plots treated with this formulation. Non-competitive mechanisms such as adaptation/habituation, camouflage, or sensory system imbalance must occur to disrupt communication in plots treated with the major component alone.
Laboratory EAGs illustrate that male moth antennal receptors adapt and become less responsive to low pheromone doses \( (5.0 \times 10^5 - 0.05 \mu g) \) after one hour of exposure to either the complete blend or to the major component alone as compared to unexposed males (Fig. 6a). Twenty-four hours in clean air after the 1 hour pre-exposure treatment allows males to fully recover (Fig. 6b). Antennal response of moths that experience the hour long pre-exposure is stronger to higher pheromone stimuli \( (0.5 - 50 \mu g) \) than antennae from the unexposed control male moths. This interesting finding indicates a shift in the pheromone response threshold, known as a classic threshold elevation (Mafra-Neto and Baker 1996). Adaptation may be a mechanism of mating disruption that can be elicited by the major component alone (Trimble 2012). However, the current study and that of D’errico et al. (2013) show that the complete pheromone blend does not induce greater levels of adaptation compared to the major component alone. The shift in response threshold at higher pheromone doses in the current study differs depending on the pre-exposure treatment. Exposure of moths to the complete blend elevates antennal response to high stimuli doses \( (5 - 50 \mu g) \) compared to moths exposed to only the major component (Fig. 6a). The elevated response of moths to pheromone stimuli after exposure to the complete blend may be due to the fact that receptors for both major and minor components exhibit a shift in threshold response. Receptors specific to the minor component are not adapted after exposure to the major component alone and thus there is less of a shift in threshold response of males exposed to the main component as compared to those exposed to the full pheromone blend (Fig. 6a). This observation could be further tested with single sensillum recordings to determine the adaptation of each receptor to both pheromone components followed by stimulation of the antennae with the major and minor components separately.

The release rate of the complete blend and major component pheromone dispensers at 20 °C on day 1 was 4.19 and 3.53 mg/day, respectively, therefore, in the 1 hour pre-exposure period moths were exposed to \( \sim 180 \mu g \) and \( \sim 150 \mu g \), respectively. The air flow rate was 66.6 ml/min and would equate to a total volume of 3.996 l/hr. Thus moths were pre-exposed to \( \sim 45.05 \mu g/l \) \( (45.05 \text{ mg/m}^3) \) of the complete blend and \( \sim 37.54 \mu g/l \) \( (37.54 \text{ mg/m}^3) \) of the major component. These concentrations are six orders of magnitude \( (\text{mg/m}^3 \text{ compared to ng/m}^3) \) above any pheromone concentrations measured in field crop mating disruption studies. Witzgall et al. (1996) directly measured the pheromone concentration in air from plots treated with twist-tie dispensers \( (333 \text{ dispensers/ha, 16.5g AI/ha}) \) in a pea field and found \( 1.3 \pm 0.5 \text{ ng/m}^3 \) and Flint et
al. (1990) measured the concentration of pheromone in a treated cotton field (≈1000 dispensers/ha, 78 g AI/ha) to be 1.4-2.0 ng/m$^3$. Furthermore, some species of moths in the field need to be exposed to high doses of pheromone for extended periods (24 h) for any reduction in subsequent pheromone-mediated behavior (Schmitz et al. 1997, Rumbo and Vickers 1997, Stelinski et al. 2003). Although antennal receptors of male *C. deauratella* were readily adapted after a 1 h pre-exposure treatment in these experiments, antennae were exposed to much more pheromone than they will likely encounter in the field cropping environment. When moths approach the dispensers in the field they would have to remain in the plume for an hour or more to receive an equivalent dose. Thus, further experiments are needed to determine the lowest dose to which adaptation can occur and if moths experience this dose in the field.

Dispenser density and the number of pheromone point sources (release sites) are known factors that can affect the efficacy of mating disruption. Generally, mating disruption increases as the number of dispensers increases (Flint and Merkle 1983; Suckling et al. 1994; Rodriguez-Saona et al. 2009; De Lame et al. 2010), which may indicate that competitive attraction is the main mechanism in these cases (Miller et al. 2006). Nevertheless, in order to optimize mating disruption and reduce application costs, the minimal dispenser density capable of successful disruption should be determined. This study was designed to distinguish between the effect of overall pheromone concentration and the number of point sources of pheromone per unit area on subsequent communication disruption. All three dispenser density treatments (1000 dispensers/ha and 500 dispensers/ha on 500 point sources, and 256 dispensers/ha on 256 point sources) significantly reduce male moth trap capture compared to that in the untreated control plots but there is no difference among the efficacy of the three treatments (Fig. 4). The most probable explanation for this finding is that the pheromone concentration threshold at which communication disruption breaks down is never reached in the current study, and further experiments are required with lower dispenser densities or pheromone release rates to determine the most economical application rate to achieve successful disruption. A second explanation could be that communication disruption in plots treated with dispensers releasing the attractive full pheromone blend acts via non-competitive mechanisms that do not rely on dispenser density as strongly as competitive attraction. Under competitive attraction, the first dispensers added to the plot will have the most impact on disruption, and as the number of dispensers is increased each dispenser has a diminishing impact on control (Miller et al. 2006, Reinke et al. 2014).
Based on the field and laboratory results presented here, we can conclude that the complete pheromone blend released from dispensers will elicit false-trail following initially in pheromone-treated plots. The attraction of males to the dispensers could cause intermittent bouts of antennal adaptation that could raise the threshold of sensitivity and thereby reduce the males’ ability to locate the natural plume of pheromone against the background (Cardé 1990). This shift in pheromone responsiveness makes competitive attraction to pheromone dispensers less common in the interior of pheromone-treated plots. Camouflage may also occur in the interior of plots as the pheromone released from dispensers could mask the females’ natural plume boundaries, and thus prevent orientation to the female. Habituation of the central nervous system may also be occurring in male moths as a result of pheromone treatment with the full pheromone blend. This possibility was not tested in the current study and further laboratory wind tunnel studies are needed to assess the role of habituation in this species.

Mechanisms of communication disruption elicited by the major component alone could include several non-competitive mechanisms. Sensory system imbalance is likely to occur in plots treated with the main pheromone component alone. Although males are capable of adaptation when exposed to the major pheromone component, males do not orient to dispensers releasing the major component, thus they will only have intermittent exposure to pheromone which may lead to infrequent adaptation. The excess major component in the atmosphere combined with intermittent adaptation most likely causes an imbalance in the perception of the ratios of pheromone components released by calling females and would lead to a reduction in orientation to females (Baker et al. 1988). Future studies that vary the concentration and ratio of pheromone on lures (Judd et al. 1995) in pheromone-baited traps are needed to confirm this explanation.

This study demonstrates the potential for successful communication disruption of *C. deauratella* using pheromone formulations that release either the attractive complete pheromone blend or the unattractive major component alone. As far as we are aware, this is the first study to demonstrate that the major component alone elicits a higher level of communication disruption than the complete pheromone blend. Adoption of mating disruption using the major component would be more economical than the complete blend; however, the potential for resistance would have to be monitored (Mochizuki et al. 2002, Evenden and Haynes 2001). Using both field and laboratory studies, we find the mechanisms of disruption to differ between attractive and
unattractive pheromone formulations. The complete blend initially attracts males through false-trail following, and then other mechanisms such as camouflage and adaptation are likely to occur, whereas the main mechanism for the major component alone appears to be adaptation leading to sensory system imbalance. Mating disruption has great potential to control *C. deauratella* and mitigate the damage caused by this debilitating pest; this is especially useful due to the current lack of insecticide control options. However, the use of pheromone twist-tie dispensers on large acreages of land used for clover seed production in Canada (average field size: ca. 65 ha) is not economically feasible due to extensive labour costs, therefore other dispenser types (i.e. pheromone puffers, or flakes) are currently under development for large-scale applications.

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**Table 1.** Individual ANOVA results of male *C. deauratella* antennal response to various doses of stimuli (complete pheromone blend) following 1-hour pre-exposure treatments to either the complete pheromone blend, the major component alone, or blank (clean air) control

<table>
<thead>
<tr>
<th>Stimuli (μg)</th>
<th>$F$</th>
<th>d.f.</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane (Control)</td>
<td>1.83</td>
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<td>0.18</td>
</tr>
<tr>
<td>0.00005</td>
<td>4.58</td>
<td>2,25</td>
<td>0.02</td>
</tr>
<tr>
<td>0.0005</td>
<td>6.87</td>
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<td>0.004</td>
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<tr>
<td>0.005</td>
<td>7.43</td>
<td>2,25</td>
<td>0.003</td>
</tr>
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<td>0.05</td>
<td>4.69</td>
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<td>0.5</td>
<td>2.00</td>
<td>2,25</td>
<td>0.16</td>
</tr>
<tr>
<td>5.0</td>
<td>4.96</td>
<td>2,25</td>
<td>0.015</td>
</tr>
<tr>
<td>50.0</td>
<td>5.01</td>
<td>2,25</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* Bold $P$-values indicate $P < 0.05$. Significant results indicate treatment effects which were further separated by Tukey’s HSD.
Figure 1. Pre-exposure apparatus: Air is filtered through a charcoal filter and passes through distilled water in a 125 ml Büchner flask to humidify the air before being split into three treatment arms. In each arm of the apparatus, air travels at 66 ml/min and enters a 250 ml Büchner flask (pheromone release chamber) which contains one of three treatments, a twist-tie dispenser releasing the complete pheromone blend, a twist-tie dispenser releasing the major component only, or a blank (clean air) control. From the pheromone release chamber, air travel to a 500 ml glass jar (moth exposure chamber). Air is vented from the moth exposure chamber via a hose to an exhaust port in a fume hood. Moths are placed individually into a moth exposure chambers containing one of the three treatments for one hour. For simplicity only one treatment arm is shown.
Figure 2. (a) Release rate ± S.D. (mg/day) of the complete pheromone blend from twist-tie dispensers determined gravimetrically at 20.0 ± 0.1°C (triangle) and 30.0 ± 1.0°C (circle). Data are the mean value of three replicates. Best fit line at 30°C (dashed): \( \text{Release Rate} = 11.16 \times \text{Day}^{-0.72} \); best fit line at 20°C (solid): \( \text{Release Rate} = 3.14 \times \text{Day}^{-0.42} \). (b) Release rate ± S.D. (mg/day) of the major component from twist-tie dispensers determined gravimetrically at 20.0 ± 0.1°C (triangle) and 30.0 ± 1.0°C (circle). Data are the mean value of three replicates. Best fit line at 30°C (dashed): \( \text{Release Rate} = 10.55 \times \text{Day}^{-0.72} \); best fit line at 20°C (solid): \( \text{Release Rate} = 3.04 \times \text{Day}^{-0.42} \).
Figure 3. Box-and-whisker plot of the total number of male *C. deauratella* captured in pheromone-baited traps in untreated (control) and pheromone-treated (complete blend or major component) plots (30 June - 21 July 2010). The midline indicates the median. The top and bottom of the boxes denote data falling within the first and third quartiles, respectively, and whiskers indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Box-and-whisker plots followed by different letters indicate significant differences (Tukey’s HSD: *P* < 0.05) in moth capture among pheromone treatments (a) Experiment 1 (b) Experiment 2.
Figure 4. Box-and-whisker plot of the total number of male *C. deauratella* captured on yellow sticky cards placed below dispensers at the edge and interior of pheromone-treated (complete blend or major component) plots (27 June - 12 July 2011). The midline indicates the median. The top and bottom of the boxes denote data falling within the first and third quartiles, respectively, and whiskers indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller.
Figure 5. Box-and-whisker plot of the total number of male *C. deauratella* captured in pheromone-baited traps in plots containing various densities of twist-tie dispensers releasing the complete pheromone blend (10:1 Z7-12:OAc:Z5-12:OAc) (18 July - 12 August 2011). The midline indicates the median. The top and bottom of the boxes denote data falling within the first and third quartiles, respectively, and whiskers indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Box-and-whisker plots followed by different letters indicate significant differences (Tukey’s HSD: *P* < 0.05) in moth capture among dispenser densities.
Figure 6. Mean (+SE) normalized EAG ($\ln(x + 1)$) responses generated from excised antennae of male Coleophora deauratella (a) 5 min and (b) 24 hr after a 1-hour pre-exposure treatment to either a twist-tie dispenser releasing the complete pheromone blend, the major component alone, or a blank (clean air) control (N=10 antennae/treatment). Means followed by different letters indicate significant differences (Tukey’s HSD: $P < 0.05$) in antennal response following a significant ANOVA.
Challenges of mating disruption using aerosol-emitting pheromone puffers in red clover seed production fields to control Coleophora deauratella (Lepidoptera: Coleophoridae)

Boyd A. Mori* and Maya L. Evenden

Department of Biological Sciences, CW405 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

*Corresponding Author: Phone: 1-780-492-3080
Fax: 1-780-492-9234
Email: bmori@ualberta.ca

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Preface

In this chapter we evaluate aerosol pheromone puffer dispensers for communication disruption of the red clover casebearer and explore the mechanisms by which mating disruption interferes with communication (Objective 1 and 2). We also evaluate whether pheromone puffers can reduce larval infestations and improve seed yield (Objective 4).

Abstract

Sex pheromone-mediated mating disruption using pheromone puffer dispensers was evaluated to control Coleophora deauratella (Lepidoptera: Coleophoridae) at three red clover seed production fields in Alberta, Canada. The objectives of the study were to determine aspects of the biology of C. deauratella which may affect successful mating disruption, evaluate the ability of aerosol-emitting pheromone puffers to reduce male trap capture in small-plot trials, and evaluate the ability of puffers to reduce male trap capture, larval numbers and damage in large-plot trials. The median longevity of male and female C. deauratella was 6 days in the laboratory with males emerging in larger numbers earlier in the emergence period than females (protandry). The male periodicity of response to pheromone peaked at sunrise and this information was used to program pheromone puffers to dispense pheromone when males are responsive. Small-plot (0.25 ha) mating disruption trials indicated that pheromone could reduce male C. deauratella orientation to traps by 60.7 ± 18.6% compared to that in untreated control plots. Reduction of male orientation to traps in large-plot (5 ha) trials over the course of the season was also successful (93.7 ± 1.6%). However, there was no corresponding decrease in larval numbers or increase in seed yield in pheromone-treated plots. Challenges of mating disruption of C. deauratella appear to be immigration of mated females combined with high population densities. In the future, mating disruption formulations (flakes or twist-ties) that work through density independent non-competitive mechanisms may work better to control high populations of C. deauratella.
Introduction

The red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae), is a severe pest of cultivated red clover, *Trifolium pratense* L. (Fabaceae), seed production fields in Canada (Ellis and Bjørnson 1996, Evenden et al. 2010). Female *C. deauratella* lay eggs on the calyx of developing red clover florets and upon hatching, larvae burrow through the calyx and feed on the developing seeds (Landry 1991, Ellis and Bjørnson 1996). The first three larval instars feed within red clover florets, whereas fourth instar larvae construct a portable case from which they feed (Landry and Wright 1993). Larvae are capable of consuming 2-3 seeds per day which can result in > 80% seed loss (Hammer 1937, Ellis and Bjørnson 1996, Evenden et al. 2010). As a result of the concealed feeding nature of larvae, infestations are difficult to control with insecticide and, presently, there are no registered insecticides for use against *C. deauratella* in Canada.

An alternative control method, pheromone-mediated mating disruption, could be advantageous against *C. deauratella* as it aims to control the mobile adult stage by preventing or delaying mating (Witzgall et al. 2010, Baker and Heath 2005). Mating disruption is achieved by treatment of the crop environment with synthetic sex pheromone which disrupts chemical communication between male and female insects (Howse et al. 1998, Witzgall et al. 2010). Four main mechanisms mediate mating disruption in most insects and are often divided into two categories: competitive and non-competitive (Miller et al. 2006). Competitive mechanisms occur via false-trail following in which males orient to a synthetic pheromone dispenser rather than a calling female (Bartell 1982, Miller et al. 2006). Non-competitive mechanisms include: sensory adaptation of the antennal receptors or habituation of the central nervous system which increases the response threshold to pheromone, camouflage of the natural female-produced pheromone plume as a result of the background of synthetic pheromone, and sensory system imbalance in which males preferentially respond to sub-optimal blends compared to the natural pheromone released by a calling female (Bartell 1982, Cardé and Minks 1995, Miller et al. 2006). These four mechanisms are not mutually exclusive and may work in concert to effectively control insect pest species (Sanders 1997). Mating disruption has successfully controlled many lepidopteran species including *Cydia pomonella* L. (Tortricidae) (Judd et al. 1996), *Keiferia lycopersicella* (Walsingham) (Gelechiidae) (Jimenez et al. 1988), *Epiphyas postvittana* (Walker) (Tortricidae) (Suckling and Shaw 1995), *Scripophaga incertulas* (Walker) (Crambidae) (Cork et al. 1998),
Lymantria dispar (L.) (Erebidae) (Cameron et al. 1974), and Pectinophora gossypiella (Saunders) (Gelechiidae) (El-Adl et al. 1988). Unlike insecticides, mating disruption is relatively species-specific and does not harm beneficial insects. This is especially important in red clover seed production fields as pollinators are needed in order to produce a high quality seed crop (Fairey 1981).

A variety of release formulations have been tested to apply pheromone in the cropping environment for mating disruption. The most popular release formulations used in commercial applications include twist-tie (rope), laminate flake, and aerosol-emitting dispensers. Hand-applied twist-tie dispensers are the most widely used mating disruption formulation worldwide (Gut et al. 2004); however, they are mainly used in high-value crops as their deployment is subject to high labour costs (Casado et al. 2014, Witzgall et al. 2010). A disadvantage of both laminate flake and twist-tie dispensers is that their pheromone release rate increases with ambient temperatures and large amounts of pheromone may enter the environment when the target species is not active (Witzgall et al. 2010). Aerosol-emitters (‘puffers’, ‘misters’, ‘microsprayers’) (hereafter, puffers) are mechanical devices that can be programmed to emit aerosolized pheromone into the air, and unlike flakes and twist-ties, pheromone is released over the course of a specific time period when the insect pest is active in the crop (Baker and Heath 2005, Suckling et al. 2007). Furthermore, puffers are applied at low densities (2-5/ha) which make them better suited for low value crops over large areas. Puffers have been used successfully in both field and tree cropping environments (Shorey and Gerber 1996a, 1996b). The high release rate of pheromone from puffers is thought to compensate for their low density of deployment (Baker and Heath 2005) and wind distributes the pheromone throughout the treated area and into the crop canopy (Gut et al. 2004). The high release rate and low density of pheromone puffers may help invoke false-trail following and habituation (Baker and Heath 2005), and recently, false-trail following was confirmed as the mechanism that disrupts C. pomonella when puffers are used (McGhee et al. 2014).

Since its identification, the sex pheromone of C. deauratella, 10:1 ratio of (Z)-7-dodecenyl acetate (Z7-12:OAc) to (Z)-5-dodecenyl acetate (Z5-12:OAc) (Evenden et al. 2010), has primarily been used for monitoring populations of this species (Mori et al. 2014). However, the potential for mating disruption to control C. deauratella should be explored. Many factors of the biology and ecology of the target species are known to influence the success of mating
disruption programs including the duration of responsiveness of males to pheromone (Cardé and Minks 1995), dispersal capacity, number of generations per year, and the adult lifespan (Gut et al. 2004). Here, we aim to identify factors that may influence the efficacy of mating disruption including the emergence pattern of C. deauratella throughout the season, male and female longevity, and the periodicity of male response to pheromone. We then explore the ability of pheromone puffer formulations to disrupt communication of C. deauratella in small-plot trials. One drawback of small-plot trials is that damage reduction is difficult to measure as mated females may immigrate into mating disruption treated plots. Thus, to reduce the effect of immigration and measure larval density and damage in the field, we also conduct a large-plot mating disruption study to determine if pheromone puffers can be used as a viable control method for C. deauratella in red clover seed production fields in Alberta.

Materials and Methods

Study sites. All field studies were conducted in the Peace River region of Alberta, Canada in 2010-2012. Study sites were located around Guy (55° 32' 54" N, 117° 7' 47" W) and Girouxville, AB (55° 45' 14" N, 117° 20' 18" W) and were conducted on red clover (T. pratense L. va. ‘Altaswede’) (Fabaceae) seed production fields (ca. 65 ha) separated by ≥ 1 km. No insecticides, herbicides or fungicides were applied to the fields during the course of these studies.

Moth emergence and longevity. To determine the emergence patterns and longevity of adult C. deauratella, field trash (stubble and leaf litter) containing overwintering larvae was collected from two sites on 4-5 May 2011. Trash was placed into cotton bags and transported in refrigerated containers to the laboratory at the University of Alberta (Edmonton, AB). In the laboratory, half the trash was removed from the bags and placed into ten plastic emergence bins (80 cm long by 40 cm wide by 45 cm high) equipped with two 500 ml emergence jars. Emergence bins were placed next to a window on a laboratory bench (22 ± 1°C) and under a fluorescent light set to a 16:8 h dark:light cycle. The other half of the trash was sorted by hand and larval cases were removed and individually placed into 30 ml cups and closed with a lid. A total of 600 larval cases were placed into cups. Cups were placed in a growth chamber (Precision Dual Program Illuminated Incubator, Thermo Scientific, Waltham, MA) under a 16:8 h dark:light cycle and temperature of 21°C ± 0.5°C. Emergence bins and cups were checked daily for moth eclosion. Emergence bins were also opened and inspected for moths that had emerged but not entered the jar, and the trash was misted with distilled water. When moths emerged from
either cups or jars, their sex was determined and recorded, and they were placed individually into 30 ml cups with access to 5% sugar water (w/v) *ad lib*. Emerged moths were placed into a separate growth chamber held under the same conditions and checked daily to determine their longevity.

*Periodicity of male response to pheromone.* To determine when males are active to pheromone sources, two 24 h-long field studies were conducted in 2010 and 2011. In both years, pheromone lures were prepared at the University of Alberta and consisted of pre-extracted grey rubber septa (Contech Enterprises Inc., Delta, BC, Canada) loaded with Z7-12:OAc (100 μg; 96.6% chemical purity) and Z5-12:OAc (10 μg: 98.6% chemical purity) (Bedoukian Research Inc., Danbury, CT) in high performance liquid chromatography grade hexane (EMD, Gibbstown NJ). Lures were stored at -20° C until transport to the field in refrigerated containers. On 29 June 2010, two green unitraps (Contech Enterprises) were placed 5 m from the field edge, 25 m apart and 35 cm above the soil surface on two red clover seed production fields separated by ca. 17 km. A strip of vaportape (10% Dicholorovos) (Hercon Environmental, Emigsville, PA) was inserted into each trap to kill captured insects. The second 24 h-long field study was conducted on 27 June 2011 on two red clover seed production fields separated by ca. 5 km. The 2011 study was conducted in the same way as 2010; however, wing traps (Contech Enterprises) were used instead of unitraps. Wing traps had a sticky bottom (capture surface: 193.6 cm²) to capture insects. Each year traps were checked once an hour for 24 h and the captured moths were counted and removed.

*Small-plot proof-of-concept experiment.* In 2010, a small-plot proof-of-concept experiment was conducted to determine if puffers that release the full pheromone blend could disrupt pheromone communication of *C. deauratella* as measured by orientation to pheromone-baited traps. Puffers were obtained from Suterra LLC (Bend, OR) and were designed to last for 21 d. A puffer is a mechanical device which consists of a plastic cabinet that contains a pressurized canister loaded with the active pheromone components and inert propellant. Each canister contained 10.44 mg of active ingredients (AI) (10:1 Z7-12:OAc:Z5-12:OAc). Puffers were set to spray 9.5 mg AI/puff once every 15 min for 12 h from 2000-0800 h.

The experiment was conducted in a pair-wise fashion in a single field and replicated in time and space. In the field, four 0.25 ha experimental plots (50 m X 50 m) were setup 25 m from the field edge and ≥ 100 m apart with two plots designated as untreated controls and two as
treated plots. The treated plots each received one pheromone puffer placed 1.25 m above the soil surface at the centre of the plot. All experimental plots contained four green unitraps baited with commercially available pre-extracted grey rubber septa lures containing 100:10 μg dose of Z7-12:OAc to Z5-12:OAc (Contech Enterprises). A strip of vaportape (10% Dicholorovos) was inserted into each trap to kill captured insects. Traps were placed 12.5 m from the centre of the plots along the cardinal directions 35 cm above the soil surface (Mori and Evenden 2013). The first two replicates of the experiment ran for 14 d (28 June to 12 July) after which all traps and puffers were removed and captured moths counted. The third and fourth replicates were conducted (12 July to 26 July) for a subsequent 14 d with re-randomized treatments applied to plots using new puffers and unitraps. At the end of the second set of replicates all puffers and traps were removed and captured moths counted. The number of moths captured in all traps in each plot per replicate was combined to give a total number of moths captured per plot. In order to determine the trap capture reduction due to pheromone treatment, the disruption index was calculated as: % DI = \left( \frac{C - T}{C} \right) \times 100\%, where C = number of males captured in the control plot, and T = number of males captured in the treated plot (Roelofs and Novak 1981).

Large-plot mating disruption experiment. After the success of the initial small-plot proof-of-concept experiment in 2010, a large plot mating disruption experiment was conducted in 2012 to determine if pheromone puffer treatment could not only reduce male C. deauratella trap capture, but also reduce larval infestation and increase seed yield. The mating disruption experiment was conducted at three red clover fields (ca. 65 ha) over the course of the entire flight period in 2012 (11 June – 20 August). On 11 June, two 5 ha (223.6 m x 223.6 m) plots were established 25 m from the field edge and >100 m apart in each of three fields. One plot in each field was designated the control plot and one the treatment plot. A 2.7 m strip was mowed around each plot four times throughout the summer to facilitate access and ease of harvest. Each treatment plot received 10 pheromone puffers (Suterra LLC) (2 puffers/ha) which contained 33.6 g of AI (10:1 Z7-12:OAc:Z5-12:OAc) and were designed to release 7.0 mg AI/puff. Puffers were set to spray one puff of pheromone every 15 minutes from 2000-0800 h each day. Twelve green unitraps baited with commercially available pre-extracted grey rubber septa lures containing 100:10 μg dose of Z7-12:OAc to Z5-12:OAc (Contech Enterprises) were deployed to assess pheromone communication in each plot (Fig. 1). A strip of vaportape (10% Dicholorovos) was inserted into each trap to kill captured insects. Pheromone puffers were placed in a grid pattern
throughout the plot, with two puffers placed at the centre (Fig. 1). Unitraps were placed throughout the plot at least 28 m from the nearest puffer and were positioned 35 cm above the soil surface (Fig. 1) (Mori and Evenden 2013). Unitraps were also placed in control plots in the same pattern as the pheromone-treated plots. Pheromone lures were replaced in the unitraps after 6 weeks. Traps were checked at two-week intervals (25 June, 9 July, 23 July, 6 August, and 20 August), their contents removed and *C. deauratella* counted. The number of *C. deauratella* captured across all traps in each plot was combined to give the total number of males captured per plot per two-week interval.

Larvae were sampled in all plots on 7 August 2012. Twenty-five samples of 50 flower heads were collected systematically around the edge and the interior of the plot. Samples at the edge of the plots were taken 5 m into the plot and 32 m apart parallel to the plot edge. In the interior, samples were taken in a square pattern 28 m from the centre of the plot and 4 m apart. The sides of the square were parallel to the edge of the plot (Fig. 1). Each 50-flower head sample was individually bagged and placed in a refrigerated container for transport back to the laboratory at the University of Alberta. In the laboratory, all flowers were dissected and the number of larvae counted. The number of larvae collected in all samples at the edge and interior were combined to give a total number of larvae at the edge and interior of each plot, respectively.

Seed yield was assessed at each field at the end of the growing season (12, 18 September, and 18 October) and was obtained from individual producers. Four strips (width varied between producer due to different machinery: 6.2-14.6 m but remained the same for treated and control plots on each field) were harvested in each plot starting along the north edge. The centers of the strips were spaced evenly throughout the plot with the final strip occurring along the south edge. The raw seed yield was obtained for each strip (kg/ha) and samples were taken to determine dockage. Dockage is a factor used to determine the overall clean seed weight. The seed yield after dockage was combined from each strip per plot to give the total seed yield per plot.

*Statistical analyses.* All data was analyzed for normality and heteroscedasticity using Shapiro-Wilks tests and visualization techniques (R Core Team 2013). If non-normal error distributions were observed, Poisson or negative binomial error distributions were used in the models. Akaike information criterion (AIC) values were compared between each model and log likelihood ratio tests were conducted to determine if a Poisson or negative binomial distribution was a better fit to the data. In the laboratory studies, a generalized linear mixed-effects model
with a Poisson distribution was used to determine if moth emergence time differed between sexes. The total number of moths emerged in the laboratory was specified as the dependent variable and sex, emergence day, and a sex X emergence day interaction were specified as independent variables and all were considered fixed effects. Collection site was specified as a random effect. To determine if the longevity of moths in the laboratory differed between the sexes a generalized linear model with a Poisson distribution was used.

The mating disruption proof-of-concept trap capture data was normally distributed; therefore a general linear mixed-effects model was used to determine if the number of moths captured in pheromone-baited traps differed between pheromone-puffer-treated and control plots. Time period was specified as a random effect and number of moths captured and treatment as fixed effects.

A repeated-measures mixed-effect model with negative binomial distributions was used to determine if trap capture differed with pheromone treatment for the large-plot mating disruption study. The number of moths captured and treatment were specified as fixed effects, and site nested within time as a repeated-measure. Larval numbers were normally distributed; therefore to determine if larval numbers differed between pheromone-treated and control plots a generalized linear mixed-effects model was used. Pheromone treatment, sample position, and a sample position X pheromone treatment interaction were specified as fixed effects and site was specified as a random effect. The interaction term was removed from the model as it was not significant. Finally, a generalized linear mixed-effects model was used to determine if seed yield differed by pheromone treatment with pheromone treatment specified as a fixed effect and site as a random effect. To determine the \( P \)-values for all fixed effects in the models, analysis of deviance tables and \( \chi^2 \) goodness-of-fit statistics (analogous to \( F \)-values) were used. All data analyses were conducted in R 3.0.1 (R Core Team 2013).

**Results**

*Moth emergence and longevity.* Very few moths emerged from larval cases placed in individual cups \((N = 10)\) potentially due to the extraction process or inadequate moisture, thus the emergence pattern is based on moths that emerged from rearing bins. There was a significant *C. deauratella* sex by emergence day interaction \((\chi^2 = 4.97, \text{df} = 1, P = 0.025)\) with males emerging earlier in the emergence period than females (Fig. 2). There was no difference in *C.*
*deauratella* longevity between the sexes ($\chi^2 = 0.57$, df = 1, $P > 0.05$). The median male and female age was 6 d and ranged from 3-11 and 3-15 d, respectively.

**Periodicity of male response to pheromone.** In both 2010 and 2011, male attraction to sex pheromone traps peaked between 0300 and 0600 h each morning. Almost all males arrived at the traps between 0000 and 1000 h (Fig. 3). In 2010, there was a moderate secondary peak of attraction between 2100 and 0000 h, but this was not observed in 2011. The captures at the primary peak of attraction were ca. 6 times greater than those at the secondary peak (Fig 3). During this time of year sunrise and sunset at the field sites occurred at 0410 and 2236 h, respectively (National Research Council of Canada [http://www.nrc-cnrc.gc.ca/eng/services/sunrise/](http://www.nrc-cnrc.gc.ca/eng/services/sunrise/)).

**Small-plot proof-of-concept experiment.** Male *C. deauratella* capture in pheromone-baited traps was reduced by 60.7 ± 18.6 % compared to untreated controls ($\chi^2 = 4.11$, df = 1, $P = 0.04$) (Fig. 4). The highest recorded number of male *C. deauratella* captured was 1,321 and 2,428 in a treated and control plot, respectively in a 2 wk period. Puffer canisters were expected to deliver 456 mg AI/d, but after recording the initial and final weights of the canisters the realized amount was 392.9 ± 23.4 mg AI/d or 8.18 ± 0.48 mg AI/puff (values are estimates based off the percent AI in each canister provided by the manufacturer).

**Large-plot mating disruption experiment.** Over the course of the entire flight period of *C. deauratella*, male capture in pheromone-baited traps was reduced by 93.7 ± 1.6% in pheromone-treated plots compared with that in untreated control plots ($\chi^2 = 368.24$, df = 1, $P < 0.0001$) (Fig. 5). Over all time periods, male moth capture in control plots was four times greater than the number of males captured in treated plots and during peak moth flight it was 25 times greater in control plots compared with treated plots. Even during peak flight (23 July check) with population densities (> 600 moths captured/trap) communication was greatly disrupted (94.8 ± 1.2%) (Fig. 5). Puffer canisters were expected to deliver 336 mg AI/d, but after recording the initial and final weights of the canisters the realized amount was 284. 7 ± 14.7 mg AI/d or 5.93 ± 0.30 mg AI/puff (values are estimates based off the percent AI in each canister provided by the manufacturer). The number of larvae per plot position varied from a high of 721 at the edge of a pheromone-treated plot, to a low of 66 in the interior of a control plot (Fig. 6). There was no significant effect of pheromone treatment on larval numbers ($\chi^2 = 0.85$, df = 1, $P = 0.36$), however there was a significant position effect with plot edges having higher numbers of larvae
than the interior ($\chi^2 = 11.54, \text{df} = 1, \text{P} < 0.001$) (Fig. 6). There was no effect of pheromone treatment on seed yield ($\chi^2 = 0.0001, \text{df} = 1, \text{P} = 0.99$). The median seed yield was 569.3 kg/ha for control and 492.2 kg/ha for treated plots and ranged from 161.8-597.7 kg/ha and 154.2-640.7 kg/ha, in control and treatment plots, respectively. The seed yield was higher in two of three control plots compared with the pheromone-treated plots at the same sites.

**Discussion**

All aspects of the biology of the target insect, pheromone chemistry and biology, and dispenser technology need to be integrated for mating disruption to be successful in the field (Witzgall 2001). Hence, we explored aspects of the biology of *C. deauratella* before conducting any large-scale mating disruption studies. *Coleophora deauratella* is univoltine throughout its North American range. Adults begin to emerge in June and larvae complete development by early September, depending on temperature (Ellis and Bjørnson 1996). In the laboratory, *C. deauratella* is slightly protandrous with males emerging in larger numbers than females early in the flight period. Evidence of protandry is further supported by sweep net samples in the field (B.A.M., unpublished data). Protandry has implications for control of pest populations by mating disruption, as early emerging males naturally experience difficulty in mate location. Pheromone treatment will further enhance this effect as males may be attracted to pheromone dispensers which could prevent them from mating before their death. The increased number of point sources releasing pheromone will directly compete with the few females that emerge early and any benefit early emerging females may have had due to reduced competition would be negated. Protandry may be an adaptation to minimize the time females remain unmated (Fagerström and Wiklund 1982), given that we also found the median lifespan of male and female *C. deauratella* was six days, the longer females are prevented from mating the more likely it is that females will die before they can deposit fertile eggs (Beroza and Knipling 1972). It is also important to know if the species is protandrous as the mating disruption treatment should be applied before males are present in the field. Moths likely emerge throughout the summer as their median longevity in the lab is only 6 days and yet they exhibit a prolonged unimodal flight period in the field (June-August). The long emergence/flight period of species can affect mating disruption as the formulation must be able to last in the field for the entire flight or be reapplied.

Knowledge on the periodicity of male response to pheromone enables pheromone puffers to be programmed to emit pheromone when males are responsive. Baker et al. (1997) observed
lower mating disruption efficacy when aerosol-emitters (Metered Semiochemical Time Release System, MSTRSTM) released pheromone against *Rhopobota naevana* (Hübner) (Lepidoptera: Tortricidae) during the night when moths were not active compared to aerosol-emitters dispensing pheromone 24-hours a day. The results of our study indicate *Coleophora deauratella* are primarily attracted to pheromone traps 1 h before and after sunrise (Fig. 3). Similarly, *C. dahurica* Flkv. (Lepidoptera: Coleophoridae) males were attracted to traps mainly between 0300-0400 h which also coincided with sunrise (Priesner and Zhang 1991). Whereas, *C. laricella* Hbn. (Lepidoptera: Coleophoridae) are attracted to pheromone traps in the afternoon and evening (Witzgall 1985). We used the findings on the periodicity of *C. deauratella* males in our subsequent mating disruption studies to time the release of pheromone during the period males were most responsive.

Female *C. deauratella* pheromone glands dissected at dusk had pheromone titers lower than the detection threshold of the gas chromatography (GC) flame ionization and GC mass spectrometry (MS) detection systems (Evenden et al. 2010). Many moths are known to produce pheromone in diel circadian rhythms (Webster and Cardé 1982, Kamimura and Tatsuki 1993, Rosén 2002) and although the male response period to pheromone is often greater than the female calling period, the response and calling period overlap. Therefore, the periodicity of male response to pheromone could be used as a proxy for the periodicity of female pheromone production and calling. Evenden et al. (2010) dissected females during the first 2-3 h of the scotophase as moths are active in the late afternoon and early evening (Evenden et al. 2010, Landry and Wright 1993). Dissection of females around sunrise (or the end of the scotophase) would probably result in an increased detection of pheromone in female glands.

Reduction in trap capture indicates that pheromone puffers are effective at reducing the ability of male *C. deauratella* to locate pheromone point sources. This may also indicate that mating within the treated area could be reduced. However, given that reduction in trap capture never reached 100%, some males may locate females and mate. In the proof-of-concept study, there was large variation in the reduction of male pheromone-baited trap capture among replicates. This was particularly emphasized by poor reduction in trap capture in one treated plot (7% reduction) compared to the reduction (> 62% reduction) in all other treated plots. Although the average level of reduction in trap capture in treated plots (60.7%) is not large, the ability of puffers to disrupt pheromone-based communication is enhanced over larger treatment areas.
(Shorey and Gerber 1996a, 1996b), and therefore large-plot mating disruption studies were carried out.

In the large-plot study there was a high reduction in trap captures (93.7%) in pheromone-treated plots, but it did not correspond with a subsequent reduction in larval numbers and an increase in seed yield. This same phenomenon of reduced trap capture, but lack of damage suppression has been noted in several other lepidopteran species under pheromone-based mating disruption including Grapholita molesta (Busck) (Tortricidae) (Kovanci et al. 2004), P. gossypiella (Saunders) (Gelechiidae) (Lykouressis et al. 2005), Spodoptera exigua (Hubner) (Noctuidae) in broccoli and lettuce (Kerns 2000), Choristoneura rosaceana Harris (Tortricidae), and Anarsia lineatella Zeller (Gelechiidae) (Baker and Heath 2005). The inability of pheromone treatment to reduce larval numbers and damage in some mating disruption studies has been attributed to immigration of mated females, high population densities, and the dispenser type used (Baker and Heath 2005, Gut et al. 2004).

The design of the large-plot study which placed two plots (control and treatment) within the same field was required as there were few red clover seed production fields in the area and it is often difficult to find plots that have similar population densities, the same clover cultivars, planting dates and phenology to act as control plots (Baker and Heath 2005). However, placement of two plots within one field, rather than treating the entire field, left large untreated areas of clover surrounding each plot. These untreated areas may have acted as a source of mated C. deauratella females that could immigrate into the plots to lay eggs. Increased larval numbers at the edge of both treatment and control plots indicate that this may have arisen. Also, the arrangement of the puffers within the 5 ha plots may have led to large areas at the plot edges with low pheromone concentrations. Low pheromone concentrations at the edge of the plots led to increased damage or larvae in other studies (Ogawa 1990, Sauer and Karg 1998). In orchards clean air (wind) entering the treated area results in depletion of pheromone up to 15 m into the plot (Milli et al. 1997). To overcome these edge effects, other studies have adopted a design in which pheromone puffers are placed only at the edge of pheromone-treated plots (Shorey and Gerber 1996, Burks and Brandl 2004). While other researchers apply a secondary treatment of twist-tie dispensers at the plot edge to increase control (Knight 2002, 2004). It is also possible that females immigrated into large plots from long distances as red clover is often found as a weed growing in ditches, pasture, and fallow fields. To date, there is no information on the
movement of *C. deauratella* within red clover fields or their dispersal ability between fields. However, immigration of mated females, rather than depletion of pheromone at the edge, is the most probable contributing factor to mating disruption failure for several reasons. First, disruption of orientation to traps placed 28 m from the edge of the plot was as successful as disruption of orientation to traps positioned further in the interior which indicates pheromone released from puffers is present and not depleted around the plot edge. Second, there were large untreated areas of clover that surrounded each plot from which mated females could enter the plots. This movement is supported by the finding that there were higher numbers of larvae at the edge than in the interior of both treatment and control plots. If pheromone depletion at the plot edge was the primary culprit of a reduced mating disruption effect, the number of larvae in the center of treated plots (where pheromone is not depleted) should be lower than that in the center of control plots. In the current study, the number of larvae recovered in the center of both control and treated plots was similar. It also seems plausible that *C. deauratella* would be capable of flying the ~111 m from the edge to the centre of the plots and future studies using this technology should test mating disruption treatment applied to an entire field (~64.7 ha).

Another contributing factor to mating disruption failure may be high *C. deauratella* population numbers. Mating disruption is commonly known to be inconsistent and often fails at high population densities (Sanders 1981, Gut et al. 2004). With high population densities, there is an increased probability that males locate females by chance through random encounters (Barclay and Judd 1995). Most successful pheromone puffer mating disruption studies are conducted under low pest population density with average trap capture in untreated control plots < 100 moths/wk (Knight 2002, 2004, Shorey and Gerber 1996a, 1996b). In the current study, densities were much higher especially during peak flight when > 300 moths/trap/wk were captured in control plots. These results coincide with work on *C. pomonella* in which high pest population densities decreased the efficacy of mating disruption using pheromone puffers (Stelinski et al. 2007).

The results of the current study are consistent with previous work that confirms mating disruption with puffers acts through the competitive mechanism of false-trail following which is density dependent (Miller et al. 2006, McGhee et al. 2014). It appears that *C. deauratella* males are attracted to pheromone puffers as all puffers had remnants of moth scales at the port of the nozzle of the plastic cabinet. Habituation has also been suggested as a possible mechanism that is
enacted by puffers (Baker and Heath 2005). *Coleophora deauratella* males are able to orient and contact pheromone puffers despite the high levels of pheromone on the puffer cabinet and surrounding the nozzle. Therefore, it appears that non-competitive mechanisms such as adaptation and habituation do not play a large role. A lack of neurophysiological effects of pheromone exposure to male *C. deauratella* might be expected as males do not exhibit a dose response over a wide range of pheromone doses (10-1000 μg) released from lures (Evenden et al. 2010). Puffers are thought to provide enough pheromone to interfere with mating even at low density by releasing high amounts of pheromone (Baker and Heath 2005). However, there is mounting evidence that mating disruption of various moth species is superior with a high density of point sources that provide even pheromone coverage throughout the crop canopy (Stelinski et al. 2007). The findings from the current study suggest that puffers do not elicit non-competitive mechanisms of mating disruption in this system. Non-competitive mechanisms are needed to control high pest population numbers in other systems (Stelinski et al. 2008, Miller et al. 2006), suggesting that pheromone puffers cannot successfully control *C. deauratella* at the population densities experienced in this study.

This study demonstrates that pheromone puffers can disrupt communication in *C. deauratella*; but treatment of large plots did not reduce larval numbers or increase seed yield. Immigration of mated females combined with high population densities most likely resulted in mating disruption failure. In the future, mating disruption formulations (flakes or twist-ties) that are applied at high point source densities and work through multiple mechanisms, including density independent non-competitive mechanisms, may work better to control high populations of *C. deauratella*.

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References


Figure 1. Large-plot pheromone puffer treatment layout indicating positions of pheromone puffers, unitraps, and larval sampling pattern. Control plots were laid out in the same way except no pheromone puffers were deployed. Plots were 5 ha (223.6 X 223.6 m).
Figure 2. Emergence of *C. deauratella* males and females from field collected trash (leaf litter and stubble) in the laboratory. Bars indicate the total number of each sex that emerged each day. There was a significant sex by emergence day interaction (*P* < 0.05).
Figure 3. Periodicity of male *C. deauratella* over 24 h to pheromone-baited traps. A) Mean (± S.E.) number of male *C. deauratella* captured in green unitraps checked every hour on 29-30 June 2010 at two sites. B) Mean (± S.E.) number of male *C. deauratella* captured in wing traps checked every hour on 27-28 June 2011 at two sites. Vertical dashed lines represent sunrise and sunset.
Figure 4. Box plot of the total number of male *C. deauratella* captured in control (grey) and pheromone-treated (white) plots in the proof-of-concept small-plot communication disruption trial. The bottom and top of the box represent the first and third quartiles, respectively, the midline indicates the median. Vertical lines extending from the box (whiskers) represent the maximum and minimum values. Box plots followed by different letters indicate significant differences ($P < 0.05$) in moth capture between plots.
Figure 5. Box plot of the total number of male *C. deauratella* captured control (grey) and pheromone-treated (white) plots in the large-plot mating disruption study over the course of the season. The bottom and top of the box represent the first and third quartiles, respectively, the midline indicates the median. Vertical lines extending from the box (whiskers) represent the maximum and minimum values. Box plots at the same time period followed by different letters indicate significant differences (*P* < 0.05) in moth capture between plots.
Figure 6. Box plot of the total number of *C. deauratella* larvae sampled at the edge and interior of control (grey) and pheromone-treated (white) plots in the large-plot mating disruption study. The bottom and top of the box represent the first and third quartiles, respectively, the midline indicates the median. Vertical lines extending from the box (whiskers) represent the maximum and minimum values. There was no effect of treatment on larval numbers, but there is a significant effect of position. Box plots followed by different letters indicate significant differences (*P* < 0.05) between larval numbers at the different sampling positions.
Mating disruption of *Coleophora deauratella* (Lepidoptera: Coleophoridae) using laminate flakes in red clover seed production fields

Boyd A. Mori* and Maya L. Evenden

Department of Biological Sciences, CW405 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

*Corresponding Author: Phone: 1-780-492-3080
Fax: 1-780-492-9234
Email: bmori@ualberta.ca

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Preface

In this chapter we evaluate sprayable laminate flake pheromone dispensers for communication disruption of the red clover casebearer and explore the mechanisms by which mating disruption interferes with communication (Objective 1 and 2). We also assess the release rate and application density required for mating disruption (Objective 3) and evaluate whether laminate flake dispensers can reduce larval infestations and improve seed yield (Objective 4).

Abstract

Red clover (Trifolium pratense L.) is an important temperate region forage and cover crop that can enhance soil quality. The red clover casebearer, Coleophora deauratella (Lepidoptera: Coleophoridae) is a significant seed predator in red clover seed production regions throughout the world. Due to the internal feeding nature of C. deauratella larvae, infestations are not controlled with insecticide. Here, we test the ability of Hercon Disrupt Micro-Flakes® releasing the complete female-produced pheromone blend to disrupt C. deauratella populations in red clover seed production fields in Alberta, Canada. Initial small-plot (0.25 ha) proof-of-concepts trials found a significant reduction (93.6 ± 2.9%) of male C. deauratella captured in pheromone-treated plots compared to untreated controls. Subsequent large-plot (5 ha) mating disruption trials found a significant reduction (72.3 ± 5.7 %) of male C. deauratella captured in pheromone-treated plots compared to untreated controls over the growing season. Furthermore, larval numbers were significantly reduced and seed yield was slightly increased in pheromone-treated large plots compared to untreated controls. A further small-plot (0.0625 ha) trial was conducted with varying flake density to determine the mechanisms by which mating disruption acts on C. deauratella when laminate flakes are used. Disruption increased as pheromone flake density increased and the resulting graphical disruption profiles matched the theoretical predictions of mating disruption by competitive attraction. This study demonstrates that mating disruption with laminate flakes can suppress C. deauratella populations and may help to reduce damage even at high pest pressure.
Introduction

Red clover, *Trifolium pratense* L. (Fabaceae), is an important temperate region forage and cover crop which can suppress weeds, reduce soil erosion and enhance soil quality through nitrogen fixation (Taylor and Quesenberry 1996, Schipanski and Drinkwater 2011). The red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae) is an important pest throughout most red clover seed production regions (Markkula & Myllymäki, 1960, Ellis & Bjørnson 1996, Evenden et al. 2010). It is native to Europe, Eastern Siberia and the Middle East and was introduced to North America more than 50 years ago (Landry 1991). In Europe, *C. deauratella* causes only minor damage with occasional but severe infestations (Markkula & Myllymäki 1960), however, in Canada *C. deauratella* infestations have led to >80% seed losses (Evenden et al. 2010, Ellis & Bjørnson 1996). Furthermore, *C. deauratella* was recently identified from the Willamette Valley in western Oregon (BAM, unpublished), the largest red clover seed production region in the world (Wong 2005).

*Coleophora deauratella* is univoltine with adults beginning to emerge in late May or early June (Landry 1991, Ellis & Bjørnson 1996, Mori et al. 2014). Eggs are laid on the calyx of developing red clover florets, and upon hatching, larvae enter the floret and feed on the developing ovules (Landry 1991, Ellis & Bjørnson 1996). The cryptic internal feeding nature of *C. deauratella* larvae make populations difficult to control with insecticides and currently there are no registered products available for use. Pheromone-mediated mated disruption may have the potential to control *C. deauratella* as it targets the mobile adult stage by permeating the crop environment with synthetic sex pheromone to interfere with chemical communication between males and females and prevent or delay their mating (Howse et al. 1998, Baker and Heath 2005, Witzgall et al. 2010). Targeting adults would be particularly useful against *C. deauratella* as this species overwinters as a fully grown larva and pupation occurs in the spring. Therefore, adults can be targeted with mating disruption before larvae feed during the growing season. Furthermore, mating disruption is relatively species specific and does not harm non-target organisms which is especially important in red clover seed production fields where managed honey bees (*Apis melifera* L. (Hymenoptera: Apidae)) and wild bumble bees (*Bombus* spp. L. (Hymenoptera: Apidae)) are needed for pollination (Forester and Hadfield 1954, Holm 1966).

The development of successful mating disruption programs often hinges on understanding the mechanisms by which mating disruption alters the mate-finding behaviour of a
particular insect pest (Cardé et al. 1998). Both competitive and non-competitive mechanisms mediate mating disruption and are thought to act either individually or collectively to disrupt mating (Sanders 1997, Miller et al. 2006). False-trail following, in which males orient to dispensers releasing synthetic sex pheromone rather than to a calling female, is a density-dependent competitive mechanism (Bartell 1982, Miller et al. 2006). Whereas, non-competitive mechanisms act in a density-independent manner and include: 1) the neurophysiological effects of adaptation of the antennal pheromone receptors or habituation of the central nervous system that result in reduced responsiveness to pheromone; 2) camouflage of the natural pheromone plume released by calling females due to high levels of synthetic background pheromone which results in the inability of a male to locate a calling female; 3) and sensory-system imbalance in which males respond to suboptimal pheromone blends that are not naturally produced by a calling female (Bartell 1982, Cardé and Minks 1995, Miller et al. 2006). Mating disruption has been successfully developed for many lepidopteran field crop pests including *Sesamia nonagrioides* Lefèbvre (Noctuidae) (Albajes et al. 2002), *Cydia nigracana* F. (Tortricidae) (Bengtsson et al. 1994), *Pectinophora gossypiella* (Saunders) (Gelechiidae) (El-Adl et al. 1988), *Keiferia lycopersicella* (Walsingham) (Gelechiidae) (Jimenez et al. 1988), and *Tecia solanivora* (Povolny) (Gelechiidae) (McCormick et al. 2012). A series of mathematical formulae and graphical disruption profiles have been developed to determine if mating disruption acts via competitive or non-competitive mechanisms and have been validated with several different insect species (Miller et al. 2006a,b, Stelinski et al. 2008, Miller et al. 2010, Rodriguez-Saona et al. 2010, Reinke et al. 2014, McGhee et al. 2014).

In the current study Hercon Disrupt Micro-Flakes® loaded with the female *C. deauratella* sex pheromone (10:1 of Z-7-dodecenyl acetate (Z7-12:OAc) to Z-5-dodecenyl acetate (Z5-12:OAc)) are used to test if mating disruption can control *C. deauratella* in red clover seed production fields in Alberta, Canada. The attractiveness of laminate flakes to male *C. deauratella* is tested in flake-baited traps to help determine if false-trail following is a potential mechanism by which mating disruption can work in this species. Small-plot trials test the capacity of laminate flakes to disrupt *C. deauratella* pheromone communication and the potential mechanism(s) by which mating is disrupted in this species. The data from small plot trials are applied to the formulae of Miller et al. (2006) to test if competitive or non-competitive mechanisms are prominent in this system. The potential to control *C. deauratella* populations by
pheromone-based mating disruption is tested in a large-plot experiment to determine if pheromone treatment can decrease male trap capture and larval numbers and increase seed yield.

**Materials and Methods**

**Pheromone formulation**

Hercon Disrupt Micro-Flake® dispensers (Hercon Environmental, Emigsville, PA) (hereafter flakes) were formulated to contain 9.5 % active ingredients (Full pheromone blend: 10:1 Z7-12:OAc to Z5-12:OAc, Evenden et al. 2010) and 90.5 % inert ingredients with a manufacturer recommended application rate of 280 g flakes/ha. The application rate was targeted to provide a release rate of 407 mg Al/ha/day for 60 days. Pheromone lures used in all experiments to bait assessment traps are commercially available (Contech Enterprises Inc., Delta, BC) and consisted of a pre-extracted grey rubber septa loaded with 100 μg of Z7-12:OAc and 10 μg Z5-12:OAc.

**Study Sites**

All studies were conducted on red clover (*Trifolium pratense* L. (Fabaceae) va. ‘Altaswede’) seed production fields (~65 ha each) in the Peace River region of Alberta, Canada. Experiments were conducted over two years (2012-2013) at study sites predominately around the town of Guy (55° 32' 54" N, 117° 7' 47" W) and Girouxville (55° 45' 14" N, 117° 20' 18" W). No insecticides, herbicides or fungicides were applied to the fields over the duration of these studies.

**Experiment 1: Attraction of pheromone flakes**

To determine the attractiveness of flakes to *C. deauratella*, wing traps (Contech Enterprises) with a sticky insert (capture surface: 193.6 cm²) to capture insects were baited with either zero, one, five, or ten flakes, or a grey rubber septa lure (positive control). Flakes and lures were attached centrally to the interior of the top of the trap using double sided sticky tape. A push pin was used to further secure the lure. Traps were placed 5 m from the field edge, 25 m apart and 35 cm above the soil surface (Mori & Evenden 2013) in a randomized-block design at six red clover seed production fields near Guy, AB. No mating disruption treatments occurred on these fields. Traps were checked weekly for two weeks (9 July – 23 July 2012), sticky inserts were removed and moths counted. The numbers of moths captured each week for each treatment was combined to give the total number of moths captured over the course of the experiment.

**Experiment 2: Small-plot communication disruption experiment**
To determine if flakes releasing the complete pheromone blend of *C. deauratella* could cause communication disruption to pheromone-baited traps, a small-plot (0.25 ha) proof-of-concept experiment was conducted. At each of three red clover seed production fields, two 0.25 ha plots (50 x 50 m) were setup 25 m from the field edge and ≥100 m apart. Each plot received four green unitraps placed 35 cm above the soil surface (Mori and Evenden 2013) and 12.5 m from the centre of the plot along the cardinal directions. One plot was randomly designated the treatment plot and received 70 g of pheromone flakes spread by hand evenly throughout the plot. The second plot remained untreated and acted as the control. The experiment was conducted for four weeks (9 July – 6 August 2012), traps were checked every two weeks and their contents removed. The number of male *C. deauratella* captured per trap after each two-week check was pooled to give the total number of males captured per plot. To determine the reduction in trap capture of male *C. deauratella* in pheromone-treated plots the percent inhibition of male trap capture was calculated as $\% \text{ inhibition} = \left(\frac{C-T}{C}\right) \times 100\%$ where $C =$ number of *C. deauratella* captured in control plots and $T =$ number of *C. deauratella* captured in pheromone-treated plots (Roelofs and Novak 1981).

**Experiment 3: Pheromone flakes density experiment**

This experiment was conducted in three red clover seed production fields, to determine the effect of pheromone flake density on communication disruption of male *C. deauratella* to pheromone-baited traps. In each field, six 0.0625 ha (25 m X 25 m) plots were setup 25 m from the field edge and 50 m apart. The dominate wind direction at each site was determined and the control plot (0 g pheromone flakes) was placed upwind of the treatment plots to prevent pheromone drift. All other plots were randomly treated with 2.1875 g (35 g/ha), 4.375 g (70 g/ha), 8.75 g (140 g/ha), 17.5 g (280 g/ha) or 35 g (560 g/ha) of pheromone flakes. One green pheromone-baited unitrap placed 35 cm above the soil surface (Mori and Evenden 2013) was placed in the centre of each plot to assess communication disruption. The experiment was conducted over eight weeks (56 days) (17 June – 12 August 2013) and traps were checked, emptied and moths counted at two week intervals. The number of *C. deauratella* captured per trap over the course of the eight week period in each plot was totalled and used in subsequent analyses. The disruption index was also calculated to determine the reduction in trap capture in pheromone-treated plots.
To determine the mechanisms by which communication disruption occurs when flakes are used to disrupt *C. deauratella*, three graphical plots were created and compared to theoretical disruption profiles (Miller et al. 2006a). The number of male *C. deauratella* captured was plotted against flake density (untransformed plot), 1/male *C. deauratella* capture against flake density (Miller-Gut plot), and male *C. deauratella* capture against flake density X male *C. deauratella* catch (Miller-de Lame plot) (Miller et al. 2006a). The shape of each of the graphical profiles (untransformed, Miller-Gut, and Miller-de Lame plots) is indicative of competitive or non-competitive mechanisms. A competitive mechanism should result in a graphical profile that is concave with the shape of an inverse function on the untransformed plot, linear with a positive slope on the Miller-Gut plot, and linear with a negative slope on the Miller-de Lame plot (Miller et al. 2006a). Whereas, non-competitive mechanisms result in a graphical profile that is linear on the untransformed plot, concave on the Miller-Gut plot, and recurved on the Miller-de Lame plot (Miller et al. 2006a).

**Experiment 4: Large-plot mating disruption experiment**

The success of the small-plot communication disruption trials warranted follow up with large-plot trials to determine if treatment with pheromone flakes can reduce *C. deauratella* trap capture and subsequent larval populations, and increase seed yield over the course of the season (70 days) (17 June – 26 August 2013). At each of three red clover seed production fields, two 5 ha plots were established 25 m from the field edge and ≥ 100 m apart. Four times throughout the summer a 2.7 m strip was mowed around each plot to delimit the plots, and for access and ease of harvest. To assess communication disruption, each plot received nine green unitraps placed 35 cm above the soil surface (Mori and Evenden 2013) in a grid pattern (Fig. 1). At each site, one plot was randomly designated the treatment plot and received 1400 g of flakes (280 g/ha) spread evenly by hand throughout the plot. The other plot was designated the control and left untreated (0 g flakes/ha). Pheromone traps were checked every two weeks throughout the flight period and the contents emptied and male *C. deauratella* counted.

To sample for larval density in the experimental plots, twenty-five samples of fifty flower heads were sampled systematically on 6-7 August at the edge and interior of each plot. Samples at the edge were taken 5 m from the edge and 32 m apart parallel to the plot edge. Interior samples were taken 28 m from the centre of the plot and 4 m apart, parallel to the edges of the plot. Each sample of fifty flower heads was placed individually into plastic sealable bags and
was transported in refrigerated containers to the laboratory at the University of Alberta (Edmonton, AB). In the laboratory, all flower heads were dissected and the number of larvae counted. The number of larvae from all samples at the edge and interior were pooled to give the total number of larvae at the edge and interior of each plot, respectively.

Seed yield from each 5 ha plot was obtained at the end of the season when individual producers harvested their fields (5, 13, 18 October 2013). Four strips from each plot (width varied with producer’s equipment: 7.62 – 10.68 m) were harvested starting from the west side. The strips were spaced evenly throughout the plot with the last strip on the east edge. Raw seed yield was obtained for each strip and samples were taken to determine the dockage. Dockage is a factor used to grade seed and takes into account any weed seed or residues in the crop seed and is used to determine the overall clean seed weight. The seed yield (kg/ha) after dockage from each strip was combined to give a total seed yield per plot based on area harvested and used in subsequent analyses.

**Experiment 5: Field-aged pheromone flake release rate**

To determine the release rate of the pheromone flakes in the field, packages of laminate flake dispensers (1 g flakes/pkg) were provided by the manufacturer (Hercon Environmental). Ten wooden stakes were placed 5 m from the field edge and 10 m apart at two of the large-plot mating disruption trial fields. Pheromone packages were stapled to the wooden stakes within the clover crop canopy (17 June 2013). One pheromone package from each site was removed every two weeks over the course of the summer. Packages were placed individually into plastic sealable containers and were placed on ice for transport back to the University of Alberta where they were stored at -20 °C. Field-aged flakes in the packages were shipped overnight on ice to Hercon Environmental to determine the amount of pheromone remaining in the flakes and the subsequent release rate.

**Statistical Analyses**

Data was checked for normality with visualization plots and Shapiro-Wilks tests. General linear mixed-effects models (GLMM) were used when the data was normally distributed and when the data was non-normal a generalized-linear mixed-effects model specifying a Poisson error distribution was used (Package **lme4**, Bates, Maechler & Bolker 2013). All GLMMs were performed in **R** x64 3.0.1 (R Core Team 2013). For Experiment 1, to determine if the number of *C. deauratella* captured differed by flake or lure treatments, a GLMM was performed with bait
treatment specified as a fixed effect and site as a random effect. A Tukey’s Honestly Significant Differences (HSD) test compared differences between the treatments \((P < 0.05)\) (R Core Team 2013). For Experiment 2, to determine if the number of male \(C. deauratella\) captured in assessment traps positioned in small-plots treated with pheromone flakes differed compared to those captured in traps in untreated control plots, a GLMM was fit with pheromone treatment specified as a fixed effect and site nested within time as a random effect.

To determine if there was an effect of flake density on disruption of male \(C. deauratella\) orientation to assessment traps in Experiment 3, a generalized-linear mixed-effects model with a Poisson distribution was performed with mating disruption treatment as a fixed effect and site as a random effect. Multiple comparisons were performed with a Tukey’s HSD test to determine significant differences between the treatments \((P < 0.05)\) (R Core Team 2013). In Experiment 4, a generalized-linear mixed-effects model specifying a Poisson distribution was used to test if moth orientation to assessment traps was disrupted in the large plot study. Pheromone treatment was specified as a fixed effect and site nested within time period was specified as a random effect. To determine if larval numbers differed by mating disruption treatment or position in the field, a GLMM was specified with pheromone treatment, sample position and pheromone treatment \(X\) position as fixed effects and site as a random effect. The interaction effect was not significant, so it was removed from the final model. Finally, to determine if seed yield differed with pheromone treatment a GLMM was performed with pheromone treatment specified as a fixed effect and site as a random effect.

Data on the release rate of the pheromone flakes was provided by Hercon Environmental as the percent (\%) AI remaining/1 g of flakes for each sample period. Non-linear regression was used to determine the relationships between the percent \(Z7-12:OAc\) and \(Z5-12:OAc\) remaining on the flakes and time in the field (SigmaPlot 12.0).

**Results**

**Experiment 1: Attraction of pheromone flakes**

All baited traps in Experiment 1 captured male \(C. deauratella\). There was a significant difference in the number of male \(C. deauratella\) captured in wing traps baited with either zero, one, five, or ten pheromone flakes, or a grey rubber septa lure \((\chi^2 = 51.3, \text{df} = 4, P < 0.001)\) (Table 1). All traps baited with flakes or a lure caught significantly more male \(C. deauratella\) than the blank control trap. There were no significant differences between the baited traps (Table...
1). The greatest capture of male *C. deauratella* occurred in traps baited with one pheromone flake followed by the lure, five pheromone flakes, and finally, ten pheromone flakes (Table 1).

**Experiment 2: Small-plot communication disruption experiment**

Capture of male *C. deauratella* in assessment traps positioned within pheromone-treated plots was reduced by 93.6 ± 2.9 % compared to captures in traps in the untreated control plots ($\chi^2 = 2163.4$, df = 1, $P < 0.001$) (Fig. 2). Trap capture in control plots in the first two-weeks of the experiment (mean ± SE, 1742.7 ± 782.8 moths/plot) was eight-fold higher than in the second two-weeks (230.0 ± 58.7 moths/plot), but significant disruption of trap capture in pheromone-treated plots was still obtained. Mean capture in treated plots was 43.7 ± 11.7 and 18.6 ± 8.4 moths/plot in the first and second two-weeks of the experiment, respectively.

**Experiment 3: Pheromone flakes density experiment**

There was a significant reduction in the number of male *C. deauratella* captured in assessment traps positioned in pheromone-treated plots with varying flake density compared to the untreated control ($\chi^2 = 29162$, df = 1, $P < 0.001$). There were also significant differences among the flake density treatments, with the number of male *C. deauratella* captured decreasing as the density of flakes increased (Fig. 3a; Table 2).

Response profiles of male *C. deauratella* captured and flake density (Fig. 3a-b) are consistent with the prediction of a competitive mechanism causing mating disruption rather than a non-competitive mechanism (Miller et al. 2006a). On the untransformed plot (Fig. 3a), moth capture decreased asymptotically with the shape of an inverse function. On the Miller-Gut plot (Fig. 3b), 1/moth capture increased linearly with increasing flake density and on the Miller-de Lame plot (Fig. 3c), moth capture decreased linearly with increasing flake density X male *C. deauratella* catch.

**Experiment 4: Large-plot mating disruption experiment**

There was a significant reduction (72.3 ± 5.7 %) in *C. deauratella* trap captures in large plots treated with laminate flake dispensers compared to captures in traps positioned in the untreated control plots across the season ($\chi^2 = 15361$, df = 1, $P < 0.001$) (Fig. 4). The reduction in trap capture decreased over the course of the season (Table 3). There was also a significant reduction in the number of larvae sampled in the pheromone-treated plots compared to the untreated controls ($\chi^2 = 12.5$, df = 1, $P < 0.001$) (Table 4), but there was no significant effect of sample position ($\chi^2 = 0.7$, df = 1, $P = 0.42$) on the number of larvae retrieved. Pheromone
treatment slightly increased seed yield compared to yield in untreated control plots, however the result was only marginally significant ($\chi^2 = 3.1$, df = 1, $P = 0.079$) (Table 4).

**Experiment 5: Field-aged pheromone flake release rate**

The total percent AI and the percent of Z7-12:OAc and Z5-12:OAc individually remaining in 1 g of flakes aged for different periods in the field were determined by Hercon Environmental and fit with a exponential decay function (Total: $% AI = 9.03 \times e^{-0.012day}$, $F_{1,5} = 108.4$, $P = 0.005$, $r^2 = 0.96$; Z7-12:OAc: $% AI = 8.17 \times e^{-0.012day}$, $F_{1,5} = 111.0$, $P = 0.0005$, $r^2 = 0.97$; Z5-12:OAc: $% AI = 0.86 \times e^{-0.014day}$, $F_{1,5} = 81.5$, $P = 0.0008$, $r^2 = 0.95$) (Fig. 5). The total percent AI decreased from a high of 9.23 % on the application date to a low of 4.17 % at the end of the season. The initial realized 9.23 % AI in the flakes equates to 25.8 g AI/ha (683 μg AI/flake) and decreased to 11.7 g AI/ha remaining by the end of the season. Using the exponential decay function for the total percent AI remaining in the field, we estimated the release rate over each collection period (Table 3). The estimated release rate decreased as the season progressed (Table 3) and over the course of the season 14.4 g AI/ha was released.

**Discussion**

This study demonstrates that mating disruption has the potential to control *C. deauratella* populations in red clover seed production fields. Initial small-plot proof-of-concept trials showed significant communication disruption to pheromone-baited traps when plots were treated with Hercon Disrupt Micro-Flakes®. The impact of pheromone treatment on larval numbers or seed yield cannot be assessed in small plots due to immigration of mated females from surrounding untreated crop areas (Rothschild 1980; Baker and Heath 2005). Large-plot studies help mitigate the effects of immigration of mated females and allow for the assessment of larval numbers and seed yield. Although not a direct measure of mating disruption, from an economics perspective, the level of infestation and damage in the crop are the most relevant criteria and indicative of successful disruption (Rothschild 1980). In subsequent large-plot trials, there was a significant disruption of male moth orientation to traps and subsequent larval densities were reduced in pheromone-treated plots. There was a marginal increase in seed yield in pheromone-treated plots.

Reduction of male *C. deauratella* trap capture across the entire season in the large plots treated with pheromone indicates that communication can be disrupted; however, the efficacy of pheromone treatment decreased as the season progressed. The decreased efficacy of the
pheromone treatment over time was most likely due to the diminished release rate of pheromone from the flakes over the course of the experiment. Flakes were applied once to the field before the beginning of the *C. deauratella* flight period and the release rate decreased over time. The disruption index fell from a high of $98.5 \pm 0.3\%$ to a low of $38.3 \pm 9.8\%$ at the end of the season. There was enough pheromone applied to the plots to successfully disrupt *C. deauratella* with one application, however the expected release rate (407 mg/ha/day) was not realized and several grams of pheromone remained in the flakes at the end of the season (11.7 g AI/ha). Our data suggest that >70% disruption can be achieved across the season with just a 25.8 g AI/ha rate (season long average: 205.2 mg AI released/ha/day). Although, 70% disruption across the season is not as high as that obtained in other studies, the amount of pheromone applied to the field is less than the 480 mg/ha/day (43.2 g AI/season) suggested for the control of *Grapholita molesta* Busck (Lepidoptera: Tortricidae) (Audemard et al. 1989), but not as little as the 10 g AI/ha used to control *Keiferia lycoperiscella* (Walsingham) (Lepidoptera: Gelechiidae) (Jenkins et al. 1990) or the extremely low two applications of 1.5 g AI/ha used to control *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) (Stelinski et al. 2008). In order to maintain a release rate that causes > 95% disruption over the season (10 week flight period), the percent AI in the flakes should be modified to achieve an increased release rate from the flakes or flakes should be applied twice in the growing season. The properties of laminate flake dispensers and the pheromone release rate required for disruption will differ by target species. Hercon laminate flakes (Disrupt® II) loaded with a higher amount of pheromone (17.9 % disparlure) maintains successful disruption of *Lymantria dispar* L. (Lepidoptera: Eberidae) for eight weeks with one application of 15 g AI/ha (Tcheslavskaia et al. 2005).

Larval numbers were reduced in large plots treated with laminate flake dispensers compared to untreated control plots, indicating pheromone treatment prevented females from mating in treated plots. Often larval density and crop damage are higher at the edge of plots and this has been attributed to immigration of mated females (Knight 1995, Gut and Brunner 1998) or lower pheromone concentration (Ogawa 1990, Karg and Sauer 1995) at the edge of pheromone-treated plots. However, in the current study, sampling position within large plots had no effect on larval numbers as a similar number of larvae were sampled at the edge and interior of the plots. This is in contrast with our previous work using pheromone puffers in large-plot trials which showed significant edge effects likely due to pheromone loss at the plot edge and/or
immigration of mated females (BAM, unpublished). The even distribution of flakes throughout
the plot most likely eliminated any pockets of untreated air around the edge of the plot and
immigration of mated females into pheromone-treated plots did not appear to be a factor.
Although larval numbers were reduced by approximately half in large plots treated with
pheromone compared to control plots, this reduction only marginally increased seed yield in
treated plots. In this study, the increase in seed yield in treated plots was on average 58.0 kg/ha
greater than in control plots. Unfortunately, this increase in seed yield would not offset the cost
of applying the pheromone treatment. It appears that an increase in the application rate or an
increase in percent AI per flake is needed to further reduce larval numbers and have a significant
effect on seed yield.

The second major objective of this study was to determine the mechanisms by which
mating disruption acts on *C. deauratella* when flakes are used to dispense pheromone. In order to
determine the mechanisms, we conducted a small-plot study in which male trap capture was
quantified across various flake densities. We then used the disruption profiles published by
Miller et al. (2006a) to differentiate the potential for non-competitive and competitive
mechanisms of mating disruption against this species. Our results indicate that competitive
attraction (false-trail following) is the main mechanism which disrupts *C. deauratella* orientation
to pheromone-baited traps when a flake formulation is used as the disruptant (Fig. 3). The
disruption profiles (Fig. 3a-c) match the theoretical predictions for competitive mechanisms
(Miller et al. 2006a). The untransformed plot is concave and approaches zero asymptotically
(Fig. 3a); the Miller-Gut plot increases linearly (Fig. 3b); and the Miller-de Lame plot decreases
linearly (Fig. 3c). Furthermore, in the flake attraction experiment, *C. deauratella* males are
attracted to individual flakes in traps positioned in untreated fields, indicating false-trail
following as a potential mating disruption mechanism. We did not directly examine the attraction
of male *C. deauratella* to flakes in pheromone-treated fields, but on two occasions males were
observed orienting to flakes in pheromone-treated plots in the early morning (BAM, pers. obs.).
If non-competitive mechanisms were invoked, males would not approach dispensers in treated
fields (Miller et al. 2006a,b).

Miller et al. (2006b) compared moth communication disruption outcomes when
competitive attraction was found to be the main mechanism and these results concur with those
of the current study. Over the season, the estimated release rate, based on the release rate curve
generated here (Fig. 5), was 0.24 μg/hr/flake with a maximum dispenser density of ~76,204 flakes/ha (based on 0.0074 g/flake and 560 g flakes/ha) tested in Experiment 3. At the maximum dispenser density the percent male trap capture inhibition was 96.8 ± 1.1%. The maximum male catch ($C_{\text{max}}$), given by the y-intercept in the Miller-de Lame plot (Fig. 3C) (Miller et al. 2006a), in untreated control plots was 5170.7 males/trap/56 days (92.3 males/trap/night). The area over which a flake can have a disruptive effect, given as dispenser activity $D_a = \text{area (ha)}$ over which one flake can theoretically reduce $C_{\text{max}}$ by 50% (Miller et al. 2006a), is equal to the absolute value of the slope of the Miller-de Lame plot (Fig. 3C) which is 0.0065 ha or 65 m$^2$. Although this seems like a large area over which 1 flake dispenser can have a disruptive effect, dispenser impact declines in a non-linear fashion asymptotically. The first few dispensers applied to the crop provide the greatest disruption per dispenser (Miller et al. 2006a), thus to increase disruption (>90%) thousands more dispensers per plot are needed.

The dispenser application activity can be calculated as $D_{\text{ia}} = D_a \times D_D$ where $D_a$ is defined as above and $D_D =$ dispenser density (dispenser/ha) (Miller et al. 2006a). Dispenser application activity is the potency of a given pheromone formulation as applied to one ha, and is argued to be an alternative measure of disruption in situations where disruption occurs by competitive attraction (Miller et al. 2006b). The maximum $D_{\text{ia}}$ for $C. \text{deauratella}$ in this study is 495, which is over two-fold higher than any $D_{\text{ia}}$ values summarized by Miller et al. (2006b) across ten studies. If we compare our results with that of data taken from Stelinski et al. (2005) on $G. \text{molestta}$ using wax droplets as pheromone dispensers (as reported in Table 1 of Miller et al. 2006b), the release rate (0.25 μg/hr), experimental plot size (0.05 ha), maximum disruption (99.6%) and $D_a$ (0.0071) are very similar to our study, however, the maximum dispensers (27,300/ha) and maximum $D_{\text{ia}}$ (194) are lower than the results of the current study. Recalculation of the maximum $D_{\text{ia}}$ in the $G. \text{molestta}$ study using the number of dispensers from the current study (76,204), results in a larger $D_{\text{ia}}$ (541) than was calculated for $C. \text{deauratella}$ in the current study. This corresponds with a higher level of mating disruption in the $G. \text{molestta}$ study than was achieved for $C. \text{deauratella}$ in this study (99.4% inhibition of males in $G. \text{molestta}$ (Stelinski et al. 2005), 96.8% inhibition of $C. \text{deauratella}$ observed in this study). Interestingly, the maximum male catch in untreated controls ($C_{\text{max}}$) was only 5.1 males/trap/night in the $G. \text{molestta}$ study (Miller et al. 2006b) compared with the 92.3 males/trap/night captured in this study. In fact, the 92.3 males/trap/night captured in the current study is the highest number of
males observed across all ten studies analysed by Miller et al. (2006b). Flint and Merkle (1983) achieved 88% disruption of *P. gossypiella* in pheromone-treated cotton fields with a *D*ₐ₀ of 6.9 when 78 males/trap/night were captured (Miller et al. 2006b). The *D*ₐ (0.0038) in the Flint and Merkle (1983) study multiplied by the dispenser density used in the current study reveals a *D*ₐ₀ of only 290. This finding in addition to the percent inhibition of males indicates that, under high population densities, mating disruption of *C. deauratella* (98.6% inhibition, *D*ₐ₀ = 495) is more successful than that of *P. gossypiella* (88% inhibition, *D*ₐ₀ = 290).

Because dispenser impact declines in an inverse fashion asymptotically with dispenser density and the first few dispensers applied to the crop provide the greatest reduction per dispenser (Miller et al. 2006a), it is possible to calculate the number of dispensers needed to cause the maximum average disruption obtained (96.8%) in the current study. If one flake can disrupt 50% of *C*ₘₐₓ over 65 m² (*D*ₐ), then 153.8 flakes can disrupt 50% of *C*ₘₐₓ/ha. Each additional 153.8 flakes applied to a 1 ha plot can only disrupt 50% of the remaining moths (50% of 50% *C*ₘₐₓ), and so forth. Thus, based on the theoretical maximum male catch in untreated controls, *C*ₘₐₓ obtained from the y-intercept of Fig. 3C, 5,107.7 moths are caught in control plots over the total experiment. The addition of 153.8 flakes to the plot should decrease capture to 1134.1 moths/plot based on the equation of the regression line in Fig. 3A. This equates to a 78.1% reduction in trap capture. A dispenser density of 24,454 flakes/ha would be needed to provide the average level of disruption found in our study (96.8%). The maximum number of flakes/ha used in our study of ~76,204 could theoretically give a disruption of 97.9% which is only slightly above the percentage obtained in the current study. These comparisons are based on theory, but demonstrate that the equations of Miller et al. (2006a) apply to the data set generated here, and support that competitive attraction is likely the main mechanism of mating disruption when flake dispensers are used against *C. deauratella*.

This study was designed to test the efficacy of communication and mating disruption on *C. deauratella* and to test the mechanisms by which mating disruption may act on *C. deauratella*. In both small and large-plot trials communication disruption occurred, and although not measured directly, we can infer mating disruption occurs in large treated plots as there is a reduction in larval numbers and an increase in seed yield in pheromone-treated fields. Furthermore, we determine that flakes disrupt *C. deauratella* competitively based on behavioural observations and disruptive response profiles under different dispenser densities that match the
specific outcomes for competitive attraction (Miller et al. 2006a). Compared to other pheromone mating disruption dispensers, laminate flakes allow for mechanical application which would be beneficial as most red clover seed production fields in Alberta, Canada are ~65 ha in size. Unfortunately, under the conditions tested here, seed yield was only marginally increased with pheromone treatment, but further refinement of the flake formulation may significantly improve seed yields.

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Grapholita molesta (Busck), using high densities of wax-drop pheromone dispensers. J. Econ. Entomology 98: 1267-1274.


Table 1. Mean male *C. deauratella* captured per trap in the point source attractiveness experiment

<table>
<thead>
<tr>
<th>Dispenser or Lure Type</th>
<th>Mean (± SE) male <em>C. deauratella</em> captured/trap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>2.0 ± 0.8a</td>
</tr>
<tr>
<td>One Pheromone Flake</td>
<td>248.2 ± 46.9b</td>
</tr>
<tr>
<td>Five Pheromone Flakes</td>
<td>191.0 ± 25.1b</td>
</tr>
<tr>
<td>Ten Pheromone Flakes</td>
<td>179.7 ± 32.3b</td>
</tr>
<tr>
<td>Contech Grey Rubber Septa Lure</td>
<td>196.0 ± 46.7b</td>
</tr>
</tbody>
</table>

Means followed by different letters are significantly different (*P* < 0.001).

Table 2. Flakes/ha and the disruption index for the various flake densities used across the eight week flake density experiment

<table>
<thead>
<tr>
<th>Flake density (g/ha)</th>
<th>Approximate Number of Flakes/ha</th>
<th>Disruption Index (±S.E.) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>4763</td>
<td>72.2 ± 12.3</td>
</tr>
<tr>
<td>70</td>
<td>9526</td>
<td>82.4 ± 6.0</td>
</tr>
<tr>
<td>140</td>
<td>19051</td>
<td>87.2 ± 7.5</td>
</tr>
<tr>
<td>280</td>
<td>38102</td>
<td>93.0 ± 2.8</td>
</tr>
<tr>
<td>560</td>
<td>76204</td>
<td>96.8 ± 1.1</td>
</tr>
</tbody>
</table>

Table 3. Large-plot mating disruption trials disruption indices and estimated release rate over the duration of the season

<table>
<thead>
<tr>
<th>Collection Period</th>
<th>Disruption Index (±S.E.) (%)</th>
<th>Estimated Release Rate (mg AI/ha/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 June – 2 July</td>
<td>98.6 ± 0.3</td>
<td>277.6</td>
</tr>
<tr>
<td>2 – 15 July</td>
<td>83.9 ± 1.5</td>
<td>234.6</td>
</tr>
<tr>
<td>15 – 29 July</td>
<td>77.3 ± 3.3</td>
<td>199.6</td>
</tr>
<tr>
<td>29 July – 12 August</td>
<td>63.8 ± 3.4</td>
<td>168.7</td>
</tr>
<tr>
<td>12 August – 26 August</td>
<td>38.8 ± 9.8</td>
<td>142.6</td>
</tr>
</tbody>
</table>

Table 4. Larval numbers and seed yield for the large-plot mating disruption trials

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (±S.E.) larvae/plot(^\text{Y})</th>
<th>Mean (±S.E.) seed yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>121.3 ± 14.8a</td>
<td>594.9 ± 126.3a</td>
</tr>
<tr>
<td>Pheromone Flakes</td>
<td>57.3 ± 12.7b</td>
<td>652.9 ± 159.3a</td>
</tr>
</tbody>
</table>

Means in the same column followed by a different letter indicate significant differences (*P* < 0.05). \(^\text{Y}\) Larval numbers at the edge and interior of each plot were combined to give a total number of larvae sampled/plot and then the mean was taken for each treatment.
Figure 1. Large-plot pheromone disruption experimental design indicating position of pheromone traps, and larval sampling positions. Control plots were the exact same design, but received no pheromone flakes (0 g/ha). Plots are 5 ha in size (223.6 X 223.6 m).
Figure 2. Box-plot of the total number of male *C. deauratella* captured in control (grey) and pheromone treated (white) plots in the small-plot proof-of-concept experiment. The midline indicates the median and the bottom and top of the box represent the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, respectively. Vertical lines extending from the box (whiskers) represent the maximum and minimum values. Letters above the box-plots indicate significant differences ($P < 0.05$) between the treated plots.
Figure 3. Plots for the flake density experiment. (A) Untransformed plot of male *C. deauratella* captured/plot by dispenser density. Letters by means indicated significant differences based on a generalized-linear mixed-effects model specifying a Poisson distribution followed by a post-hoc Tukey’s HSD test (*P* <0.05), (B) Miller-Gut plot of 1/(male *C. deauratella* captured/plot) by dispenser density, (C)Miller-de Lame plot of male *C. deauratella* captured/plot by dispenser density X male *C. deauratella* captured/plot.
Figure 4. Box-plot of the total number of male *C. deauratella* captured in control (grey) and pheromone treated (white) plots in the large-plot season long mating disruption experiment. The midline indicates the median and the bottom and top of the box represent the 25th and 75th percentiles, respectively. Vertical lines extending from the box (whiskers) represent the maximum and minimum values. Letters above the box-plots indicate significant differences (*P* < 0.05) between the treated plots.
Figure 5. Percent active ingredients remaining in one gram of field aged flakes. The individual pheromone components (Z7-12:OAc and Z5-12:OAc) and the total components are displayed. Total (black triangle): %AI = 9.03 \times e^{-0.012\text{day}}, r^2 = 0.96; Z7-12:OAc (open circle): %AI = 8.17 \times e^{-0.012\text{day}}, r^2 = 0.97; Z5-12:OAc (black circle): %AI = 0.86 \times e^{-0.014\text{day}}, r^2 = 0.95).