Following the plume: Development of a pheromone-based monitoring and management program for *Coleophora deauratella* (Lepidoptera: Coleophoridae)

by

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Abstract

The reliance of moths on pheromone-mediated communication for mate location makes sex pheromones ideal candidates for exploitation in integrated pest management (IPM) programs. Pheromones make sensitive tools that can detect invasive species at low population densities and in new habitats. The red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae), is an invasive pest of clover throughout North America. In Canada, infestations of *C. deauratella* in red clover seed production fields can cause > 80% seed loss and insecticides have been ineffective against this pest. Recently, the sex pheromone produced by *C. deauratella* females was identified as a 10:1 ratio of (*Z*)-7-dodecenyl acetate and (*Z*)-5-dodecenyl acetate. This identification allows synthetic pheromones to be used for detection and management of this invasive pest.

In this thesis, I investigated several non-pheromone factors that can influence the efficacy of pheromone-baited traps. I found that green unitraps placed 35 cm above the soil surface and 5 m from the field edge maximized capture of *C. deauratella* and minimized by-catch of non-target organisms. I then used this optimized trapping system to develop a pheromone-based monitoring program that can monitor male *C. deauratella* population density and flight phenology. Male *C. deauratella* captures were significantly and positively related to larval abundance and proportion of seed damage even with moderate to high population densities. A phenological model based on degree days (base 11.7 °C) more consistently described the median flight period of male *C. deauratella* compared to the ordinal date model. Further, the model found that 258.3 $DD_{11.7}$ from January 1 are needed for median flight to occur.

In addition, I tested pheromone-mediated mating disruption formulations to determine if treatment interferes with mate finding, reduces larval abundance, and increases seed yield.

Results from small-plot (0.25 ha) field experiments with rope, aerosol-emitter, and laminate-flake pheromone dispensers demonstrated male *C. deauratella* orientation to pheromone-baited traps was reduced 60-99% in treated compared to non-treated control plots. In large-plot (5 ha) experiments, aerosol-emitting pheromone dispensers reduced male *C. deauratella* orientation to pheromone-baited traps, but no reduction in larval numbers or increase in seed yield was found compared to untreated control plots. However, a significant reduction in *C. deauratella* captures in pheromone-baited traps and larval numbers and an increase in seed yield were observed using laminate flakes.

I also investigated the mechanisms through which pheromone treatment acts to disrupt mating by *C. deauratella*. Complete and partial pheromone formulations were compared to determine the capacity and mechanisms of both formulations to cause disruption. The partial pheromone formulation was as successful as the complete pheromone blend for reducing male *C. deauratella* orientation to pheromone-baited traps. Small-plot field trials, combined with laboratory electroantennograms, determined that both competitive and non-competitive mechanisms may cause mating disruption when the complete pheromone blend is used, whereas non-competitive mechanisms were primarily invoked with the partial formulation. Additionally, studies on laminate flake dispensers found theoretical models of competitive attraction to be supported.

In order to identify the source of *C. deauratella* populations and determine possible routes of invasions in North America, I used pheromone-baited traps to collect specimens throughout North America and Europe. I isolated and characterized four polymorphic microsatellite loci for *C. deauratella* and combined their use with mitochondrial DNA to examine genetic diversity and population differentiation. Most genetic differentiation was

observed between continents; little differentiation and no evidence of isolation-by-distance were found between populations within each continent, suggesting *C. deauratella* has been readily transported across large distances. The low number of haplotypes observed in North America, combined with clustering analyses suggest only two genetic clusters are present, and indicate that a limited number of invasion events occurred in North America. The initial *C. deauratella* invasion most likely occurred in southern Ontario or adjacent American states followed by subsequent transport throughout the continent, however, the precise source population could not be inferred by this study.

Ultimately, these investigations could form the basis of a comprehensive IPM program that can detect, monitor and manage this pest throughout North America. Mating disruption will provide growers with a non-insecticidal control option to help mitigate damage caused by *C. deauratella*. Furthermore, determination of the origins of *C. deauratella* in North America may help focus the search for biocontrol agents to control the introduced populations.

Preface

A version of Chapter 2 of this thesis has been published as: Mori BA and Evenden ML (2013) Factors affecting pheromone-baited trap capture of male *Coleophora deauratella*, an invasive pest of clover in Canada. Journal of Economic Entomology 106: 844-854. I contributed to the experimental design and was responsible for data collection, analysis and manuscript composition. Maya Evenden also contributed to the experimental design and planning of experiments, and was involved with the concept formation and manuscript editing.

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List of Abbreviations

mtDNA – Mitochondrial Deoxyribonucleic acid

COI – Cytochrome Oxidase 1 gene

GC-EAD – Gas chromatographic-electroantennographic detection

*Z*7-12:OAc – (*Z*)-7-dodecenyl acetate

*Z*5-12:OAc – (*Z*)-5-dodecenyl acetate

*Z*5-12:OH – (*Z*)-5-dodecenol

$DD_{11.7}$ – Degree days (base 11.7 °C)

AI – Active ingredients

EAG – Electroantennogram

HPLC – High performance liquid chromatography

AIC – Akaike information criterion

GLMM – General linear mixed-effects model

ANOVA – Analysis of variance

AMOVA – Analysis of molecular variance

Chapter 1

General Introduction

Sex pheromone communication in moths

Sex pheromones are intraspecific chemical signals used by many species to locate a potential mate (Baker and Heath 2005). In Lepidoptera, specifically moths, most females release sex pheromones and males detect and respond to the calling female by upwind oriented flight along the pheromone plume (Tamaki 1985). The majority of female-produced lepidopteran sex pheromones are a blend of straight-chain 10- to 18- carbon compounds composed of primary alcohols and their derivatives (acetates and aldehydes) (Ando et al. 2004). Female lepidopteran pheromones are relatively species specific with species specificity maintained by varying the components and ratios of the compounds (Cardé and Haynes 2004). Females release minute quantities (picograms, Hansson 1995) of pheromone which has led to the evolution of a finely tuned neurosensory system in males (Bengtsson and Löfstedt 2007) due to intense competition to be the first to locate a calling female (Cardé and Haynes 2004).

Male moth antennae house olfactory sensilla with highly specific pheromone receptor cells which can detect minute quantities of pheromone molecules released by a calling female (Hansson 1995). The dendrites of pheromone receptor cells are bathed in sensillar lymph in the lumen of the sensillum (Hansson 1995). After initial pheromone adsorption onto the surface of the antennae, hydrophobic pheromone molecules diffuse into the sensillar lymph via tiny pores on the cuticular surface (Stengl et al. 1992). Within the sensillar lymph, pheromone molecules are bound to pheromone-binding proteins which deliver the pheromone to specific receptors on the dendritic membrane of the pheromone-receptor neurons (Prestwich 1993, Kaissling 2009).

This triggers neuronal signals that travel along the axons of the pheromone-receptor neuron and terminate in the macroglomerular complex in the antennal lobe of the male moth's brain (Breer 1997, Christensen 1997). Information is subsequently processed in the macroglomerular complex and transmitted to higher centers of the brain for final interpretation and output (Christensen 1997). Behavioural response to the odour is initiated as male moths fly upwind within the pheromone plume. If males lose contact with pheromone they slow their upwind flight and cast side to side in a zig-zag pattern across the pheromone plume to locate the source. Frequent contact with the plume and decreasing clean air pockets between pheromone strands results in surges of upwind flight and eventual source location (Vickers and Baker 1994). Turbulence within moving air causes the pheromone plume to break up into strands of odour-laden air interspersed with clean air pockets. It is this pattern of odour-laden air followed by clean air that is needed for males to sustain upwind flight to locate the pheromone source (Vickers and Baker 1994, Mafra-Neto and Cardé 1994).

The reliance of moths on pheromone-mediated communication for mate location makes sex pheromones among the most widely used management tools in integrated pest management (IPM) (Baker and Heath 2005). Two tactics are commonly employed in IPM – pheromone-based monitoring programs and mating disruption – to directly control populations with pheromone application.

Pheromone-based monitoring programs

Pheromone-based monitoring is used for many different applications including monitoring for invasive species, early warning of pest incidence, surveys to define an infested area, and to time pest management tactics (Howse et al. 1998, Gut et al. 2004). Pheromone-

monitoring programs can be used in risk assessment, in which trap catch is quantitatively related to population density or damage levels (Howse et al. 1998). Some pheromone-monitoring programs have shown accurate relationships between male moth capture and pupal, larval or egg stages within forestry and agricultural settings (Ngollo et al. 2000, Jones et al. 2007, Cross et al. 2009). In these studies, pheromone-traps were placed in fields to monitor male flight, and sampling for immature stages and damage was used to determine if trap catch could predict pest densities. In agriculture, pheromone-based monitoring has been an effective tool for predicting immature densities and damage for *Ostrinia nubilalis* Hübner, (Lepidoptera: Crambidae) (Ngollo et al. 2000), *Mamestra configurata* Wlk., (Lepidoptera: Noctuidae) (Turnock 1987), and *Dasineura mali* Keiffer (Diptera: Cecidomyiidae) (Cross et al. 2009). Moreover, phenological models that can predict the appearance time of specific insect life-stages can incorporate pheromone-based trap catch with a measure of physiological time (i.e. degree days) or ordinal date (Hardman 2012). Phenological models based on pheromone-baited trap capture can be used to time specific life-stage sampling and cultural, biological or chemical control tactics (Hardman 2012). Phenological models that use first male moth captured in pheromone-baited traps as a "bio-fix" have been created for several lepidopteran agricultural pests including *Cydia pomonella* L. (Tortricidae) (Glen and Brain 1982), *Podosesia syringae* (Harris) (Sessiidae) (Potter and Timmons 1983), *Grapholita molesta* L. (Tortricidae) (Rice et al. 1984), *Chilo paratellus* (Swinhoe) (Pyralidae) (Unnithan and Saxena 1990), *Lobesia botrana* (Denis and Schiffermüller) (Tortricidae) (Gallardo et al. 2009), and *Acrobasis nuxvorella* Nuenzig (Pyralidae) (Knutson and Muegge 2010).

In order to create a successful monitoring program, the parameters that influence trap capture of male moths need to be identified. Pheromone lures need to be attractive to male moths

throughout the flight period and the trap must retain attracted moths. Pheromone lures come in many different shapes, and sizes, and are composed of a variety of materials. Not only do they emit pheromone, but most lures also protect the pheromone from degradation by ultraviolet light and oxidation (Howse et al. 1998). Thus, the choice of lure is very important. For instance, the release of the same pheromone loaded on to red and grey rubber septa varied with lure age and attracted different numbers of *Plutella xylostella* L. (Lepidoptera: Plutellidae) (Mayer and Mitchell 1999). When designing a pheromone-based monitoring program, trap design and height at which traps are deployed in the field need to be considered. Different traps may provide different visual cues to male moths (Knight and Fisher 2006) and create turbulence that can alter the pheromone plume structure and affect moth capture (Foster et al. 1995, Quartey and Coaker 1992). As well, numerous field studies show that trap type can alter the number of insects retained in the trap (Ameline and Frerot 2001, Athanassiou et al. 2008). The position of the trap within the crop or forest canopy can also affect the number of insects caught. Dorhout and Rice (2008) found traps placed 1.2 and 1.8 meters above the soil surface caught significantly more western bean cutworm, *Striacosta albicosta* Smith (Lepidoptera: Noctuidae), than traps placed at 0.6 meters. In order to create a successful monitoring program that can be used for risk assessment or to create phenological models, the trapping system must be carefully chosen to ensure optimal insect trap capture.

Tracing invasions with pheromone traps and population genetics

Pheromone-baited traps can be useful for detecting invasive species at low population densities and in new habitats (Leibold and Tobin 2008). They are used in surveys to determine the presence of invasive pests and are routinely deployed around high-risk sites (i.e. airports, harbours, border crossings) to detect potential introductions of new invasive species (Gut et al.

2004). Several studies have used pheromone-baited traps to monitor for invasive species (Brockerhoff et al. 2006, Kriticos et al. 2007, Sharov et al. 2002, Carruthers 2003, Kikkert et al. 2006, Augustin et al. 2004) and used the captured specimens for phylogeographic studies to determine the origin of the captured insects (Kim and Sappington 2004, Ciosi et al. 2008).

Phylogeography is the study of the geographical distribution of phylogenetic lineages and combines population genetics with biogeography (Avise et al. 1987). In the context of invasive species, phylogeography has been used to trace the invasion of new species and establish where new species originated. Mitochondrial DNA (mtDNA) and particularly, the cytochrome oxidase 1 gene (COI), has been widely adopted for phylogeographic studies due to its simple genetic structure, straightforward mode of inheritance, and fast mutation rate (Avise et al. 1987). Increasingly, multiple genetic markers are being used to enhance the accuracy of observed relationships (Dupuis et al. 2012) and mtDNA is often used in combination with microsatellite loci (Meixner et al. 2002, Valade et al. 2009). Microsatellites are short-sequence repeats that are widely used in molecular ecology (Selkoe and Toonen 2006) due to their neutral variation, large number of alleles per locus, and rapid mutation rate which can enhance the resolution in phylogenetic studies (Selkoe and Toonen 2006, Lukoschek et al. 2008).

By using molecular markers to analyze invasive insects captured in pheromone-baited traps, the number of introductions can be assessed (Meixner et al. 2002, Laffin et al. 2005, Simonsen et al. 2008, Ciosi et al. 2008), as well as genetic diversity (Grapputto et al. 2005, Ciosi et al. 2008), and population structure (Hartfield et al. 2011, Tooman et al. 2011). Invasive species are often subject to a founder effect that results in a genetic bottleneck and loss of genetic diversity (Sakai et al. 2001, Allendorf and Lundquist 2003). Determination of a founder effect in new populations may have implications on the ability of invasive species to adapt to their new

environment (Reed and Frankham 2003). Furthermore, by determining the number of introductions and the source population of the sampled invasive species, possible biological control agents can be collected from source populations (Le Roux and Wieczorek 2009).

Pheromone-mediated mating disruption

Pheromone-mediated mating disruption has proven to be an effective management tool for many lepidopteran pests (Cardé and Minks 1995, Witzgall et al. 2010). This technique interferes with sexual communication between male and female insects by flooding the cropping area with synthetic sex pheromone (Howse et al. 1998). The first experimental evidence of mating disruption was demonstrated by Gaston et al. (1967) in which cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), males were not captured in pheromone-baited traps in plots treated with synthetic pheromone compared with 102 captured moths in traps positioned in similar untreated control plots. Since this time, mating disruption, using the complete or partial pheromone blend of the target insect, has been an effective management tool for many agricultural lepidopteran pests including *C. pomonella* L. (Judd et al. 1996), *Rhopobota naevana* Hübner (Tortricidae) (Fitzpatrick et al. 2004), *Keiferia lycopersicella* Walsingham (Gelechiidae) (Jimenz et al. 1988), *Epiphyas postvittana* Wlk. (Tortricidae) (Suckling and Shaw 1995), and *Pectinophora gossypiella* Saunders (Gelechiidae) (Lykouressis et al. 1995).

The efficacy of pheromone-mediated mating disruption is dependent on the target species biology, population dynamics, cropping environment and pheromone formulation (Cardé and Minks 1995, Witzgall et al. 2010). High insect population densities can cause mating disruption to fail especially if mating disruption dispensers compete with calling females (Sanders 1981, Suckling and Angerelli 1996, De Lame et al. 2010). Migration of mated females into

pheromone-treated areas can decrease the effectiveness of mating disruption (Cardé and Minks 1995) and generally area-wide mating disruption treatments are recommended for best control (Ogawa 1990). Variability in crop canopy, wind speed and direction (Cardé and Minks 1995) as well as adsorption and release by crop foliage (Suckling et al. 1996) can also impact mating disruption. As well, the completeness of the pheromone blend, the emission rate, and the distribution of dispensers can affect mating disruption (Minks and Cardé 1988, Evenden et al. 1999, Reinke et al. 2014). Limitations of mating disruption may be overcome with more knowledge on mechanisms by which it affects mate-finding behaviour (Cardé et al. 1998, Evenden et al. 1999).

Over the last several decades, many different mechanisms have been proposed to explain how mating disruption works (Shorey 1977, Bartell 1982, Cardé and Minks 1995, Sanders 1997, Miller et al. 2006). However, five main mechanisms that impact male behavior predominate in the literature. The five main mechanisms that mediate mating disruption can be classified into two categories: competitive, and non-competitive (Miller et al. 2006). False-trail following (competitive attraction) in which males orient to dispensers baited with synthetic pheromone rather than calling females is the only competitive mechanism (Bartell 1982, Miller et al. 2006). Whereas non-competitive mechanisms include: sensory adaptation of the pheromone receptors on the antennae; habituation of the central nervous system to process pheromone; 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 (Shorey 1977, Bartell 1982, Cardé and Minks 1995, Sanders 1997, Miller et al. 2006). These mechanisms are not mutually exclusive and can work in concert depending on the pheromone formulation (Evenden et al. 1999), the dispenser types used to release the pheromone

and the insect's behaviour (Cardé and Minks 1995, Baker and Heath 2005, Witzgall et al. 2010). Mating disruption by competitive attraction requires an attractive pheromone blend, whereas non-competitive mechanisms can use partial or off-ratio pheromone blends (Evenden et al. 1999, Miller et al. 2006). Non-competitive mechanisms have been implicated in mating disruption of several agricultural pest species including *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (Evenden et al. 1999), *Campylomma verbasci* (Hemiptera: Miridae) (Judd et al. 1995), *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) (Stelinski et al. 2008), and *Tecia solanivora* (Lepidoptera: Gelechiidae) (McCormick et al. 2012).

Recent evidence suggests that pheromone-mediated mating disruption may also affect female reproductive behaviours (Kuhns et al. 2012). Pre-exposure of sex pheromone to females of *G. molesta* and *Pandemis pyrusana* Kearfott (Lepidoptera: Tortricidae) reduced the proportion of females that copulated, whereas females of *C. pomonella* and *C. rosaceana* were unaffected by pheromone pre-exposure (Kuhns et al. 2012). This suggests that, depending on the species, mating disruption application may not only affect male behaviour, but that of the female as well. Furthermore, mating disruption may not always prevent mating of the target insect, but it may cause delayed mating which can affect the fitness and population dynamics of the insect (Jones and Aihara-Sasaki 2004, Baker and Heath 2005, Mori and Evenden 2013). Pheromone-mediated mating disruption treatment imposed mating delay has been postulated to be another way in which mating disruption treatments can regulate insect populations (Barclay and Judd 1995). Theoretical models of mating disruption found that a delay in mating always reduced net reproductive rate (Barclay and Judd 1995). Mori and Evenden (2013) followed up on the effects of delayed mating by conducting a meta-analysis on 24 experimental studies and found that across the Lepidoptera delayed mating resulted in a decrease in fecundity, fertility, and pre-

oviposition period and an increase in female longevity. It is difficult to measure the effects of delayed mating imposed by pheromone-mediated mating disruption treatment in the field. However, using a combination of field and laboratory studies Knight (1997) demonstrated that females remained unmated for longer periods of time in pheromone-treated plots, and females in the lab prevented from mating for the same length of time had a large reduction in the number of viable offspring. Thus, it appears a further mechanism of mating disruption may be the result of pheromone-mediated mating disruption treatment imposed mating delay on female insects, which could result in further population declines.

Pheromone-based mating disruption formulations are most often applied through the use of hand-applied reservoir-type rope dispensers (Judd et al. 2005), thus limiting their use in large scale field cropping systems due to cost and time constraints. However, other formulations such as aerosol release devices and laminate flake formulations may be cheaper and easier to apply (Shorey and Gerber 1996, Baker and Heath 2005). Pheromone release from each type of dispenser may act to disrupt mating through several mechanisms, depending on the formulation and rate of pheromone released (Appendix 1-1). Reservoir type rope dispensers that release moderate dosages of the complete attractive pheromone blend are thought to act primarily through false-trail following (Miller et al. 2006) with additional mechanisms of camouflage, adaptation and habituation occurring depending on the location of the moth with respect to the pheromone source in the treated crop canopy (Cardé et al. 1998). Laminate flakes release similar amounts of pheromone as females and are thought to act as mating disruptants through false-trail following (Stelinski et al. 2008). The mechanisms of mating disruption invoked by aerosol dispensers are less well studied. It is postulated that males follow the large plume released from the aerosol dispensers so that mating is disrupted primarily by false-trail following (Stelinski et

al. 2007). Mating disruption by competitive attraction has recently been confirmed via matching response profiles (Miller et al. 2006) when aerosol dispensers are used to disrupt *C. pomonella* (McGhee et al. 2014). The mechanisms of mating disruption associated with different dispenser types and pheromone formulations will inevitably vary even within the same species. Examining various dispensers and the mechanisms by which they act may lead to new discoveries on how the mechanisms work with the various dispensers and may determine which dispenser type and formulation works best for a given pest species.

Clover seed-feeding *Coleophora*

Coleophoridae (Lepidoptera: Gelechioidea) contains 1,340 described species in five genera: *Augasma* Herrich-Schäffer 1853, *Coleophora* Hübner 1822, *Goniodoma* Zeller 1849, *Metriortes* Herrich-Schäffer 1853, and *Ischnophanes* Meyrick 1981 (Bauer et al. 2012). However, it is estimated that less than 50% of the Nearctic species of Coleophoridae have been described (Baldizzone et al. 2006). *Coleophora* is the largest genus within Coleophoridae and contains all but 16 described species in the family (Bauer et al. 2012). Moths within the family Coleophoridae are commonly called the 'casebearers' because larvae construct a portable case in which most of their larval stages occur (Landry and Wright 1993, Bucheli et al. 2002, Bauer et al. 2012). Most described species of *Coleophora* originate in the Palearctic region, but several hundred species are found in North America (Landry and Wright 1993). *Coleophora deauratella* Leinig and Zeller, *C. mayrella* (Hübner) and *C. trifolii* (Curtis), in the *frischella* group, are introduced into North America and feed upon clover (Fabaceae), an introduced plant (Landry and Wright 1993).

Coleophora deauratella, also known as the red clover casebearer, was only identified from North America collections within the last 25 years (Landry 1991). It was previously confused with specimens of *C. mayrella*, and *C. trifolii* (Landry and Wright 1993). The earliest known record of *C. deauratella* in North America is from Ithaca, NY in 1962 (Landry 1991). *Coleophora deauratella* feeds principally on the seeds of red (*Trifolium pratense* L.) and alsike (*T. hybridium* L.) clover (Landry 1991). *Coleophora mayrella* is known to have been present in North America since 1860 and feeds only on white clover (*T. repens* L.) (Landry and Wright 1993). In contrast, *C. trifolii* has been present in North America since the 1960's and feeds upon sweet clover (*Melilotus* sp.). All three species can be distinguished based on morphological characters and mtDNA sequences (Landry and Wright 1993, Bauer et al. 2012).

The female-produced sex pheromones of *Coleophora* spp. are not well known, with pheromones identified from only 43 described species (El-Sayed 2014). It appears that the components of the fatty acid synthesis pathway from which pheromones are produced are well conserved between species (Cardé and Hayne 2004), with most species' pheromone components being similar or closely related (El-Sayed 2014). A variety of pheromone components are attractive to *Coleophora* spp. in Europe. A blend of (*Z*)-5-decenal acetate and (*Z*)-5-decenol is attractive to *C. albidella* Herrich-Schäffer, *C. amellivora* Baldizzone, *C. anatipennella* Hübner, *C. artemisicolella* Bruand, *C. coracipennella* Hübner, *C. granulatella* Bruand, *C. juncicolella* Stainton, *C. laripennella* Zeller, *C. lineolea* Haworth, and several others (Sziraki et al. 1980, Priesner et al. 1988). Other compounds including (*Z*)-7-dodecenol, (*Z*)-9-tetradecenyl acetate, and (*Z*)-3-decenyl acetate are also attractive to a variety of *Coleophora* spp. in varying blends and doses (Subchev et al. 1990, Toth et al. 1992, Ostrauskas 2003).

Initial studies on *C. deauratella*, conducted in Hungary showed that male *C. deauratella* were attracted to mixtures of (*Z*)-5-decenyl acetate, (*Z*)-5-decenyl aldehyde and (*Z*)-7-dodecenyl acetate and to mixtures of (*Z*)-5-decenal acetate, (*Z*)-5-decenol and (*Z*)-7-dodecenyl acetate (Toth et al. 1992). These compounds were all shown to be attractive in field studies, but the female-produced signal was not known as pheromone gland extracts were not analyzed in this study. Evenden et al. (2010) examined female *C. deauratella* pheromone gland extracts using gas chromatographic-electroantennographic detection (GC-EAD) and found three antennally-active compounds in gland extracts: (*Z*)-5-dodecenyl acetate, (*Z*)-7-dodecenyl acetate and (*Z*)-7-dodecenol. Field studies in the Peace River Region of Alberta with these compounds showed that mixtures of (*Z*)-5-dodecenyl acetate and (*Z*)-7-dodecenyl acetate were attractive to male *C. deauratella*, but (*Z*)-7-dodecenol did not enhance attraction to the other two components. It was subsequently determined that (*Z*)-7-dodecenyl acetate and (*Z*)-5-dodecenyl acetate are the major and minor pheromone components of this species, respectively, and a 10:1 ratio at a 100:10 μg dose is most attractive to male moths (Evenden et al. 2010).

Since its introduction into North America, *Coleophora deauratella* has become a severe pest of red clover grown for seed in Ontario and Alberta, Canada (Ellis and Bjørnson 1996, Evenden et al. 2010). *Coleophora deauratella* is native to Europe, eastern Siberia and the Middle East (Landry 1991, Landry and Wright 1993). It was thought to be restricted to eastern Canada, but it was discovered in the Peace River Region of Alberta in 2001, and a significant outbreak began in 2005 (Alberta Agriculture and Rural Development 2009). Red clover is the preferred host plant of *C. deauratella*, however, it has been recorded on stone clover (*T. arvense* L.), alsike clover (*T. hydridum* L.), zig-zag clover (*T. medium* L.) and white clover (*T. repens* L.) (Hammer 1937, Markkula and Myllmäki 1960). It overwinters as mature fourth instar larvae within a

sealed case (Ellis and Bjørnson 1996). Pupation occurs within the case in the spring and adults emerge in early June in the Peace River Region with flight lasting until mid-August. Adults mate and females lay eggs on the calyx of clover florets (Landry 1991). Upon hatching, larvae bore through the corollas and feed on the developing ovules (Landry 1991). They can tunnel between adjacent florets, destroying up to three florets per day (Landry 1991, Ellis and Bjørnson 1996, Hammer 1937). Larvae construct their portable cases out of floret petals and silk and continue to feed from within their case, and enlarge it as they grow (Landry 1991, Ellis and Bjørnson 1996). In late summer, mature larvae move to the ground, seal their case with silk and prepare to overwinter (Landry 1991). *Coleophora deauratella* is univoltine with flight occurring before damage in the field, making it an ideal candidate for pheromone monitoring and mating disruption.

In Europe, *Coleophora deauratella* causes only minor damage (Ellis and Bjørnson, 1996); however, in Canada *C. deauratella* causes severe seed loss in red clover. In Ontario in 1989, 80% seed loss was estimated (Ellis and Bjørnson 1996) and in Alberta in 2006, >99% seed loss occurred due to *C. deauratella* infestations (Evenden et al. 2010). Red clover is widely grown for forage, honey and seed production, and for use in crop rotations for soil improvement (Fairey 1981). In the Peace River Region, red clover is primarily grown for seed because it is especially adapted to the region's Gray Luvisol soil (Alberta Agriculture 1988).

Currently, there are no approved insecticides or monitoring tools available for *C. deauratella*, and producers are beginning to discontinue growing red clover. Initial insecticide trials with both Decis™ and Success 480 SC™ applied at 5% and 50% flower, showed an increase in seed yield (4.64-5.85 g/m^2), but it was still far below the industry expectation (28-39 g/m^2) (J. Otani, pers. com.). The increase in yield did not offset costs of applying the insecticide.

As well, the application of insecticides could harm pollinators and other beneficial insects and negatively affect honey production in the area.

An integrated pest management program needs to be developed for *C. deauratella* and the development of a pheromone-based monitoring program is the first step towards determination of an economic threshold for this invasive species. Pheromone-mediated mating disruption for control of *C. deauratella* populations should be considered as it could reduce *C. deauratella* populations without impacting natural enemy and pollinator populations. Further, a phylogeographic study should be conducted to determine the origin of *C. deauratella* populations in North America to identify regions where biological control agents could be identified.

Thesis objectives

In this thesis, I use the recently identified *C. deauratella* sex pheromone to monitor and manage this invasive pest. In Chapter 2, a series of field trapping studies identify the factors that affect *C. deauratella* trap capture by testing various lure and trap types, trap deployment height and position, and lure longevity. I also assess the effect of trap type on the capture of non-target *Bombus* spp. After identification of the optimal trap characteristics for *C. deauratella*, I use this trapping system to determine the relationships between pheromone-baited trap capture and larval numbers and damage in the field (Chapter 3). Phenological models are created based on pheromone-baited trap capture and both degree-days and ordinal date to determine which measurement (degree-days or ordinal date) is better at describing the median (50%) flight period each year (Chapter 3). In Chapter 4, I explore the mechanisms of mating disruption in small-plot and laboratory studies with hand-applied reservoir type rope dispensers. Rope dispensers that

release the complete pheromone blend and the major component alone are compared with an untreated control plot to determine if the major component is as successful as the complete pheromone blend at disruption of sexual communication of *C. deauratella* assessed through pheromone-baited trap capture. Yellow sticky cards placed directly below the pheromone dispensers in the field assess whether male *C. deauratella* are attracted to dispensers and if false-trail following to dispensers occurs. Further, laboratory electroantennograms after male moth exposure to a dispenser releasing the complete pheromone blend, the major component alone or untreated (blank) air are conducted to determine if antennae adapt to high levels of pheromone. The potential for aerosol and laminate flake dispensers to disrupt *C. deauratella* sexual communication is tested in small and large-plot studies in Chapters 5 and 6, respectively. The initial small-plot proof-of-concept studies determine if the different pheromone dispensers releasing the complete pheromone blend can disrupt communication of male *C. deauratella* assessed as reduction of trap capture in pheromone-baited traps. In large-plot studies, larval numbers and seed yield are assessed in pheromone-treated and untreated control plots to determine if the dispensers tested can reduce larval numbers and increase seed yield. By varying the density of laminate flake dispensers in small-plot trials the mechanisms of mating disruption invoked by this dispenser type are also assessed in Chapter 6 and compared with the mathematical predictions of competitive or non-competitive mechanisms (Miller et al. 2006). In Chapter 7, I develop microsatellite markers and sequence a portion of the mtDNA COI gene to determine the origins of North American populations of *C. deauratella*. I use the data generated from both marker types on North American and European populations of *C. deauratella* to conduct a phylogeographic analysis. With these observations and analyses, I aim to provide a more complete understanding of *C. deauratella*'s life history and pheromone biology and

develop tools that can be used to monitor, manage and track this invasive species which may aid in saving the red clover forage seed industry in North America.

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Appendix 1-1. Mating disruption dispenser types, relative release rates, plot sizes and dispenser densities tested against *C. deauratella* throughout the mating disruption studies (Chapter 4-6).

Dispenser Type	Relative Release Rate	Plot Size Tested (ha)	Dispenser Density (dispensers/ha)
Ropes	Moderate	0.25	1,000
		0.25	500
		0.25	250
Aerosol-emitter (Puffer)	High	0.25	4
		5	2
Laminate flakes	Female-equivalent	0.0625	4,763
		0.0625	9,526
		0.0625	19,051
		0.0625	38,102
		0.0625	76,204
		0.25	38,102
		5	38,102

Chapter 2

Factors affecting pheromone-baited trap capture of male *Coleophora deauratella*, an invasive pest of clover in Canada

Introduction

The red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae), is an invasive pest of clover, *Trifolium* spp. L. (Fabaceae), in Canada (Landry 1991, Ellis and Bjørnson 1996, Evenden et al. 2010). It is native to Europe, Eastern Siberia and the Middle East and was accidently introduced to eastern North America at least 45 years ago (Landry and Wright 1993). As of 1993, *C. deauratella* is known to have been introduced to Québec, Ontario, Maryland, Massachusetts, Michigan, New Hampshire, New York, Vermont and Ohio and the first specimens were discovered from Alberta in 2006 (Landry and Wright 1993, Evenden et al. 2010). Since its introduction in Canada, *C. deauratella* has become a significant pest of red clover (*Trifolium pratense* L.) in forage seed production areas. Seed loss due to larval feeding over the growing season can be upwards of 80% in Ontario (Ellis and Bjørnson 1996) and 99.5%, in second year stands, in Alberta (Evenden et al. 2010). High larval densities which result in poor seed yield prevent many Alberta producers from cultivating red clover for seed as there are no registered control options. These extreme yield losses, combined with the lack of control options, have led Agriculture and Agri-Food Canada to rank *C. deauratella* as "A Priority Without Solution" on the national pest priority ranking system (Agriculture and Agri-Food Canada (http://www4.agr.gc.ca/)).

Coleophora deauratella females lay eggs on the calyx of red and alsike (*Trifolium hybridium* L.) clover florets. Upon hatching, larvae bore through the corollas and feed on developing ovules (Landry 1991). Larvae tunnel between adjacent florets and are capable of

destroying up to three seeds per day (Landry 1991, Ellis and Bjørnson 1996). The first three larval instars live within the floret and are difficult to sample and control (Ellis and Bjørnson 1996). Fourth instars continue to feed from within a portable case which they construct out of florets and silk (Landry 1991, Ellis and Bjørnson 1996). In late summer, mature larvae move to the ground, seal their case, and prepare to overwinter (Landry 1991). Pupation occurs in early spring and moths emerge in early summer (Ellis and Bjørnson 1996). In Alberta, *C. deauratella* are on the wing from the beginning of June to mid-August with peak flight occurring in mid-July. Red clover begins to bud by mid-June in Alberta and flowers remain in bloom until approximately the beginning of August when they begin to senesce (B. Mori, personal observation).

Pheromone-based monitoring programs can be used to survey and detect the presence of pest populations and form the basis of integrated pest management programs (Baker and Heath 2005). A pheromone-based monitoring program to determine the presence and seasonal activity of adult *C. deauratella* would be a valuable tool for growers because larvae are difficult to sample due to their internal feeding behavior. Pheromone-based monitoring of *C. deauratella* would allow for population assessment before damage is incurred so producers can make proactive decisions on whether to keep the crop for seed, hay, or green manure. Furthermore, a pheromone-monitoring program can help monitor the invasion of this insect in North America (Ellis and Bjørnson 1996, Evenden et al. 2010). Early detection of *C. deauratella* will be particularly important in the Willamette Valley in western Oregon, the primary red clover seed production region in the world (Steiner and Alderman 2003).

Mate location behavior of *C. deauratella* is mediated by a female-produced sex pheromone (Evenden et al. 2010). Females produce three compounds, (*Z*)-7-dodecenyl acetate

(*Z*7-12:OAc), (*Z*)-5-dodecenyl acetate (Z5-12:OAc) and (*Z*)-5-dodecen-1-ol (Z5-12:OH), that elicit an electrophysiological response in the male antennae (Evenden et al. 2010). However, only two of the three identified compounds, a 100:10 µg ratio of Z7-12:OAc to Z5-10:OAc, are required to attract thousands of male *C. deauratella* to traps in western Canada (Evenden et al. 2010).

The efficacy of pheromone-based monitoring systems can be affected by several parameters that influence trap capture of male moths. These characteristics include the substrate or lure type from which pheromone is released (Kehat et al. 1994, Mayer and Mitchell 1999, Athanassiou et al. 2004, Hoddle et al. 2011), the pheromone dose (Kehat et al. 1994, Kovanci et al. 2006, Evenden et al. 2010), the longevity of the pheromone lure throughout the monitoring period (Mitchell et al. 1989, Kehat et al. 1994, Francis et al. 2007), the type and volume of the trap (Mitchell et al. 1989, Kehat et al. 1994, Kovanci et al. 2006, Francis et al. 2007, Athanassiou et al. 2008, Chen et al. 2010, Reddy et al. 2011), and the position of the trap within the cropping system (Athanassiou et al. 2004, Kovanci et al. 2006, Hoddle et al. 2011, Reddy et al. 2011). Also, an effective pheromone-based monitoring program should minimize by-catch as this can increase processing time and be harmful to pollinators and other beneficial insects (Hendrix and Showers 1990, Gross and Carpenter 1991, Francis et al. 2007).

In the present study, a series of field trapping experiments test the effect of lure type and longevity, and trap type, height and field position on the response of male *C. deauratella* to pheromone-baited traps. This builds on a previous study that assessed the attractiveness of different doses of pheromone to male *C. deauratella* (Evenden et al. 2010). Information leading to improvements of trap performance will allow for the development of a pheromone-based monitoring program to detect and monitor *C. deauratella* throughout Canada and the world.

Materials and Methods

Study sites

All field experiments were carried out on red (cv. Altaswede, Belle or Taifun) or alsike (cv. Aurora) clover fields in the Peace River region of northwestern Alberta, Canada. Study site was considered the block in all experiments, and each treatment was replicated once per block. All study sites were separated by ≥ 1 km in all experiments and were concentrated around the town of Falher (55.737, -117.203) in 2009, around Falher, Rycroft (55.756, -118.705) and Beaverlodge (55.209, -119.427) in 2010, and around Falher and Debolt (55.216,-118.027) in 2011. The Peace River region runs along the confines of the Peace and Smoky Rivers and is composed primarily of Dark Grey Chernozemic and Luvisolic soils most of which are or have the potential to be arable land (Broersma et al. 1996). *Coleophora deauratella* has been severely infesting red clover throughout this region since 2006 (Evenden et al. 2010).

Pheromone lure preparation

In 2009, pre-extracted red and grey rubber septa (Contech Enterprises Inc., Delta, BC, Canada) were loaded with Z7-12:OAc (100 µg; >99% pure) (Pherobank, Wageningen, The Netherlands) and Z5-12:OAc (10 µg; >99% pure) (Pherobank) in high performance liquid chromatography (HPLC) grade hexane (110 µl) (EMD, Gibbstown, NJ, USA) at Simon Fraser University (Burnaby, BC, Canada). Lures were placed in sealed glass jars and shipped to the University of Alberta (Edmonton, AB, Canada) on ice, where they were placed in a -20°C freezer until they were transported to the field in refrigerated containers.

In 2010, grey rubber septa (Contech) pheromone lures were prepared at the University of Alberta following the same procedures as 2009. In 2011, lures were prepared as in 2010, except

Z7-12:OAc and Z5-12:OAc (≈95% pure) from Bedoukian Research Inc. (Danbury, CT, USA) were used instead of pheromone components from Pherobank, as traps baited with lures loaded with Bedoukian pheromone components attracted a similar number of moths to those baited with the more pure Pherobank pheromone components (B.A.M., unpublished data).

Experiment 1 – Evaluation of trap type and pheromone lure substrate – biweekly inspection

Experiment 1 tested the hypothesis that the type of trap and the substrate from which pheromone is released influences trap capture of male *C. deauratella* (Table 2-1). It was conducted over a two week period (30 July – 11 August 2009) at eight sites. Four commercially-available trap types baited with *C. deauratella* pheromone released from either a red or grey rubber septa lure were tested. Three saturating trap types, Wing traps (capture surface: 193.6 cm^2) (Contech), Delta traps (capture surface: 351.5 cm^2) (Contech), Diamond traps (capture surface: 480.1 cm^2) (Contech,) were tested in comparison to a non-saturating green plastic Unitrap™ (Contech). A strip of Hercon Vaportape II (10% Dichlorovos) (Hercon Environmental, Emigsville, PA, USA) killed captured insects in the Unitraps; all other traps had sticky inserts to capture insects. At each site, two traps of each type were baited with either a grey or red rubber septa pheromone lure for a total of eight traps per site. Traps were placed on a linear transect 25 m apart and 5 m from the field edge approximately 35 cm above the soil surface in a randomized block design (Table 2-1). At the end of the two-week period, traps were inspected and moths brought back to the laboratory for counting and identification.

Experiment 2 – Evaluation of trap type and color – weekly inspection

Experiment 2 tested the hypothesis that male moth capture would be influenced by trap type and color (Table 2-1). It was conducted over a two-week period (21 June – 5 July 2010) at

ten sites to compare the ability of seven commercially-available trap types to catch male *C. deauratella*. This experiment was conducted using the same four traps types as in 2009 but they were inspected on a weekly basis to determine if trap saturation was an issue with the two-week long trap inspection interval used previously. The effect of color on capture of male *C. deauratella* was evaluated by including differently colored Unitraps. Three saturating trap types: Wing traps (Contech), Delta traps (Contech), and Diamond traps (Contech), were tested in comparison to nonsaturating Unitraps that were green (Contech), yellow, white (AgBio Inc, Westminster, CO, USA.) or multicolored (green top, yellow funnel, white bucket) (Contech). A strip of Hercon Vaportape II killed captured insects in the Unitraps; all other traps had sticky inserts to capture insects. One trap of each trap type was baited on site with a grey septa pheromone lure and positioned along a linear transect 25 m apart and 5 m from the field edge approximately 35 cm above the soil surface in a randomized block design (Table 2-1). Traps were inspected, moths were collected, and trap inserts were changed on a weekly basis. Moths were brought back to the laboratory for counting and identification. In addition to counting and identifying *C. deauratella*, the number of *Bombus* spp. (Hymenoptera: Apidae) in each trap was recorded to determine if trap type or color influenced by-catch of beneficial insects in the clover system (Table 2-1).

Experiment 3 – Evaluation of pheromone lure longevity

Experiment 3 tested the hypothesis that lure age would influence male *C. deauratella* capture in pheromone-baited traps (Table 2-2). It was conducted over a two week period (6-21 July 2010) at nine sites to evaluate the length of time lures remain attractive to male *C. deauratella* in the field. Lures were aged for 0, 2, 4, 6, or 8 weeks before being deployed in traps in the field. Lures were aged by suspending freshly-loaded lures from a Wing trap (Contech) that

was positioned outdoors at the University of Alberta (maximum temperature= 33.5°C, minimum temperature= 0.7°C, mean temperature= 14.3°C). Experimentally-aged lures were placed in glass jars and transported to field sites in refrigerated containers. At each site, five green Unitraps (Contech) were each baited with a differently-aged lure and placed along a linear transect 25 m apart and 5 m from the field edge approximately 35 cm above the soil surface in a randomized block design (Table 2-2). A strip of Hercon Vaportape II killed captured insects. At the end of the two-week period, traps were inspected and moths were brought back to the laboratory for counting and identification.

Experiment 4 – Evaluation of trap height and field position

Experiment 4 tested the hypothesis that the height and position of pheromone-baited traps would influence capture of male *C. deauratella* (Table 2-3). It was conducted over a two week period (20 June – 4 July 2011) at seven sites. At each site, green Unitraps (Contech) were baited with grey rubber septa pheromone lures and placed 25 m apart on a linear transect at three trap heights (ground level, 35 cm, or 1 m) and at two different field positions (5 m and 50 m from the field edge) in a randomized block design (Table 2-3). At the end of the two-week period traps were inspected and moths were brought back to the laboratory for counting and identification.

Data analysis

Trap catch data were analyzed to check for normality and heteroscedasticity through visualization of the data and Shapiro-Wilks tests (R Core Development Team 2011). Generalized linear models with negative binomial distributions were used due to non-normal error distributions and overdispersion of the data (Veneables and Ripley 2002). A negative binomial

distribution is appropriate for count data with overdispersion (Zuur et al. 2009). Models were designed for each experiment with the total number of male *C. deauratella* caught per trap specified as the response variable and lure type, lure age, trap type, trap height and field position as dependent variables in the respective experiments (Table 2-4). An additional model for Experiment 2 was developed that specified the total number of bumble bees (*Bombus* spp.) caught as the response variable and trap type as the dependent variable (Table 2-4). In each model, site was specified as a block (random factor). When appropriate, initial models included all interaction terms (Experiment 1 included a trap type*lure type interaction term; Experiment 4 included a trap height*trap position interaction term); any non-significant terms were removed from the model. Multiple comparisons to determine significant differences among treatments within the dependent variables were conducted using the *z*-statistics ($p<0.05$) given in the model output. For multiple comparisons, all reported p-values were corrected using a false-discovery rate procedure (Benjamini and Hochberg 1995). All statistical analyses were performed in R 2.12.2 (R Core Development Team 2011).

Results

Experiment 1 – Evaluation of trap type and pheromone lure substrate – biweekly inspection

Pheromone released from grey and red septa lures was equally attractive to male *C. deauratella* regardless of trap type (Table 2-4; Fig. 2-1). Different trap types captured varying numbers of male *C. deauratella* (Table 2-4; Fig. 2-1). The non-saturating, green Unitraps caught the largest number of male *C. deauratella* of all trap types and caught significantly more moths than Diamond ($z = -3.38$, $P < 0.01$) and Wing traps ($z = -4.81$, $P < 0.01$), but not Delta traps ($z =$

-1.88, $P = 0.09$) (Fig. 2-1). Delta traps caught significantly more moths than Wing traps ($z = -2.92$, $P < 0.01$) (Fig. 2-1).

Experiment 2 – Evaluation of trap type and color – weekly inspection

Trap types were differentially effective in the capture of male moths (Table 2-4; Fig. 2-2). Multi-colored Unitraps caught significantly more male *C. deauratella* than Diamond traps ($z = -3.58$, $P < 0.01$), and Wing traps ($z = -2.88$, $P = 0.04$), but not green Unitraps ($z = -2.72$, $P = 0.05$), white ($z = -1.80$, $P = 0.23$) and yellow Unitraps ($z = -1.94$, $P = 0.22$) (Fig. 2-2). The number of male *C. deauratella* caught in each trap type was highly variable among sites, but white Unitraps caught the largest number of moths over the two week period. Overall, Unitraps, regardless of color, caught numerically more male *C. deauratella* over the two-week period than any of the saturating trap types (Delta, Diamond and Wing traps) (Fig. 2-2).

There was a significant difference among trap types in the number of *Bombus* spp. caught throughout the experiment (Table 2-4; Fig. 2-3). White and yellow Unitraps caught significantly more *Bombus* spp. than all other trap types (white-Delta: $z = -5.83$, $P < 0.01$, white-Diamond: $z = -5.85$, $P < 0.01$, white-green Unitraps: $z = -4.47$, $P < 0.01$, white-multi-colored Unitraps: $z = -3.11$, $P < 0.01$, white-Wing: $z = -5.83$, $P < 0.01$; yellow-Delta: $z = -6.69$, $P < 0.01$, yellow-Diamond: $z = -6.75$, $P < 0.01$, yellow-green Unitraps: $z = -5.77$, $P < 0.01$, yellow-multi-colored Unitraps: $z = -4.51$, $P < 0.01$, yellow-Wing: $z = -6.69$, $P < 0.01$) (Fig. 2-3). Multi-colored and green Unitraps caught significantly more *Bombus* spp. than Delta (multicolored: $z = -3.81$, $P < 0.01$, green: $z = -2.67$, $P = 0.01$), Diamond (multicolored: $z = -3.73$, $P < 0.01$, green: $z = -2.55$, $P = 0.01$) and Wing traps (multicolored: $z = -3.81$, $P < 0.01$, green: $z = -2.55$, $P = 0.01$) (Fig. 2-3).

Of the colored Unitraps tested, yellow Unitraps caught numerically more *Bombus* spp. than all other traps, followed by white, multi-colored and green Unitraps (Fig. 2-3).

Experiment 3 – Evaluation of pheromone lure longevity

There was a trend toward lower trap capture of male *C. deauratella* in traps baited with older lures, but it was not significant (Table 2-4; Fig. 2-4).

Experiment 4 – Evaluation of trap height and field position

There was no significant difference in the number of male *C. deauratella* caught in traps placed either 5 or 50 m from the field edge (Table 2-4; Fig. 2-5). Traps placed 35 cm above the soil surface caught significantly more male *C. deauratella* than traps placed either at ground level (z = -2.24, P = 0.04) or 1 m above the soil surface (z = -3.36, P = 0.02) (Fig. 2-5).

Discussion

This study shows that a number of non-pheromone factors can influence the attractiveness and efficacy of pheromone-based trapping for the invasive moth pest, *C. deauratella*. These factors are important as adoption of pheromone-based monitoring in integrated pest management programs is facilitated by ease of handling and longevity of the monitoring tool. Pheromone released from both red and grey rubber septa lures was equally attractive to male *C. deauratella*. The substrate from which pheromone is released can alter the release rate and longevity of the lure and influence its attractiveness to the target pest (Howse et al. 1998). Traps baited with grey rubber septa lures capture significantly more *Plutella xylostella* L. (Lepidoptera: Plutellidae) than those baited with red rubber septa lures when loaded with the same mixture of pheromone (Mayer and Mitchell 1999, Miluch 2010). Whereas, pheromone

released from red rubber septa lures is more attractive to male *Palpita unionalis* Hübner (Lepidoptera: Pyralidae) than that released from white rubber septa lures (Athanassiou et al. 2004). The difference between the lures is attributed to differential release rate of pheromone components from the lures or the presence of chemicals that react with aldehyde pheromone components in the lure (Steck et al. 1979, Athanassiou et al. 2004, Miluch, 2010). *Coleophora deauratella* pheromone does not contain aldehydes and the rubber septa used in this study were pre-extracted to remove impurities. All subsequent experiments were carried out with grey rubber septa.

All four trap types tested in Experiment 1 captured high numbers of male *C. deauratella*. Green Unitraps captured numerically more males than Delta, Diamond or Wing traps (Fig. 2-2). Numerous studies have found that trap type can affect the number of insects captured in pheromone-baited traps (Francis et al. 2007, Athanassiou et al. 2008, Strong et al. 2008, Chen et al. 2010, Reddy et al. 2011). Trap type can alter the pheromone plume structure and can provide different visual cues to male moths (Knight and Fisher 2006). The trap types tested in Experiment 1 were differentially effective at capturing male *C. deauratella* (Fig. 2-1). This difference may be driven by the long, two-week trap inspection interval used in the first experiment. Delta, Diamond and Wing trap types can become less efficient over time as the sticky surface saturates with male moths and scales, especially at high moth densities (Sanders 1986, Athanassiou et al. 2002, Athanassiou et al. 2004). Whereas, Unitraps can retain thousands of moths and do not become saturated. However, when traps were inspected weekly in Experiment 2, Unitraps still captured greater numbers of moths compared to the different types of sticky traps (Fig. 2-2), but the difference in trap capture was not as profound as in Experiment 1 (Fig. 2-1). Traps that capture attracted insects on a sticky surface can accumulate debris and

non-target insects which can lead to trap saturation and increased processing time of the target insect, especially with small insects like *C. deauratella* (wingspan 9.5-15.5 mm) (Vincent and Simard 1986, Adams et al. 1989, Landry 1991, Francis et al. 2007). The current study supports the use of Unitraps to monitor C. *deauratella* due to ease of handling and trap capacity that does not become saturated over a two-week trap inspection interval.

There was no significant difference in the number of moths caught in the four differently colored Unitraps tested (Fig. 2-3). Trap color is an important factor that influences response to pheromone-baited traps most commonly in highly visual or diurnal species (Mitchell et al. 1989, Suckling et al. 2005). *Coleophora deauratella* are reportedly most active in the late afternoon and near dusk (Landry 1991). However, capture of male *C. deauratella* peaks in pheromone-baited traps at dawn (unpublished data) and trap color may not be a major factor that influences male moth capture in this species. Although trap color only marginally dictates the capture of male *C. deauratella* in pheromone-baited traps, it does significantly influence by-catch of beneficial bumble bees (*Bombus* spp.). Significantly more bumble bees are captured in yellow and white Unitraps compared with multicolored or green Unitraps (Fig. 2-3). Although not significant, multicolored unitraps caught numerically more bumble bees than green unitraps (Fig. 2-3). Bumble bees are highly visual generalist flower visitors with trichromatic vision capable of seeing from 300 nm (UV) to 600 nm (Briscoe and Chitka 2001). These receptors peak around wavelengths of 330 nm, 430 nm, and 540 nm which correspond to maximal reflectance in the ultraviolet, blue-green and yellow spectra (Peitsch et al. 1992). Bumble bees should therefore be able to discriminate among the Unitrap colors used in this study. Similar to our findings, bumble bee capture is greater in yellow than green or white funnel traps positioned in corn fields (Hendrix and Showers 1990, Gross and Carpenter 1991). Bumble bees are native pollinators

throughout Canada and are excellent pollinators of red clover (Holm 1966). To avoid significant by-catch and impact on pollination services provided by bumble bees, green Unitraps should be used to monitor *C. deauratella*.

Trap capture of male *C. deauratella* is unaffected by lure age for pheromone lures aged 0-8 weeks (Fig. 2-4). However, there is a trend of reduced trap capture in older lures which is particularly noticeable in traps baited with lures aged for six weeks. Pheromone release rates from rubber septa decrease exponentially over time and are affected by environmental conditions (Bierl-Leonhardt et al. 1979, Butler and McDonough 1981, McDonough 1991, Walton et al. 2004). As lures age, they may become less attractive due to a decrease in the amount of pheromone released from each lure (Butler and McDonough 1981). The half-life of Z7-12:OAc, the major pheromone component of *C. deauratella*, released from rubber septa is ≈ 35 days (Butler and McDonough 1979). The minor component, Z5-12:OAc is likely to have a slightly shorter half-life than Z7-12:OAc because of the more centrally-located double bond (Olsson et al. 1983). Due to the location of the double bond, Z-5-dodecenyl acetate will fold differently than Z7-12:OAc and will have a smaller molecular size, a higher vapor pressure, and a shorter half life than Z7-12:OAc (Olsson et al. 1983). Because lures used in this study have a ten-fold lower amount of Z5-12:OAc than Z7-12:OAc applied to the lure, the amount of Z5-12:OAc should be the critical factor in the pheromone lure remaining attractive to *C. deauratella.* This slight difference in vapor pressure may change the blend ratio over time due to differential loss of pheromone components (Môttus et al. 1997, Jones et al. 2009). Given that pheromone lures aged up to 8 weeks (56 days) are as attractive as fresh lures to *C. deauratella*, both components must still be present and actively emitted from the lure as *C. deauratella* males are not attracted to Z7-12:OAc alone (Evenden et al. 2010). Ultimately, environmental conditions, especially

temperature, will have the greatest impact on pheromone release rates and should be monitored to determine when and if lure replacement is needed (Mayer and Mitchell 1999). Lure age may not be a major factor influencing trap capture of *C. deauratella* because, unlike some other moths (i.e. *P. xylostella* (Evenden and Gries 2010)), doses of pheromone over a wide range does not impact lure attractiveness to male *C. deauratella* (Evenden et al. 2010). An equal number of male *C. deauratella* are captured in traps baited with fresh lures containing 10-1000 µg of pheromone and trap catch significantly declines only when the pheromone dose is reduced to 1 µg per septum (Evenden et al. 2010). Thus in combination with our previous work (Evenden et al. 2010), this study demonstrates that a 100 µg dose of pheromone remains attractive for at least 8 weeks under field conditions.

Significantly more male *C. deauratella* are caught in traps placed 35 cm above the soil surface within the clover canopy than those placed at ground level or at 1 m above the soil surface. Trap height can affect the capture of various insects in a cropping environment. More *Grapholita molesta* L. (Lepidoptera: Tortricidae) are captured when pheromone-baited traps are placed in the upper rather than lower tree canopy in an orchard agroecosystem (Kovanci et al. 2006). Traps placed within the canopy of an avocado orchard capture significantly more *Stenoma catenifer* Walsingham (Lepidoptera: Elachistidae) than those placed either below or above the canopy (Hoddle et al. 2011). In contrast, *Prays oleae* Bernard and Lesne (Lepidoptera: Yponomeutidae) males are captured in equal numbers in traps variously positioned within olive orchards (Kavallieratos et al. 2005). Male *C. deauratella* may restrict their flight activity to within the clover canopy to increase the chances of encountering a calling female or for protection from natural enemies. Although moths are caught in traps positioned at ground level and 1 m above the ground, significantly more moths are captured in traps positioned at 35 cm

above the ground. This trap height should be used for trap deployment in a pheromone-based monitoring system targeting *C. deauratella*.

Trap position within the clover fields does not affect male *C. deauratella* capture. Similarly, trap position has no effect on capture in pheromone-baited traps of another field crop pest, the pink bollworm, *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) (Athanassiou et al. 2002). Traps placed at the edge of an almond orchard capture significantly more male *Palpita unionlalis* Hübner (Lepidoptera: Pyralidae) than those placed in the interior of the orchard possibly because males migrate into the orchards in the evenings searching for females (Athanassiou et al. 2004). It is unlikely that male *C. deauratella* move in and out of individual clover fields because the surrounding land use is variable and there can be large distances between clover fields. Further studies over the course of the season could determine if moth movement between and within the field influences trap capture of male *C. deauratella*. Future lab and field experiments are needed to assess the dispersal capacity of this invasive moth.

This study tested the effect of lure type and longevity, trap type, height and field position on the attractiveness of *C. deauratella* to pheromone-baited traps. Green Unitraps baited with either grey or red rubber septa lures placed 35 cm above the soil surface and 5 m from the field edge minimize by-catch and capture large numbers of male *C. deauratella*. This system is simple for growers to assemble and monitor over the course of the growing season and these findings should be considered when developing a pheromone-monitoring program. A pheromone-monitoring program would be a valuable tool to determine the presence of this invasive pest and to monitor the rates of infestation.

Table 2-1. Experimental design and mean *C. deauratella* and *Bombus* spp. (± SE) captured in various pheromone-monitoring traps for tests of trap type and pheromone lure substrate (Experiment 1) and trap type and color (Experiment 2).

Experiment	Sites	Trap Type	Lure Substrate	Mean *C. deauratella*/trap (SE)	Mean *Bombus* spp./trap (SE)
1 Trap and Lure Type	8	Wing	Red	236.25 (25.91)	-
		Delta	Red	519.88 (60.78)	-
		Diamond	Red	304.63 (35.46)	-
		Green Unitrap	Red	1621.63 (782.41)	-
		Wing	Grey	239.88 (37.33)	-
		Delta	Grey	520.50 (80.97)	-
		Diamond	Grey	330.00 (32.88)	-
		Green Unitrap	Grey	1118.63 (600.99)	-
2 Trap Type and Color	10	Wing	Grey	244.50 (25.43)	0.50 (0.50)
		Delta	Grey	356.20 (30.99)	0.30 (0.21)
		Diamond	Grey	193.90 (21.49)	0.60 (0.60)
		Green Unitrap	Grey	701.10 (344.53)	3.20 (1.77)
		Yellow Unitrap	Grey	885.00 (458.35)	24.80 (6.45)
		White Unitrap	Grey	1077.90 (601.77)	13.90 (5.91)
		Multicolored Unitrap	Grey	1071.00 (346.74)	7.30 (4.20)

At each site, each trap and lure combination was tested. Traps were placed in the interior of the field, 5 m from the field edge, 25 m apart along a linear transect, and 35 cm above the soil surface.

Table 2-2. Experimental design and mean *C. deauratella* (± SE) captured in pheromone-monitoring traps for the pheromone lure longevity experiment (Experiment 3).

Experiment	Sites	Trap Type	Lure Age (Weeks)	Lure Substrate	Mean *C. deauratella*/trap (SE)
3 Lure Longevity	9	Green Unitrap	0	Grey	1308.67 (331.68)
		Green Unitrap	2	Grey	1075.11 (260.74)
		Green Unitrap	4	Grey	1277.22 (410.85)
		Green Unitrap	6	Grey	856.56 (475.58)
		Green Unitrap	8	Grey	685.67 (204.48)

At each site, one green Unitrap was loaded with each of the variously aged lures. Traps were placed in the interior of the field, 5 m from the field edge, 25 m apart along a linear transect, and 35 cm above the soil surface.

Table 2-3. Experimental design and mean *C. deauratella* (± SE) captured in pheromone-monitoring traps for the trap height and field position experiment (Experiment 4).

Experiment	Sites	Trap Type	Trap Ht. (cm)	Trap Field Position (m)	Mean *C. deauratella*/trap (SE)
4 Trap Height and Position	7	Green Unitrap	0	5	39.86 (9.16)
		Green Unitrap	35	5	105.86 (27.72)
		Green Unitrap	100	5	62.43 (26.99)
		Green Unitrap	0	50	78.29 (16.59)
		Green Unitrap	35	50	117.43 (36.43)
		Green Unitrap	100	50	64.71 (22.80)

At each site, one green Unitrap was positioned at each of the various heights and field positions. Traps were placed in the interior of the field, 5 m or 50 m from the field edge, 25 m apart along a linear transect, and at the soil surface (0 cm), 35 cm, or 100 cm above the soil surface.

Table 2-4. Results of the generalized linear models with a negative binomial distribution of total trap capture (*C. deauratella* or *Bombus* spp.) in response to various dependent variables used in each experiment.

Experiment	Response Variable	Dependent Variables	df	Explained Deviance	Residual df	Residual Deviance	P-value
1 Trap and Lure Type	*C. deauratella* count	Null			63	161.063	
		Trap Type	3	64.612	60	96.452	$P < 0.001$
		Lure Type	1	0.158	59	96.294	$P > 0.05$
		Site	7	27.44	52	68.854	$P < 0.001$
2 Trap Type and Color	*C. deauratella* count	Null			69	250.139	
		Trap Type	6	63.873	63	186.266	$P < 0.001$
		Site	9	111.62	54	74.646	$P < 0.001$
	Bombus spp. count	Null			69	282.903	
		Trap Type	6	154.052	63	128.85	$P < 0.001$
		Site	9	65.967	54	62.833	$P < 0.001$
3 Lure Longevity	*C. deauratella* count	Null			44	155.45	
		Lure Age	4	8.81	40	146.64	$P = 0.066$
		Site	8	99.583	32	47.06	$P < 0.001$
4 Trap Height and Position	*C. deauratella* count	Null			41	58.39	
		Trap Height	2	10.584	39	84.398	$P = 0.01$
		Trap Position	1	2.181	38	82.217	$P > 0.05$
		Site	6	37.415	32	44.802	$P < 0.001$

Null models contain only an intercept. No interactions were significant for any models. Site was included as a block (random factor) in the model.

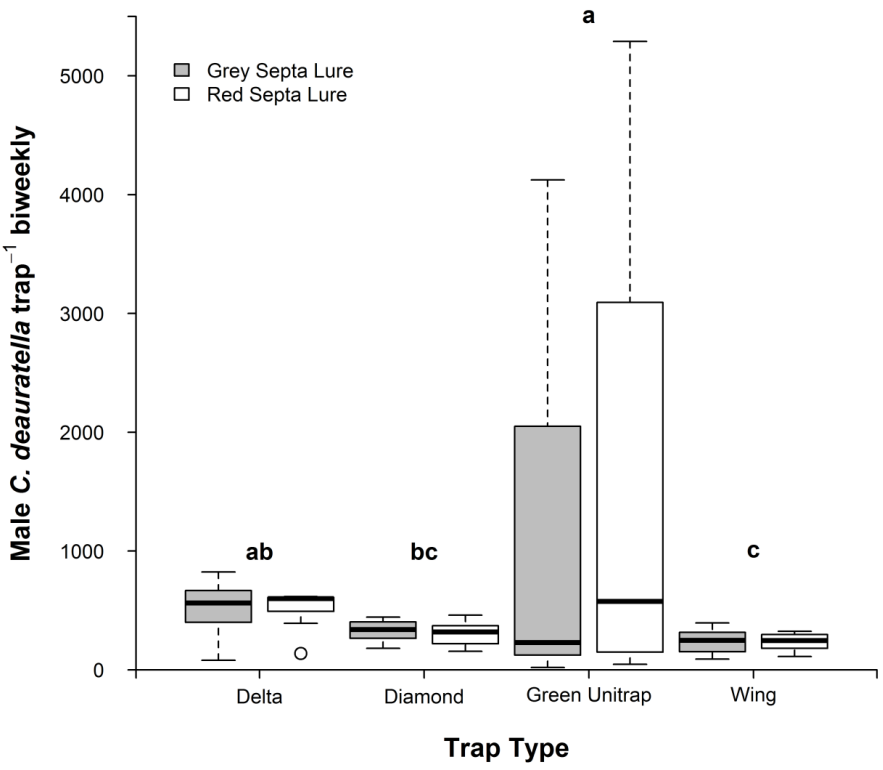

Figure 2-1. Box plot of the number of male *C. deauratella* caught in four different trap types over a two-week trapping period in 2009. The midline indicates the median and the bottom and top of the box indicate the first and third quartiles, respectively. The vertical lines, or whiskers, indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Outliers are represented by points above or below the whiskers (Crawley 2007). There was no significant difference between grey and red rubber septa lures in each trap type. Box plots followed by different letters indicate significant differences ($p < 0.05$) in moth capture among trap types.

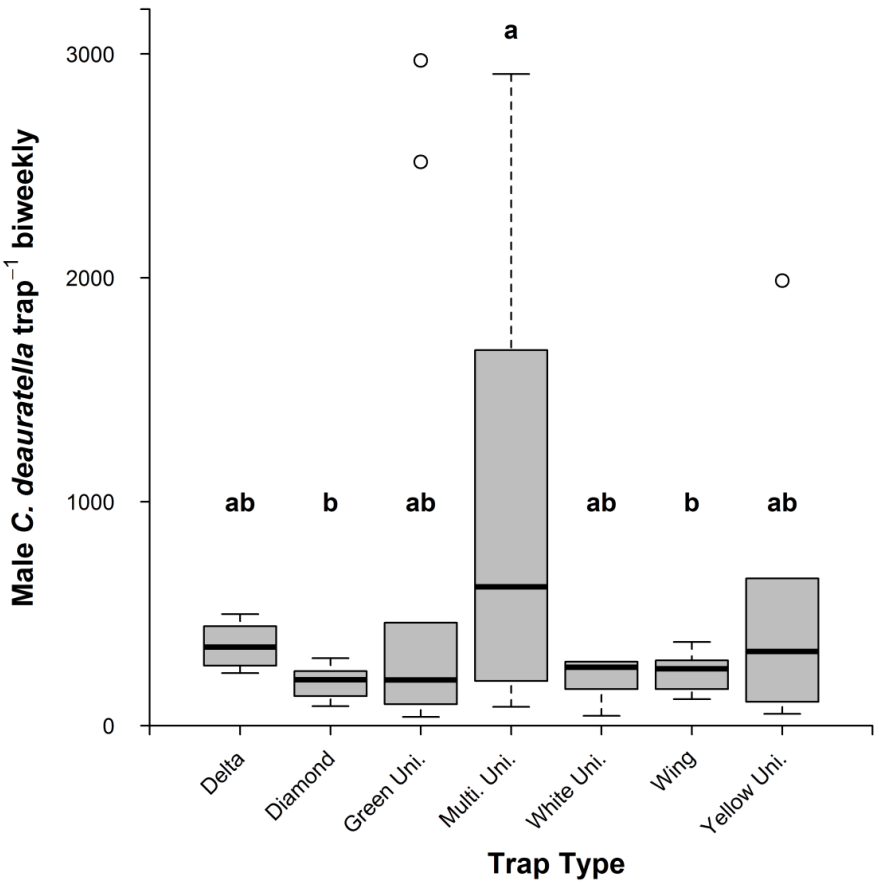

Figure 2-2. Box plot of the number of male *C. deauratella* caught in seven different trap types over a two-week trapping period in 2010. The midline indicates the median and the bottom and top of the box indicate the first and third quartiles, respectively. The vertical lines, or whiskers, indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Outliers are represented by points above or below the whiskers (Crawley 2007). Outliers for white Unitraps (5474 and 3725 moths captured) and yellow Unitraps (4669 moths captured) were removed from the figure for clarity. Box plots followed by different letters indicate significant differences ($p < 0.05$) in moth capture among trap types.

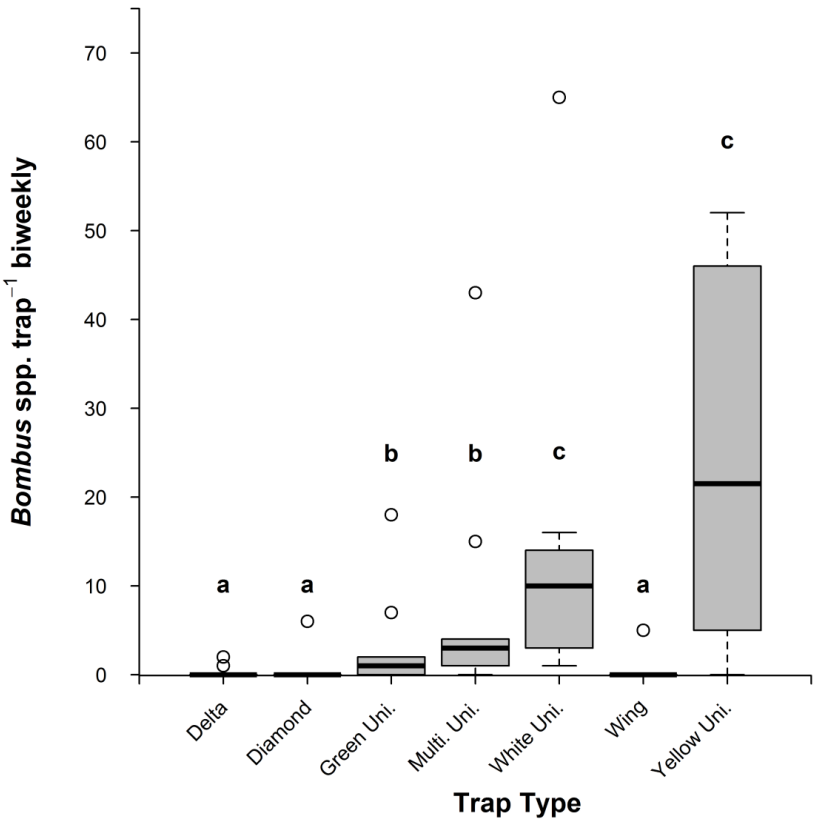

Figure 2-3. Box plot of the number of bumble bees (*Bombus* spp.) caught in seven different trap types over a two-week trapping period in 2010. The midline indicates the median and the bottom and top of the box indicate the first and third quartiles, respectively. The vertical lines, or whiskers, indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Outliers are represented by points above or below the whiskers (Crawley 2007). Box plots followed by different letters indicate significant differences ($p < 0.05$) in bumble bee capture among trap types.

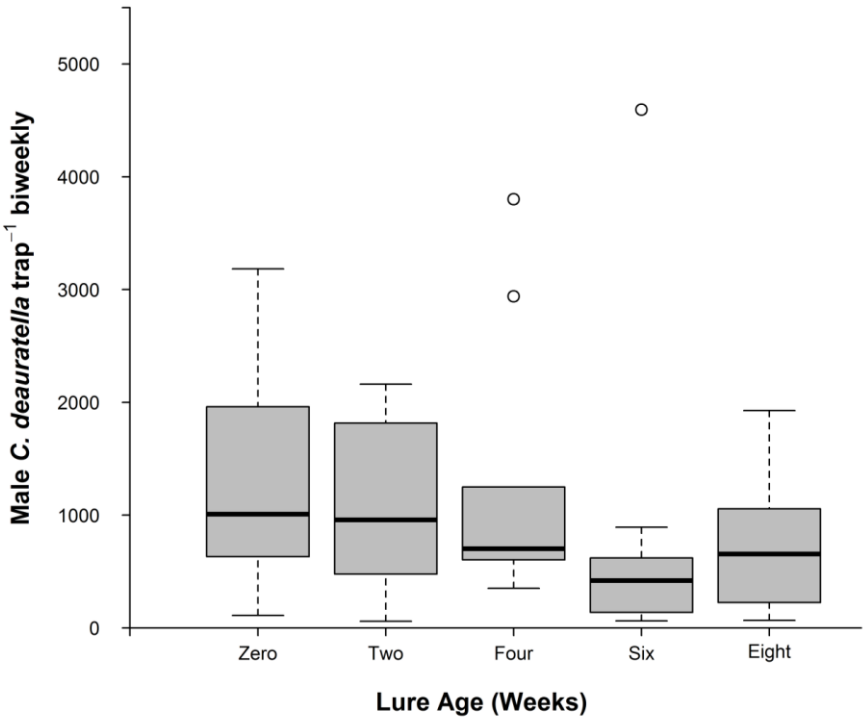

Figure 2-4. Box plot of the number of *C. deauratella* caught in green Unitraps over a two-week trapping period in 2010 baited with variously aged lures. The midline indicates the median and the bottom and top of the box indicate the first and third quartiles, respectively. The vertical lines, or whiskers, indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Outliers are represented by points above or below the whiskers (Crawley 2007). There were no significant differences in moth capture in traps baited with variously-aged lures.

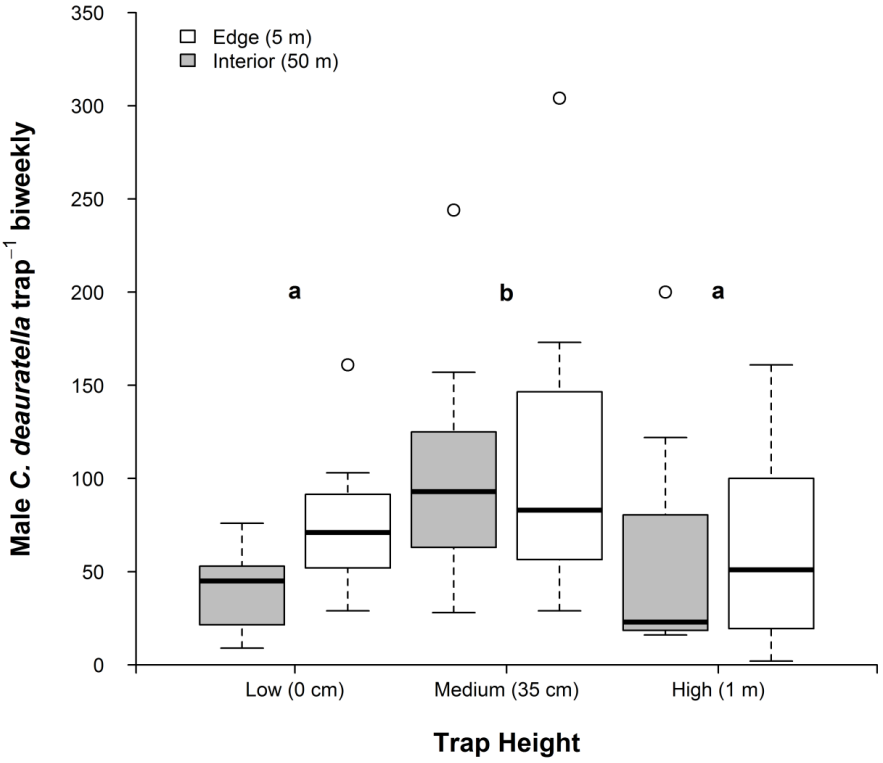

Figure 2-5. Box plot of the number of *C. deauratella* caught in green Unitraps over a two week trapping period in 2011 placed at two different field positions and three different trap heights. The midline indicates the median and the bottom and top of the box indicate the first and third quartiles, respectively. The vertical lines, or whiskers, indicate the maximum value, or 1.5 times the interquartile range, whichever is smaller. Outliers are represented by points above or below the whiskers (Crawley 2007). There was no significant difference in trap capture by field position. Box plots followed by different letters indicate significant differences ($p < 0.05$) in moth capture among trap heights tested.

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Chapter 3

Relationships among male *Coleophora deauratella* (Lepidoptera: Coleophoridae) pheromone-baited trap capture, larval abundance, damage and flight phenology

Introduction

Pheromone-baited traps are among the most widely employed monitoring tools in lepidopteran pest management. They are used for many applications including early warning of pest incidence, surveys to define an infested area, and timing of pest management tactics (Howse et al. 1998). Pheromone-based monitoring of insect populations provides information on migration, flight activity, and the periodicity of adult male emergence (Reidl et al. 1976). Consequently, pheromone-based monitoring is useful for risk assessment, in which trap catch is quantitatively related to population density or damage levels in a managed ecosystem (Howse et al. 1998). Within agricultural and forestry settings, some pheromone-based monitoring programmes have been developed to provide accurate relationships between male moth capture and pupal, larval, or egg stages and crop damage (Turnock 1987, Latheef et al. 1991, Evenden et al. 1995, Ngollo et al. 2000, Hillier et al. 2004, Jones et al. 2009, Miluch et al. 2013). In these studies, pheromone traps are used to monitor male flight, and sampling for immature stages and damage at the same sites is used to determine if trap catch can predict pest densities. In many cases, these estimates are incorporated into integrated pest management (IPM) programmes and are used to time control measures in the field.

Other types of models can combine pheromone-based monitoring with a measure of physiological time (i.e. degree days) or ordinal date to create phenological models. The objective of a phenological model is to predict the time of appearance of specific insect life stages to improve the timing of biological, chemical, or cultural control techniques (Hardman 2012) which

is often used in conjunction with a risk assessment model. Phenological models based on pheromone-baited trap capture are used to time specific life stage sampling and application of control measures for a variety of lepidopteran pests of managed ecosystems including *Cydia pomonella* (Tortricidae) (Glen and Brain 1982), *Podosesia syringae* (Sessiidae) (Potter and Timmons 1983), *Grapholita molesta* (Tortricidae) (Rice et al. 1984), *Chilo paratellus* (Pyralidae) (Unnithan and Saxena 1990), *Lymantria dispar* (Lymantriidae) (Régnière and Sharov 1998), *Lobesia botrana* (Tortricidae) (Gallardo et al. 2009), and *Acrobasis nuxvorella* (Pyralidae) (Knutson and Muegge 2010).

Here, we present results on the flight phenology and relationships between pheromone-baited trap capture of the red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae), and subsequent larval density and damage of red clover, *Trifolium pratense* L. (Fabaceae), grown for seed. Red clover is an important nitrogen-fixing legume grown for animal forage, honey production, and for use in crop rotations for soil improvement (Fairey 1985). Seed production occurs primarily in the United States, Canada and the European Union (Wong 2005). *Coleophora deauratella* is native to Eastern Siberia, the Middle East, and Europe (Landry and Wright 1993). It was first identified in Canada in 1991, as its presence had gone unnoticed or it was misidentified since the late 1960's (Landry 1991). In Europe, *C. deauratella* causes only minor damage, with most damage occurring sporadically in rare, but severe infestations (Ellis and Bjørnson 1996, Markkula and Myllymäki 1960). However, in Canada larval feeding by *C. deauratella* causes major damage and can result in ≥80% seed loss (Ellis and Bjørnson 1996, Evenden et al. 2010).

Coleophora deauratella is univoltine. Mature larvae overwinter in a case (Landry 1991) and pupation occurs in the early spring, and moths emerge in early summer (Ellis and Bjørnson

1996). Females lay eggs on the outer surface of the calyx of red and alsike (*Trifolium hybridum* L.) clover florets. Larvae hatch and either bore through the corolla or enter the tip of the floret and feed on the developing ovules (Landry 1991, Ellis and Bjørnson 1996). Larvae can destroy 1-3 seeds/day as they pass through four larval instars (Landry 1991). During the fourth instar, larvae create a case from silk and floret petals in which they overwinter in the soil (Ellis and Bjørnson 1996).

Due to the internal feeding behaviour of *C. deauratella* larvae, infestations are difficult to identify and control. Consequently, a pheromone-based monitoring programme would be a valuable tool for producers to determine the presence, and to monitor the spread and seasonal abundance of *C. deauratella*. Furthermore, implementation of an IPM programme requires reliable tools to monitor pest population densities and time control measures. To date there are no registered insecticides available for use against *C. deauratella*, however, a pheromone-based monitoring programme which can determine the seasonal activity and abundance of *C. deauratella* may help time the application of insecticides or other control measures (i.e. pheromone-based mating disruption). Pheromone-baited monitoring traps can also be used to detect *C. deauratella* as it spreads throughout North America. A pheromone-monitoring programme for risk assessment would be particularly useful for growers in the Willamette Valley of western Oregon, USA, the primary red clover seed production region in the world (Steiner and Alderman 2003), where *C. deauratella* was recently detected (B. Mori, unpublished).

The recent identification of the sex pheromone of *C. deauratella* as a 100:10 ratio of *Z*-7-dodecenyl acetate (*Z*7-12:OAc) to *Z*-5-dodecenyl acetate (*Z*5-12:OAc) (Evenden et al. 2010), and the development of an optimized pheromone-based trapping system (Mori and Evenden 2013), provides the foundation for the development of a pheromone-based monitoring

programme to evaluate populations of *C. deauratella*. The purpose of the current study is to develop a pheromone-based monitoring programme that can relate adult male trap capture to the temporal and spatial occurrence of *C. deauratella* larvae and damage in monitored fields. Moth capture in pheromone-baited traps is also used to determine the seasonal flight phenology of *C. deauratella* males and to develop phenological models based on degree days and ordinal dates.

Materials and Methods

Study area

Study sites encompassed square red clover seed production fields (64.75 ha in size) (*Trifolium pratense* L. (Fabaceae) va. 'Altaswede', 'Belle', or, 'Taifun') in the Peace River region of northwestern Alberta, Canada (Table 3-1). The Peace River region runs along the confines of the Peace and Smoky rivers and is dominated by flat, expansive agricultural land with intermittent clumps of forest vegetation. The region is the world's second largest grass and forage seed production region (by area), only behind the Willamette Valley in western Oregon, USA (Wong 2010). Study sites were scattered throughout an area of approximately 19, 000 km^2 and ranged from the towns of Hines Creek (56.248, -118.601) in the North, to Beaverlodge (55.210, -119.426) in the West and South, and Falher (55.737, -117.199) in the East. Each experimental site was separated by ≥1 km from all other experimental sites and other clover fields. No herbicides, fungicides or insecticides were applied to any of the study sites during the course of these experiments. *Coleophora deauratella* was first found causing extensive damage to red clover fields in this region in 2006 (Evenden et al. 2010).

Lure preparation

In all study years, *Z*7-12:OAc (96.6% chemical purity) and *Z*-5-12:OAc (98.6% chemical purity) pheromone components were obtained from Bedoukian Research Inc. (Danbury, CT, USA) (Purity was obtained from the manufacturer). Extracted grey rubber septa were purchased from Contech Enterprises (Delta, BC) and loaded with *Z*-7-dodecenyl acetate (100 µg) and *Z*5-12:OAc (10 µg) in HPLC grade hexane (110 µl) (EMD, Gibbstown, NJ, USA). Lures were placed in sealed glass jars and stored at -20°C until they were transported to the field in refrigerated containers.

Sampling methods

Three types of data were collected each year at each site, including male moth trap capture (number of male *C. deauratella* captured in pheromone-baited traps), larval abundance (number of larvae per inflorescence), and proportion seed damage. Pheromone-monitoring traps were deployed at eight, seven and six sites, respectively, from7 June to 16 August in 2010, 6 June to 16 August in 2011 and 11 June to 20 August in 2012. Traps were placed at each site to maximize trap capture of *C. deauratella* (Mori and Evenden 2013). At each site, two green Unitraps (Contech Enterprises) were positioned on stands ~35 cm above the ground, 5 m into the crop and 25 m apart parallel to the field edge. A strip of Hercon Vaportape II (10% dichlorovos) (Hercon Environmental, Emigesville, PA, USA) was inserted into each trap to kill captured insects. Pheromone lures were replaced after six weeks in the field. Unitraps were inspected, emptied and captured moths counted at approximately two-week intervals for the duration of the experiment in each field season.

In all years, larval sampling was conducted once (2-3 August 2010, 1-2 August 2011, and 6 August 2012) at all sites. At each site, 25 red clover plants were sampled every 5 m while walking along a zigzag pattern approximately 20 m from the field edge (15 m from the trap line) parallel to the trap line. Plants were collected, placed individually into plastic resealable bags, and kept in refrigerated containers for transport to the laboratory. In the laboratory, plants were removed from the individual bags, and all inflorescences counted, examined and dissected to determine the presence and number of larvae. Due to the variation in the number of inflorescences per plant, larval abundance was measured as larvae/inflorescence for each site.

Larval feeding damage was assessed towards the end of the growing season in all years from each site (17 August 2010, 16 August 2011, and 20 August 2012). At one site in 2011, the producer plowed the field for green manure before damage could be assessed. At all other sites, three 1 m^2 plots approximately 25 m from the field edge (20 m from the trap line), parallel to the trap line were randomly chosen. In each plot, all red clover plants were harvested and placed into cotton bags. Bags were placed in refrigerated containers for transport to the laboratory. In the laboratory, bags were suspended from the ceiling for approximately 1 month (temp: 21°C ± 3°C) to allow the clover plants to dry. After drying, the plants from each plot were threshed and the seed was cleaned by hand. One-hundred randomly chosen seeds from each plot were assessed for larval feeding damage (assessed as damaged or undamaged). The number of seeds damaged across all three plots was combined and the total proportion seed damage was calculated for each site. Producers were also contacted and the approximate seed yield (kg/ha) achieved at harvest from some fields was obtained. Due to the intermittent nature of data on seed yield at harvest, no statistical models were created with these data.

Degree days

To calculate cumulative degree days, a developmental threshold of 11.7°C was specified based on the pupal development threshold for *Coleophora spissicornis* (=*mayrella*) (Pearson 1975), a closely related sympatric species that belongs to the same monophyletic species group (*Frischella*) as *C. deauratella* (Landry and Wright 1993, Bauer et al. 2012). Like *C. deauratella*, *C. spissicornis* (=*mayrella*) is introduced from Europe to the Nearctic region and feeds on clover (Landry and Wright 1993). The adult moths and larvae are almost indistinguishable from *C. deauratella* except for a few small external morphological and genitalic characters (Landry and Wright 1993). Cumulative degree days ($DD_{11.7}$) throughout the trapping period were calculated using the single sine method (Zalom et al. 1983) in the 'DegDay' programme (Snyder 2005). Degree days were accumulated starting from January 1st through the last sample period each year. In order to obtain a complete data set, all daily maximum and minimum temperatures were taken from a nearby Environment Canada weather station located in the town of Peace River (56.227, -117.447) (http://www.climate.weatheroffice.gc.ca/climateData/canada_e.html). All sites were within 100 km of the weather station, except one site located near Beaverlodge, AB which was ~165 km from the weather station.

Predictive risk assessment models – statistical analyses

All data were analyzed for normality and heteroscedasticity using visual techniques and Shapiro-Wilks tests (R Core Development Team 2011). When non-normal errors were observed, data were transformed with the appropriate transformation (male moth trap capture: $\ln(x+1)$, larval abundance: $\sin^{-1}\sqrt{x}$) (Zar 1999). Separate general linear models were used to test whether catches of male *C. deauratella* can be used to predict larval abundance and proportion

seed damage. The total number of male moths captured over the course of each season was specified as the independent variable and larval abundance or proportion seed damage was specified as the dependent variable. An overall model which combined years was evaluated, however, due to significant interactions (year*moths captured) each year was evaluated separately.

Secondly, general linear models were used to test whether larval abundance predicts proportion seed damage. Due to a significant interaction (year*larval abundance), models were generated separately for each year. Larval abundance was specified as the independent variable and proportion seed damage as the dependent variable. All predictive general linear models were fit using R 2.12.2 (R Core Development Team 2011).

Seasonal flight phenological models – statistical analyses

The number of male moths captured per site was converted to a cumulative proportion of total male *C. deauratella* captured over the season. A three-parameter non-linear logistic regression model was fit using the cumulative proportion of captured males as the dependent variable and degree days or ordinal date as the independent variable in JMP 10.0.2 (SAS Institute, Cary, NC, 2012). The logistic model is expressed by the following equation: $y = \frac{c}{(1+e^{(-a(x-b))})}$ where y is the proportion of males captured, x is either the cumulated $DD_{11.7}$ or the ordinal date, a is the growth rate, b is the inflection point, and c is the asymptote. If there were no significant differences in the models' parameters between years, data from all years were combined and an overall logistic regression model was created. The inflection point of the logistic regression is equal to the median point of male captures based on $DD_{11.7}$ and ordinal date and was used to estimate the flight period in which 50% of males had flown in each year.

Results

Predictive risk assessment models

In all three years of this study, thousands of moths were captured (Table 3-1) and there was a significant relationship between the number of male *C. deauratella* captured over the growing season and larval abundance (Table 3-2, Fig. 3-1). An increase in the number of male *C. deauratella* captured corresponds with an increase in larval abundance indicating that pheromone-baited traps can be used to assess population densities (Table 3-2, Fig.1). The strength of the relationships varied among years with the weakest relationship occurring in 2010, and the strongest in 2012 (Table 3-2).

There was also a significant relationship between the number of male moths captured and the proportion of seed damage sampled at each site in 2011 and 2012 but not in 2010 (Table 3-2, Fig. 3-2). There was also a significant relationship between larval abundance and the proportion seed damage in 2011 and 2012 but not in 2010 (Table 3-2, Fig. 3-3). Both relationships were stronger in 2012 than in 2011 (Table 3-2).

Seasonal flight phenological models

The number of accumulated $DD_{11.7}$ was variable over the ~10 week course of the experiments from June to mid-August in each year (Table 3-1). The parameters of the logistic regression models based on $DD_{11.7}$ did not differ significantly among years (Table 3-3) therefore an overall model which combined years was created (Table 3-3, Fig. 3-4). Based on the predicted accumulated $DD_{11.7}$ of the combined-year model, 258.39 $DD_{11.7}$ (95% C.I. 251.74-265.04) (Fig. 3-4) are needed for the median male moth flight to occur which corresponds with the 11-14 July in 2010, 24-26 July in 2011, and 14-16 July in 2012. No overall ordinal date model could be

created as there were large variations in model parameters among years (Table 3-3). There were significant differences in the ordinal date for the median male flight period between 2010 and 2011, and 2011 and 2012, but not 2010 and 2012 (Fig. 3-4). Based on individual ordinal date models, the actual ordinal date for median male flight was between 10-13 July in 2010, 23-26 July in 2011, and 13-18 July in 2012.

Discussion

In the current study, we develop a pheromone-based monitoring programme that can be used to create risk assessment and phenological flight models for *C. deauratella*. Risk assessment models examine the ability of pheromone-trap capture to predict larval abundance and proportion seed damage in the field. Male *C. deauratella* trap capture was positively related to larval abundance within each study year which suggests that pheromone-baited traps can monitor adult and larval population densities. Interestingly, the relationship between moth trap capture and larval numbers holds even at extremely high adult male densities at some sites (>25,000 moths at one site in 2011, and two sites in 2012) indicating pheromone trap captures can be useful even at outbreak population densities. This is in contrast to *Plutella xylostella* (Lepidoptera: Plutellidae) (Miluch et al. 2013) and *Malacosoma disstria* (Lepidoptera: Lasiocampidae) (Jones et al. 2009) in which trap captures decline at high population densities. When very few larvae were collected in 2011, the relationship between male *C. deauratella* trap capture and larval abundance was still strong (Table 3-1, 3-2). This indicates that pheromone-monitoring traps are sensitive to both low and high populations of *C. deauratella* and can predict larval abundance over a range of densities as occurs in *Lymantria dispar* (Lepidoptera: Lymantriidae) (Thorpe et al. 1993). These results are encouraging as high population densities in

other systems can cause predictive models based on pheromone trap capture to fail (Riedl and Croft 1974).

There was large variability in the relationship between the proportion of seed damage and the number of moths captured in pheromone-baited traps across years (Table 3-1, 3-2). The proportion of seed damage was lowest in 2011, followed by 2010 and 2012 (Table 3-1). There was no significant relationship between proportion of seed damage and moth capture in 2010 whereas moth capture was positively related to seed damage in 2011 and 2012 (Table 3-2). Larval abundance was also positively related to proportion seed damage in 2011 and 2012 (Table 3-2, Fig. 3-3). Based on larval models, 1 larvae/10 inflorescences can cause 0.034 proportion seed damage, and 1 larvae/inflorescence can cause 0.20 proportion seed damage. The seed damage found in Alberta (Table 3-1) was lower than the proportion of seed damage (0.064 ± 0.024 SE) in Ontario in 1990 (Ellis and Bjørnson 1996). The measure of seed damage used in the current and previous (Ellis and Bjørnson 1996) studies severely underestimates actual damage as it does not include the number of seeds that are completely consumed over the growing season. Pheromone-baited trap capture still indicates the potential damage in the field before the crop is harvested and the seed yield determined.

Average larval abundance ranged from 0.05 to 0.22 (1/20 to 1/4.6) larvae/inflorescence across years in this study, which is similar to <1/20 larvae/inflorescence sampled at most locations in southwestern Finland in 1959 (Markkula and Myllmaki 1960). In Finland >1/4 larvae/inflorescence was considered a severe infestation, which is similar to the average larval abundance of ~1/4.75 and ~1/4.5 larvae/inflorescence sampled in Alberta in 2010 and 2012, respectively. In all three years of this study, we never came across any sites where larval numbers were as high as those measured in Ontario in 1989-90 (1.0-2.4 larvae/inflorescence)

(Ellis and Bjornson 1996). In Alberta, the sites sampled in 2010 and 2012 had higher larval abundance and lower seed yield than in 2011 when larval abundance was quite low and seed yield high (Table 3-1). Although larval abundance in this study was generally lower than that recorded in Ontario (Ellis and Bjornson 1996), yield losses associated with this insect in Alberta (up to 99.5% loss) (Evenden et al. 2010) are greater than those reported from Ontario (80% loss) (Ellis and Bjornson 1996). This leads us to believe that other factors such as growing conditions may interact with *C. deauratella* feeding to contribute to red clover seed yield loss attributed to this insect.

The $DD_{11.7}$ model more consistently describes the median male flight period among years than the ordinal date model (Table 3-3). Because no significant differences were found among years based on a $DD_{11.7}$ development threshold, a combined model was created which predicts 258.39 $DD_{11.7}$ from 1 January need to accumulate for median male flight to occur each year (Table 3-3). No combined-year model could be created based on ordinal date as the model parameters in each year were significantly different. The cooler and wetter weather in 2011 resulted in the ordinal date for median male flight to be ≥8 days later in 2011 compared to the other study years (Table 3-1). The use of a degree-day model for *C. deauratella* male flight, which is based on physiological time and can vary with climatic conditions (i.e. temperature), is more reliable than ordinal dates, which are standard and do not differ with climatic conditions. The use of temperature and time to describe poikilotherm development has been recognized for several decades (Davidson 1944) and as a result many degree-day models have been created which are more consistent at predicting flight than ordinal date models (Potter and Timmons 1983, Riedl et al. 1976, Rice et al. 1984, Hoffman and Dennehy 1989). Being able to predict when adult eclosion and flight occurs can improve sampling effort for larvae. In the future, work

on the phenological model needs to be expanded to assess oviposition and larval stages in the crop in order to best time control tactics.

The variation between years in both risk assessment and phenological models may be due to several factors including competition with calling females, predation or parasitism, and environmental conditions (i.e. weather). Female calling at high population levels can compete with pheromone-baited traps as found in several species including *C. pomonella* (Reidl et al. 1976), and *Heliothis virescens* (Lepidoptera: Noctuidae) (Witz et al. 1992). However, if competition occurred between calling females and pheromone-baited traps the linear increase in trap capture which corresponds with larval abundance would be suppressed (Hillier et al. 2004). There was no suppression of the relationship between male *C. deauratella* trap capture and larval abundance which indicates females are not outcompeting pheromone-baited traps for males.

Mortality from predation and parasitism can also adversely affect relationships between adult male trap capture and larval abundance. The impact of predation and parasitism of eggs and early instar *C. deauratella* is unknown. Only one specimen of *Gelis tenellus* (Ichneumonidae: Hymenoptera) (NIS det lot. 2009-193) was reared from hundreds of overwintered *C. deauratella* larvae collected during the course of this study. These parasitoids likely have minimal impact on the larval abundance determined in this study because they appear to have low parasitism rates and do not emerge from larvae until the following year, thus any parasitized larvae sampled would still have been sampled and counted.

Environmental conditions (i.e. temperature, precipitation) can affect the relationships between moth trap capture and larval abundance and proportion seed damage. The average precipitation in 2011 was approximately three and two times greater than in 2010, and 2012,

respectively and there were fewer $DD_{11.7}$ in the growing season in 2011 compared to 2010, and 2012 (Table 3-1). The difference in precipitation probably contributed to the higher seed yield and lower larval abundance and subsequent damage in 2011 compared to the other years of the study (Table 3-1). Similarly, *Apion* spp. (Brentidae: Coleoptera) larval abundance in red clover inflorescences was lowered due to precipitation during the female oviposition period (Lundin et al. 2012). Water stress can also reduce red clover seed yield (Olivia et al. 1994), thus the reduced seed yields in 2010 and 2012 compared to 2011 may be partially attributed to water stress in addition to the impact of *C. deauratella* larval feeding. It does not appear that the wet weather led to poor pollination and subsequently lower seed yields, as occurred in Sweden (Lundin et al. 2012), as the wettest year (2011) had the highest seed yield (Table 3-1).

The use of both risk assessment and phenological models can be incorporated into different pest management strategies for *C. deauratella*. Control options can be appropriately timed using phenological models and the necessity for control can be determined with risk assessment models. Insecticides have yet to be tested in large scale applications against *C. deauratella* in Canada. As red clover is highly dependent on honey bees (*Apis mellifera* L.) and bumble bees (*Bombus* spp. Latreille) for pollination (Forester and Hadfield 1954, Holm 1966), any potential insecticide-control tactics need to be compatible with bee safety. The phenological and risk assessment models proposed here may be able to determine if control is necessary and time future low-risk or microbial insecticide applications to maximize *C. deauratella* exposure and minimize the impact on pollinators. Another control strategy, pheromone-based mating disruption, has proven to be an effective management tool for many lepidopteron pests (Cardé and Minks 1995) and has been successfully tested in clover to control *C. deauratella* (Mori BA, unpublished). This technique interferes with sexual communication between male and female

insects by flooding the cropping area with synthetic sex pheromone (Howse et al. 1998) while having minimal impact on non-target organisms. Mating disruption is usually applied before 5% of the flight occurs (Hardman 2012) so timing of application is less important than assessment of need of control using this expensive control tactic. Finally, inundative and classical biological control agents for *C. deauratella* are currently being researched for importation into Canada (Otani J, pers. comm.). All of these potential control tactics may benefit from the risk assessment and phenological models developed here to appropriately time their application/release.

This study contributes to the development of a pheromone-based monitoring programme to monitor flight phenology and predict the population density of *Coleophora deauratella* in red clover seed production fields in Alberta, Canada. Risk assessment models determined the number of male *C. deauratella* captured in pheromone-baited traps is positively related to larval abundance and proportion seed damage at moderate and high population levels and partially validates the models as monitoring tools. Future work should discern the relationship between trap capture and seed yield while accounting for various other factors that may affect it (i.e. temperature, precipitation, pollination services, etc.). The models presented here are most likely regionally specific and should be further validated before they are adopted for IPM programmes for *C. deauratella* in other areas of the world. In future studies, the phenological model could be expanded to include larval stages and may be used with the risk assessment model to time sampling and address possible control strategies for this devastating pest.

Table 3-1. Site characteristics and the number of *C. deauratella* larvae and adults sampled across all study years.

Variable	Year		
	2010	2011	2012
Total no. of sites (n)	8	7	6
Trapping Period (calendar date)	7 June - 17 Aug.	6 June - 16 Aug.	11 June - 19 Aug.
Trapping Period (ordinal date)	158-229	157-228	163-232
Total $DD_{11.7}$ accumulated from January 1*	457.7	348.6	498.0
Total precipitation accumulated from June 1 (mm)	136.3	447.2	234.2
Pheromone-baited trap sampling			
Total no. of male *C. deauratella* captured	38454	49589	99225
Mean no. of male *C. deauratella* captured per site	4807	7084	16538
Standard Error	1796	3151	7831
Larval Sampling			
Total no. of larvae	1245	153	1245
Total no. of inflorescence	5871	3125	4990
Larval abundance (larvae/inflorescence/site)	0.22	0.05	0.21
Standard Error	0.07	0.02	0.09
Seed Damage			
Mean proportion seed damage per site	0.042	0.016	0.043
Standard Error	0.006	0.006	0.018
Seed Yield			
Mean seed yield per site (kg/ha)	125.2¥	574.4†	329.4‡
Standard Error	9.9	80.5	38.5

* Total $DD_{11.7}$ accumulated from January 1st until the end of the last collection period each year. Seed yields reported by producers and are based on 3 sites in 2010 (¥), 4 sites in 2011 (†), and 6 sites in 2012 (‡).

Table 3-2. Relationships between pheromone-baited trap capture of adult *C. deauratella* males, larval abundance (larvae/inflorescence) and seed damage (proportion seed damage) in 2010, 2011 and 2012.

Year	Independent Variable (x)	Dependent Variable (y)	Regression	F	d.f.	P
2010	Adult males captured	Larval abundance	$\sin^{-1}\sqrt{y} = -1.13 + 0.19\ln(x+1)$	9.38	1,6	**0.022**
	Adult males captured	Seed damage	$y = -0.014 + 0.0070\ln(x+1)$	1.111	1,5	0.34
	Larval abundance	Seed damage	$y = 0.039 + 0.014\sin^{-1}\sqrt{x}$	0.22	1,5	0.66
2011	Adult males captured	Larval abundance	$\sin^{-1}\sqrt{y} = -0.42 + 0.073\ln(x+1)$	10.44	1,5	**0.023**
	Adult males captured	Seed damage	$y = -0.084 + 0.013\ln(x+1)$	10.46	1,5	**0.023**
	Larval Abundance	Seed damage	$y = -0.0077 + 0.13\sin^{-1}\sqrt{x}$	7.72	1,5	**0.039**
2012	Adult males captured	Larval abundance	$\sin^{-1}\sqrt{y} = -1.93 + 0.26\ln(x+1)$	39.03	1,4	**0.0034**
	Adult males captured	Seed damage	$y = -0.29 + 0.036\ln(x+1)$	35.20	1,4	**0.0041**
	Larval Abundance	Seed damage	$y = -0.0090 + 0.13\sin^{-1}\sqrt{x}$	14.56	1,4	**0.018**

P-values in bold indicate significance ($p < 0.05$).

Table 3-3. Non-linear logistic regression parameters for degree day and ordinal date phenological models.

Measurement Unit	Year	Parameters (95% C.I.)		
		Growth Rate (a)	Inflection Point (b)	Asymptote (c)
Degree Days	2010	0.032 (0.025-0.038)	252.80 (243.30-262.31)	1.00 (0.95-1.05)
	2011	0.043 (0.035-0.050)	261.85 (256.32-267.38)	1.03 (0.97-1.09)
	2012	0.021 (0.014-0.028)	269.01 (247.91-290.10)	1.02 (0.93-1.10)
	Overall	0.031 (0.026-0.035)	258.39 (251.74-265.04)	1.00 (0.96-1.04)
Ordinal Date	2010	0.17 (0.14-0.21)	192.98 (191.26-194.69)	1.01 (0.96-1.06)
	2011	0.15 (0.13-0.18)	206.21 (204.61-207.80)	1.01 (0.93-1.10)
	2012	0.15 (0.10-0.21)	196.94 (194.32-199.57)	1.01 (0.93-1.09)

Logistic regression model: $y = \frac{c}{(1+e^{(-a(x-b))})}$

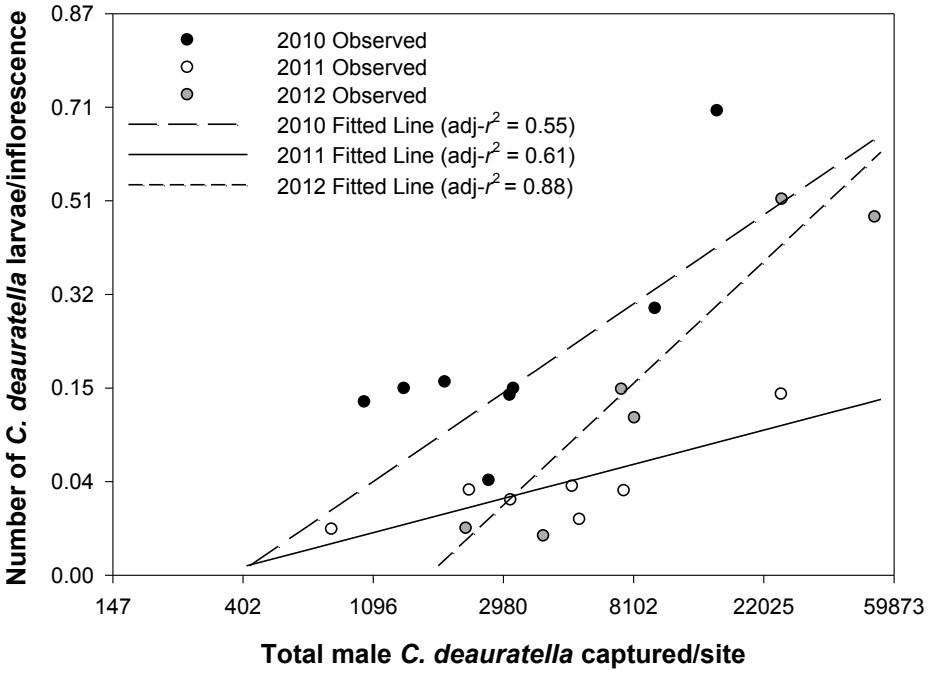

Figure 3-1. Relationship between season-long pheromone-baited trap capture of male *C. deauratella* and larval abundance (larvae/inflorescence) in 2010, 2011, and 2012. The axes have been back-transformed.

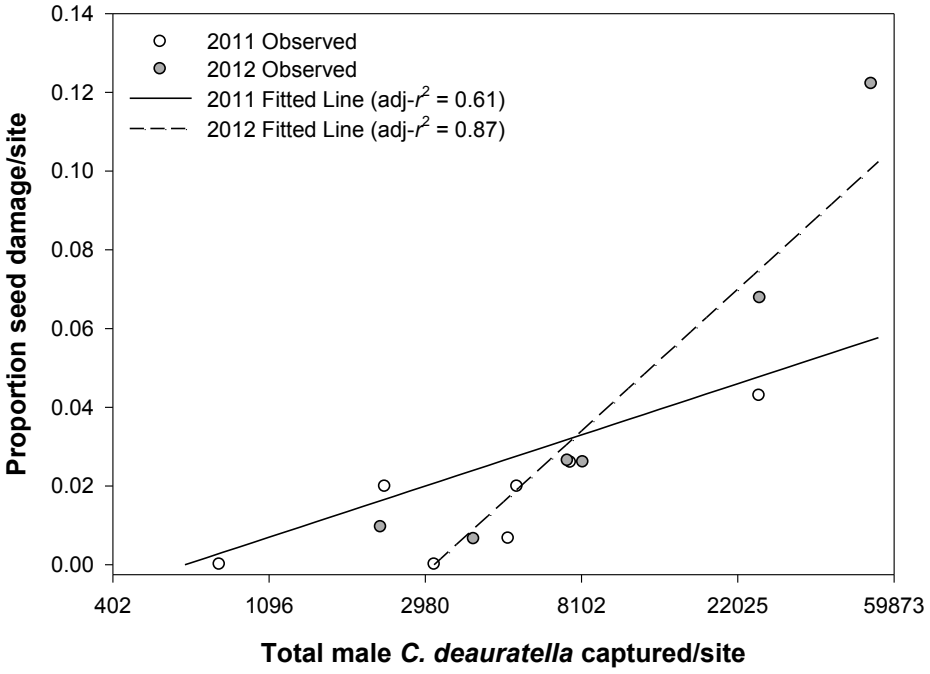

Figure 3-2. Relationships between season-long pheromone-baited trap capture of male *C. deauratella* and proportion seed damage in 2011 and 2012. There were no significant relationships in 2010. The axes have been back-transformed.

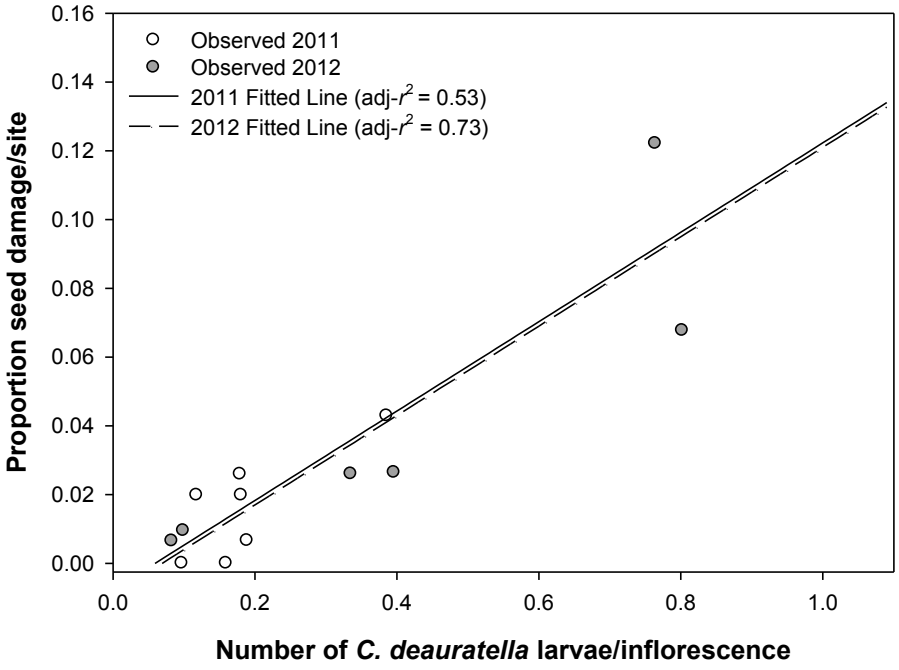

Figure 3-3. Relationships between *C. deauratella* larval abundance (larvae/inflorescence) and proportion seed damage in 2011 and 2012. There were no significant relationships in 2010. The axes have been back-transformed.

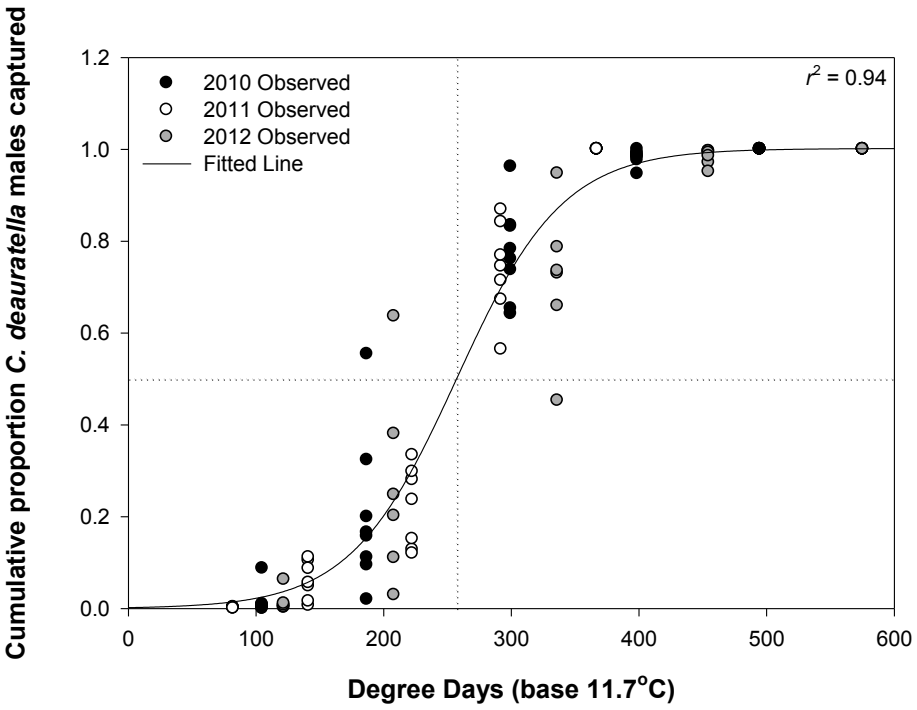

Figure 3-4. Cumulative trap capture of male *C. deauratella* in pheromone-baited traps during 2010, 2011, and 2012 plotted against degree days (base 11.7°C from January 1). The vertical line indicates the average degree days ($DD_{11.7}$) accumulated corresponding to the median (50%) male flight period over the flight season. Regression line: $y = \frac{1.00}{\left(1+e^{(-0.031(x-258.39))}\right)}$

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Chapter 4

Efficacy and mechanisms of communication disruption of the red clover casebearer moth (*Coleophora deauratella*) with complete and partial pheromone formulations

Introduction

Pheromone-mediated 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 behavior 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 (Epstein et al. 2006). The development of successful mating disruption

programs is sometimes hindered by a lack of knowledge on the mechanisms by which mate-finding behavior 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). False-trail following (competitive attraction) in which males orient to dispensers baited with synthetic pheromone rather than to calling females is the only competitive mechanism and requires an attractive pheromone blend (Bartell 1982, Miller et al. 2006). Non-competitive mechanisms can be invoked by the complete attractive pheromone blend, off-ratio blends or single components 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 formulations against various insect species, it may be useful to understand the mechanisms (competitive vs. non-competitive) by which the formulations act 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 strategies to control *C. deauratella* 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 (*Z*7-12:OAc) to (*Z*)-5-dodecenyl acetate (*Z*5-12:OAc) (Evenden et al. 2010) allows the potential for mating disruption to be explored. Evenden et al. (2010) found *C. deauratella* males were highly attracted to pheromone-baited traps with lures containing 100:10 µg of *Z*7-12:OAc to *Z*5-12:OAc (complete blend), whereas traps baited with 100 µg *Z*7-12:OAc (major component) captured very few moths. 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: Gelechiidae) (McCormick et al. 2012), do not need the complete pheromone blend to maintain effective disruption. As the major pheromone component alone is less attractive to *C. deauratella* males than the complete blend, 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 quantified 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 there is the potential for pheromone-based disruption of mating of *C. deauratella* and adds to the growing body of literature on the possible mechanisms that are invoked by treatment with complete and partial 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 each site was separated by at least 1 km. 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 center 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 *Z*7-12:OAc to *Z*5-12: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 of 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 hand-applied reservoir-type rope dispensers (hereafter, rope dispensers) in treated plots. In 2011, wooden stakes were placed in a uniform grid pattern at 500 stakes/ha in the treated plots only.

Pheromone dispensers and release rates

For the initial proof-of-concept experiment in 2010, rope dispensers (Shin-Etsu Chemical Co., Tokyo, Japan) were formulated to release the complete pheromone blend (10:1 ratio of *Z*7-12:OAc to *Z*5-12:OAc). In 2011, rope dispensers (Shin-Etsu Chemical Co.) were formulated to

release the complete pheromone blend as in 2010, and other rope dispensers were formulated to release the major component (*Z*7-12: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 dispensers were hung in a climate controlled chamber with continuous air flow (Contech Enterprises). Every 1-4 days, dispensers 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 active ingredients.

Experiment 1 – Proof-of-concept

In 2010, a small-plot proof-of-concept study was conducted to determine if treatment with the attractive complete 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 grid pattern 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, rope 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. Treatments 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 were 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).

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 at 500 dispensers/ha and the final plot was left as an untreated control. To determine if *C. deauratella* males landed near the 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 captured moths 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, releasing the complete pheromone blend, 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. The growth chamber 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. There was no effect of collection method or age on electroantennogram (EAG) responses of control *C. deauratella* males that were not pre-exposed to pheromone (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 rope 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, ropes 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. 4-1). Air was pumped through each treatment arm of the apparatus at 66 ml/min via the pressurized building air supply (Earth Sciences Building, University of Alberta. Edmonton, AB). Air was first filtered via an ARS charcoal filter (#ADS-STD-C2F, Analytical Research Systems Inc., Gainesville,

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 rope dispenser releasing the complete blend, a rope 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. 4-1). 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 ºC 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 *Z*5-12:OAc (98.6% chemical purity) (Bedoukian Research Inc., Danbury, CT, USA) serially diluted in HPLC grade hexane to obtain solutions between 1.0 x 10^{-6} µg to 1.0 µg/µl hexane. Fifty microliters 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 (>95% chemical purity, Aldrich Chemical Co., WI, USA) (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). Each 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 (see Appendix 4-1 to 4-3 for raw EAG dose response curves of male antennae stimulated 5 minutes after a 1 hour pre-exposure treatment).

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: $\ln(Release\ Rate) = m \times \ln(Day) + b$, where m = slope and b = y-intercept. The benefit of this equation is that the back-transformation equates to a power function ($y = ax^b$, where a = a constant and b = slope) and allows us to fit a line to the untransformed data using the equation: $Release\ Rate = e^b \times (Day)^m$ (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 nested generalized linear mixed-effects model was used with replicates nested within time period specified as a random effect 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 by 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 by 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 by pre-exposure 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

A power function curve was fit to the mean pheromone release rates from dispensers containing the complete blend and major component at 20 ºC and 30 ºC (20 ºC: complete blend dispenser $F_{1,12} = 246.8$, adj-$r^2 = 0.95$, $P < 0.001$, major component dispenser $F_{1,12} = 387.4$, adj-r^2

= 0.97, $P < 0.001$, 30 ºC: complete blend dispenser $F_{1,12} = 167.5$, adj-$r^2 = 0.93$, $P < 0.001$, major component dispenser $F_{1,12} = 188.9$, adj-$r^2 = 0.94$, $P < 0.001$, Fig. 4-2a,b). 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. The mean release rate of the major-component 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 plots treated with the full pheromone blend was reduced by 99.6 ± 0.2 % compared to that in untreated control plots ($\chi^2 = 113.7$, $df = 1$, $P < 0.001$) (Fig. 4-3a). 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, pheromone communication in *C. deauratella* was disrupted. Male moth captures in control plots were ≥ 3 408 compared to ≤ 31 in the pheromone-treated plots over the 14-day period of the second trial. Based on trap capture in control plots in the second trial, there was no evidence of contamination of crop foliage from treatments in the first trial.

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. 4-3b). Interestingly, pheromone-baited trap captures were lower in plots treated with the major component alone than in those positioned in plots

treated with the complete blend (Fig. 4-3b). Both treatments reduced pheromone-baited trap capture by > 99.9% (complete blend: 99.94 ± 0.06 %, major component: 99.98 ± 0.01 %).

The ability of *C. deauratella* to orient to and land near dispensers was also evaluated with yellow sticky cards, but due to wind damage, 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 ($\chi^2 = 44.2$, $df = 1$, $P < 0.001$) (Fig. 4-4). There was also a significant treatment by position interaction ($\chi^2 = 7.1$, $df = 1$, $P = 0.005$). Yellow sticky cards positioned below dispensers on the edge of the plots treated with the complete pheromone blend captured more moths than those in the interior of the same plot. Whereas, there was no difference in the number of moths captured on yellow sticky cards at the edge or interior of plots treated with the major component (Fig. 4-4).

Experiment 3 – Pheromone dispenser density

All three density treatments of dispensers releasing the complete blend significantly reduced the number of male *C. deauratella* captured in pheromone-baited traps compared to untreated controls ($\chi^2 = 783.5$, $df = 3$, $P < 0.001$ followed by Tukey's HSD test $P < 0.05$). No treatment effect was observed when the number of pheromone dispensers was doubled while the number of point sources was maintained constant (Fig. 4-5). Trap capture was reduced by > 99.9 ± 0.08 % in all treatment plots compared to untreated control plots, even at the lowest level of dispensers (256 dispensers/ha) tested in this study (Fig. 4-5).

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 4-1, Fig. 4-6). 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 5.0×10^{-5} µg to 0.05 µg as compared to moths exposed to clean air prior to measurement. There was no significant difference between both pheromone pre-exposure treatments and the control at the 0.5 µg stimulus dose (Table 4-1, Fig. 4-6). 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 4-1, Fig. 4-6).

After a 1 hour pre-exposure period followed by a 24 hour recovery period, there were no significant treatment or treatment by dose interaction effects on the normalized mV antennal response (treatment: $F_{2,26} = 1.0$, $P = 0.38$, treatment by 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 capture of male *C. deauratella* in pheromone traps in red clover seed production fields can be reduced by treatment with pheromone formulations releasing either the complete pheromone blend or the major component alone. Field trials combined with laboratory EAGs demonstrate that different mechanisms of disruption may occur when *C. deauratella* are exposed to the complete blend or major component. We conclude that both competitive and non-competitive mechanisms occur when the complete blend is used, whereas mainly non-competitive mechanisms occur when the major component is used.

In the initial proof-of-concept experiment, rope dispensers releasing the complete pheromone blend significantly reduced the number of males captured in pheromone-baited traps (Fig. 4-3a). 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). Treatment of pea fields with synthetic pheromone of *Cydia nigricana* (Fabricius) (Lepidoptera: Tortricidae) reduced male moth capture in pheromone-baited traps, but did not reduce damage in the field (Saucke et al. 2014). Ultimately, treatment of red clover fields with synthetic *C. deauratella* sex pheromone will only be successful if a reduction in female mating, oviposition and subsequent seed damage occurs.

This study shows that pheromone-based communication disruption of *C. deauratella* can occur 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. 4-3a). Since male and female *C. deauratella* emerge in approximately equal numbers around peak flight (Mori BA, 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. Non-competitive mechanisms are thought to drive treatment efficacy in the few instances when mating disruption is successful under high pest population pressures (Stelinski et al. 2008, Miller et al. 2006), thus when high population densities are present treatments should maximize non-competitive mechanisms.

Pheromone treatment with rope dispensers releasing either the complete pheromone blend or the major component results in a reduction in the number of males captured in pheromone-baited traps compared to that in the untreated control plots (Fig. 4-4). These results correspond with previous studies on *Agrotis segetum* Dennis and Schiffermüller (Lepidoptera: Noctuidae) (Svensson et al. 1995), *C. rosaceana* (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 disrupted pheromone-based communication to the same degree as more attractive complete pheromone blends. Interestingly, in this study, it appears communication is disrupted to a slightly greater degree in plots treated with dispensers releasing the major component than in those treated with the complete pheromone blend (Fig. 4-4). Similarly, Lapointe et al. (2009) found the single triene pheromone component of *P. citrella* was more successful at reducing male trap capture than the complete pheromone blend in small plot field trials. In other pest species, the major

component or off-ratio 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. There were large untreated areas of red clover surrounding each semiochemical-treated plot which may have acted as source populations from which males could have been attracted to the complete pheromone blend. Male *C. deauratella* were captured on yellow sticky cards positioned above dispensers releasing the complete pheromone blend in pheromone-treated plots and more males were captured on cards positioned at the edge of plots than the interior (Fig. 4-5). 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 less attractive 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 and determine if the major component is actually more successful at causing disruption than the complete blend.

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*. We are not aware if dispensers releasing the major component elicit false-

trail following behavior further downwind, but the lack of captures on yellow sticky cards indicate moths do not contact or land nearby the dispensers. 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, Löfstedt et al. 1985, Evenden and Gries 2010, Vacas et al. 2013), *C. deauratella* is a moth species that does not exhibit a strong dose response to pheromone (Evenden et al. 2010) and males are capable of flight to the high-release dispensers in this study. 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 captured in traps 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* outside the treated plot 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 numerous and overlapping plumes 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 occur if distinct pheromone plumes can be located, but upon subsequent exposure to pheromone other mechanisms such as adaptation/habituation or camouflage could occur. Given the high densities of *C. deauratella* in our plots and the lower number of moths that orient to dispensers in the interior of plots treated with the complete blend, non-competitive mechanisms may play a more prominent role in communication disruption than competitive mechanisms. Non-competitive mechanisms are more conducive to effective control of target insects at high population densities where competitive mechanisms alone generally fail (Miller et al. 2006, Reinke et al. 2014, Stelinski et al. 2008). As male moth capture on yellow sticky cards positioned below dispensers releasing the major pheromone component alone was low, we contend 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 may 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 ($5.0 \times 10^{-5} – 0.05$ μg) after one hour of exposure to either the complete blend or to the major component as compared to unexposed males (Fig. 4-6a). Twenty-four hours in clean air after the 1 hour pre-exposure treatment allows males to fully recover (Fig. 4-6b). Antennal response of moths that experience the hour-long pre-exposure is stronger to higher pheromone stimuli (0.5 – 50 μg) 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. 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 µg) compared to moths exposed to only the major component (Fig. 4-6a). 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 may not be adapted after exposure to the major component alone and thus there is a reduced shift in the threshold response of males exposed to the main component as compared to those exposed to the full pheromone blend (Fig. 4-6a). 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 ºC on day 1 was 4.19 and 3.53 mg/day, respectively, therefore, in the 1 hour pre-exposure period moths were exposed to ~180 µg and ~150 µg, 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 ~45.05 µg/l (45.05 mg/m^3) of the complete blend and ~37.54 µg/l (37.54 mg/m^3) of the major component. These concentrations are six orders of magnitude (mg/m^3 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 rope dispensers (333 dispensers/ha, 16.5g AI/ha) in a pea field and found 1.3 ± 0.5 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 hour 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. 4-4). There are two probable explanations for our findings. The first 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 complete 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). Recently, non-competitive mechanisms were found to dominate when rope dispensers releasing the complete pheromone blend of *G. molesta* were used in small-plot trials (Reinke et al. 2014). Reinke et al. (2014) observed male *G. molesta* orienting to dispensers but not traps and conclude that desensitization that did not first require attraction was the dominant non-competitive mechanism. The same non-competitive mechanisms could occur on *C. deauratella* in this study, as few moths were captured in pheromone-baited traps, however some still oriented to the dispensers based on yellow-sticky card capture.

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 occur 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. Competitive attraction, leading to landings near the dispenser, does not appear to be a main mechanism of mating disruption when the major component alone is used. Although males are capable of adaptation when exposed to the major pheromone component, males do not land nearby dispensers releasing the major component and therefore are less likely to be exposed to concentrations needed to cause adaptation (Fig. 4-6a). The excess major component in the atmosphere combined with intermittent adaptation may cause an imbalance in the perception of the ratios of pheromone components released by calling females and could 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 complete pheromone blend or the major component alone. This study demonstrates that the major component alone elicits a higher level of communication disruption than the complete pheromone blend. However, increased disruption with the major component alone may be an artefact of the small-plot design as males were attracted to plots treated with the complete blend, but not the major component. 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 that more mechanisms of disruption may be elicited when a complete pheromone formulation is used compared to the partial pheromone formulation, but the success of both formulations at high pest densities most likely depends on non-competitive mechanisms. The complete blend may act through both competitive and non-competitive mechanisms by initially attracting males through false-trail following, and then other mechanisms such as camouflage and adaptation likely occur. False-trail following leading to close range landing does not occur to the major component alone. Thus, the main mechanisms for the major component alone appear to be non-competitive. Adaptation can occur to the major component and may lead to sensory system imbalance, but we cannot rule out habituation or camouflage. 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 insecticidal-control options. However, the use of pheromone rope 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 labor costs, therefore other dispenser types (i.e. aerosol-emitting or flake dispensers) are currently under development for large-scale applications.

Table 4-1. 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.

Stimuli (μg)	*F*	d.f.	*P**
Hexane (Control)	1.83	2,25	0.18
0.00005	4.58	2,25	**0.02**
0.0005	6.87	2,25	**0.004**
0.005	7.43	2,25	**0.003**
0.05	4.69	2.25	**0.02**
0.5	2.00	2,25	0.16
5.0	4.96	2,25	**0.015**
50.0	5.01	2,25	**0.015**

* Bold *P*-values indicate $P < 0.05$. Significant results indicate treatment effects which were further separated by Tukey's HSD.

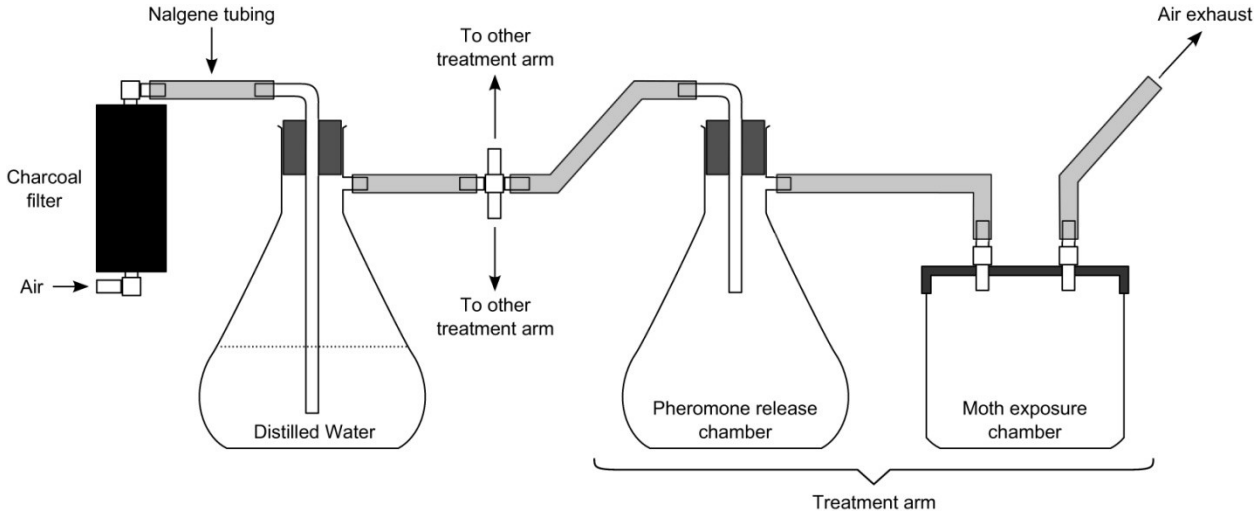

Figure 4-1. Pre-exposure apparatus: Air is pumped through a charcoal filter and distilled water in a 125 ml Büchner flask to humidify the air before being split into three treatment arms. In each arm of the apparatus, air travels at 66 ml/min and enters a 250 ml Büchner flask (pheromone release chamber) which contains one of three treatments, a rope dispenser releasing the complete pheromone blend, a rope 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.

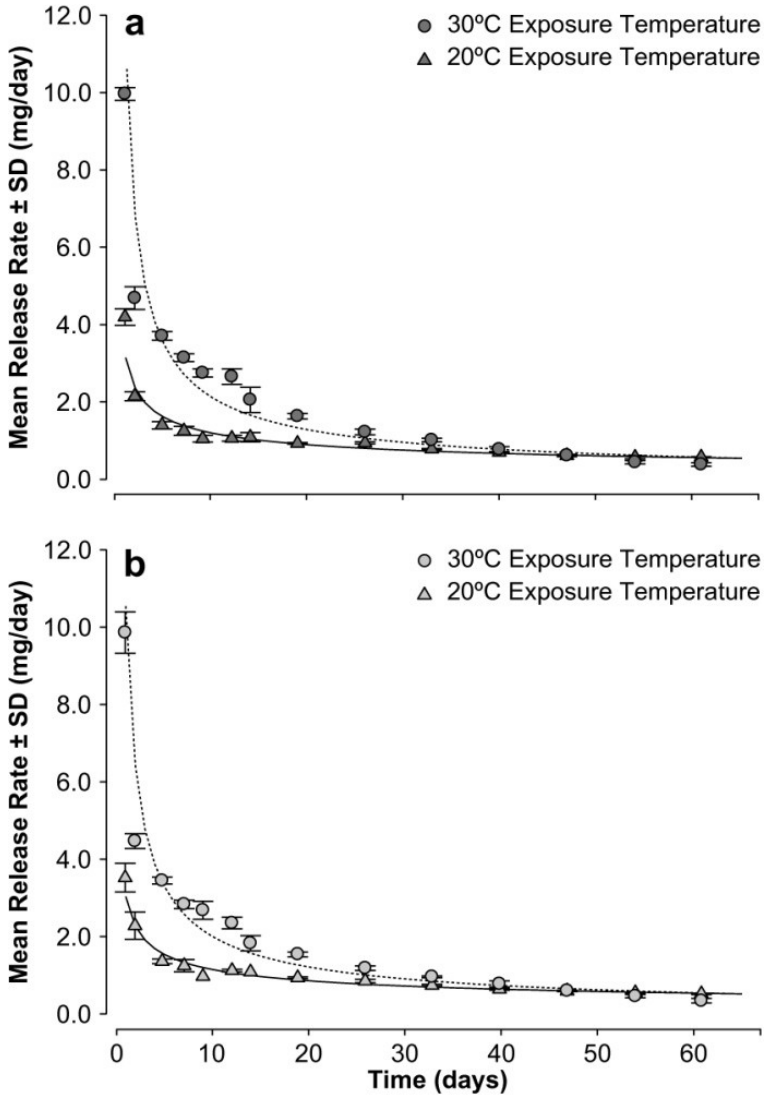

Figure 4-2. (a) Release rate ± S.D. (mg/day) of the complete pheromone blend from rope dispensers determined gravimetrically at 20.0 ± 0.1°C (triangle) and 30.0 ± 1.0°C (circle). Data are the mean value of three replicates. Best fit line at 30ºC (dashed): $Release\ Rate = 11.16 \times Day^{-0.72}$; best fit line at 20ºC (solid): $Release\ Rate = 3.14 \times Day^{-0.42}$ (b) Release rate ± S.D. (mg/day) of the major component from rope 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 30ºC (dashed): $Release\ Rate = 10.55 \times Day^{-0.72}$; best fit line at 20ºC (solid): $Release\ Rate = 3.04 \times Day^{-0.42}$.

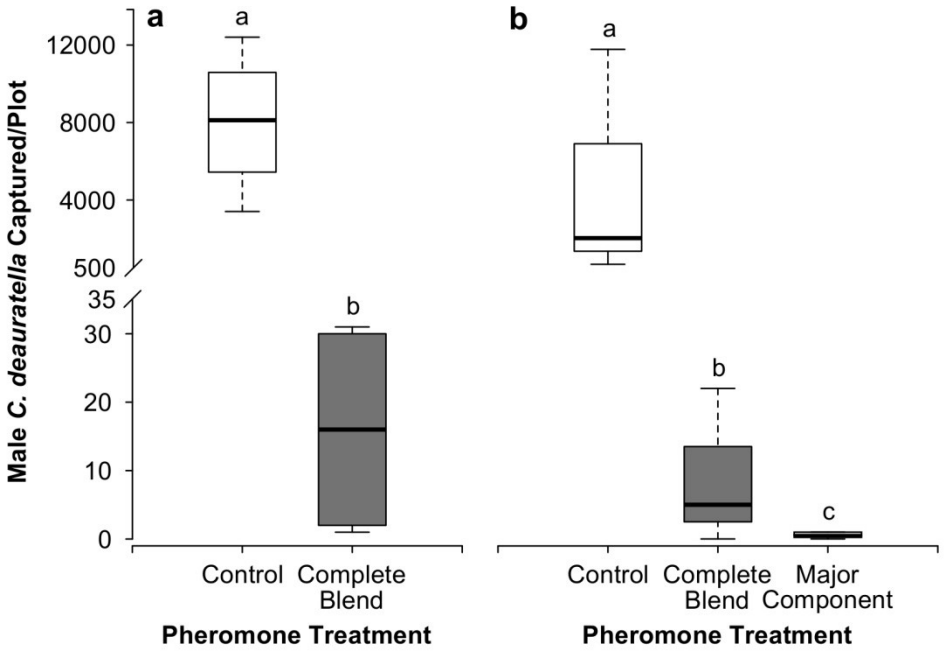

Figure 4-3. Box-and-whisker plot of the total number of male *C. deauratella* captured in pheromone-baited traps in untreated (control) and pheromone-treated (complete blend or major component) plots (30 June - 21 July 2010). The horizontal midline indicates the median. The top and bottom of the boxes 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 topped by different letters indicate significant differences (Tukey's HSD: $P < 0.05$) in moth capture among pheromone treatments (a) Experiment 1 (b) Experiment 2.

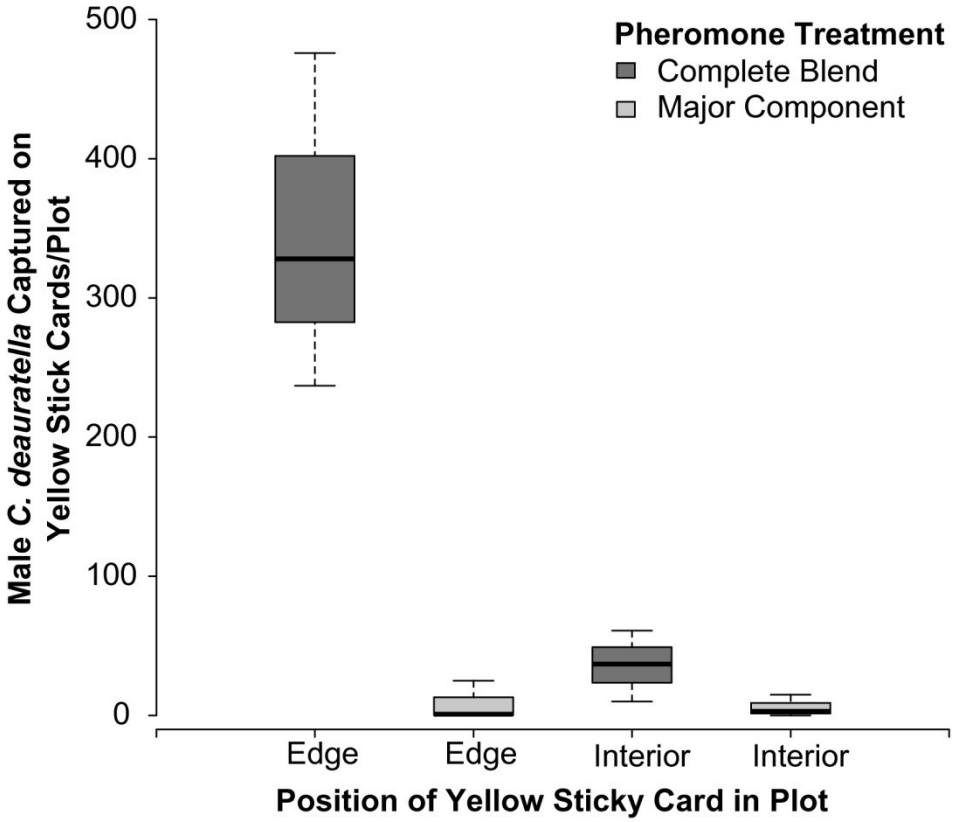

Figure 4-4. Box-and-whisker plot of the total number of male *C. deauratella* captured on yellow sticky cards placed below dispensers at the edge and interior of pheromone-treated (complete blend or major component) plots (27 June - 12 July 2011). The horizontal midline indicates the median. The top 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.

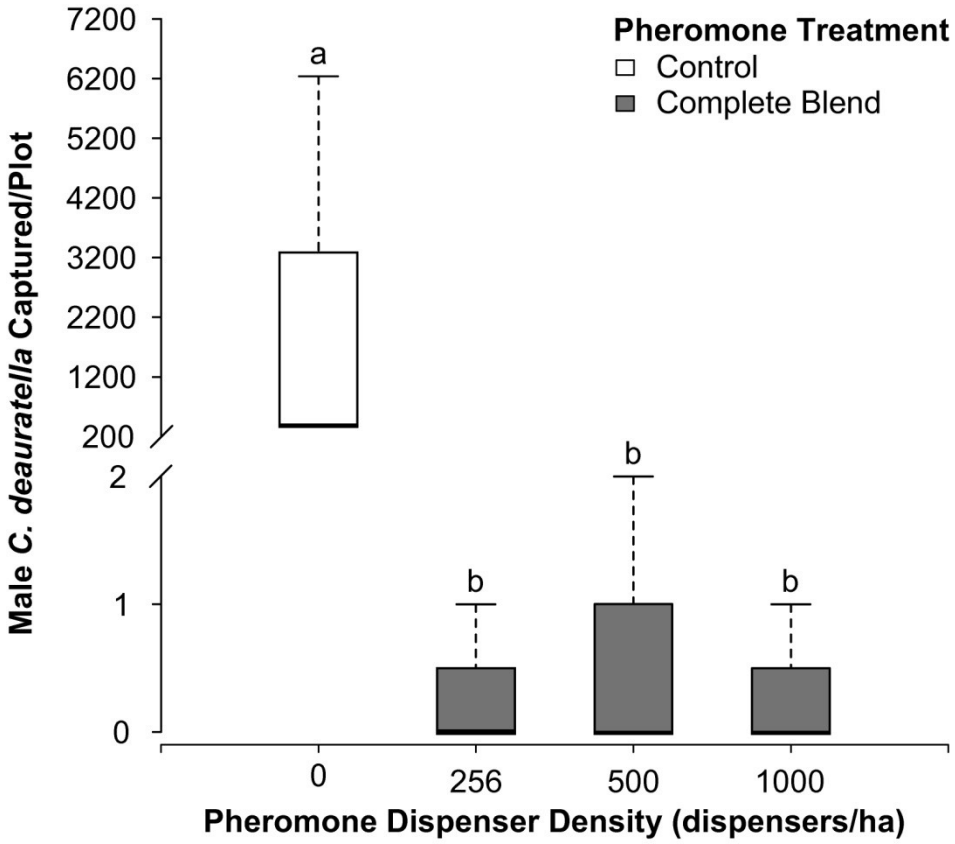

Figure 4-5. Box-and-whisker plot of the total number of male *C. deauratella* captured in pheromone-baited traps in plots containing various densities of rope dispensers releasing the complete pheromone blend (10:1 *Z*7-12:OAc:*Z*5-12:OAc) (18 July - 12 August 2011). The horizontal midline indicates 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 topped by different letters indicate significant differences (Tukey's HSD: $P < 0.05$) in moth capture among dispenser densities.

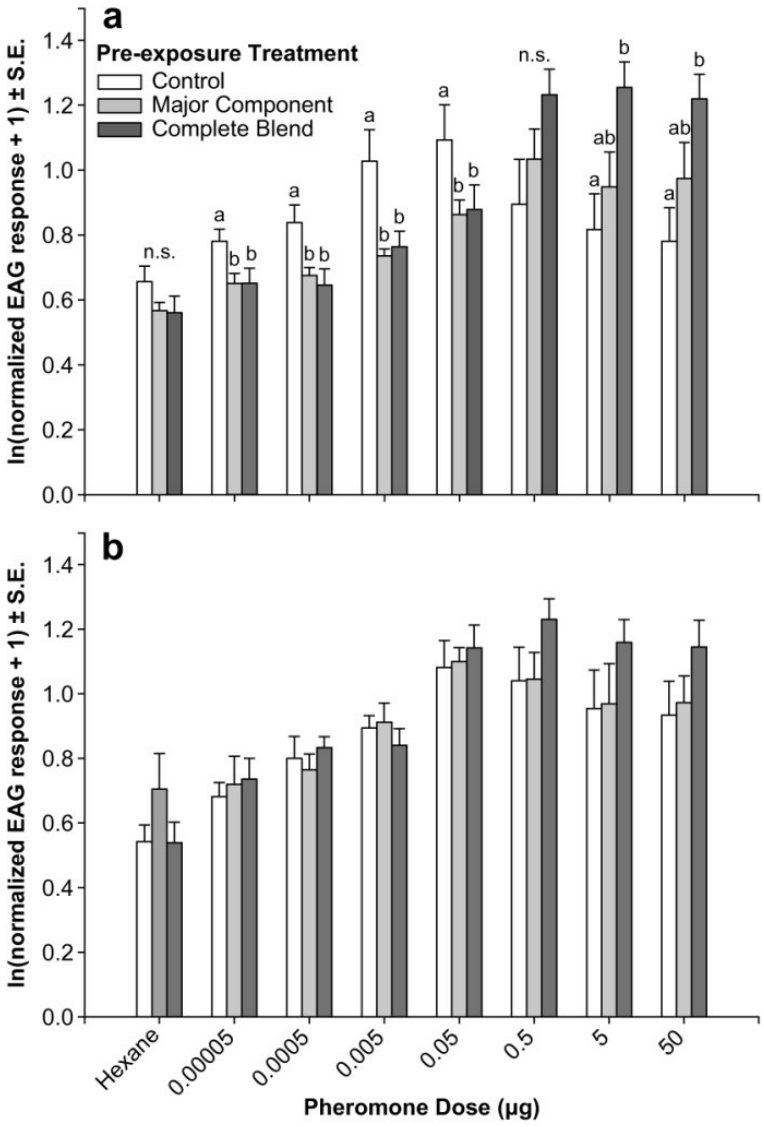

Figure 4-6. Mean (+SE) normalized EAG ($\ln(x+1)$) responses generated from excised antennae of male *Coleophora deauratella* stimulated with various doses of the complete pheromone blend (10:1 ratio of *Z*7-12:OAc to *Z*5-12:OAc) (**a**) 5 min and (**b**) 24 hr after a 1-hour pre-exposure treatment to either a rope dispenser releasing the complete pheromone blend, the major component alone, or a blank (clean air) control (*N*=10 antennae/treatment). Means topped by different letters indicate significant differences (Tukey's HSD: $P < 0.05$) in antennal response following a significant ANOVA.

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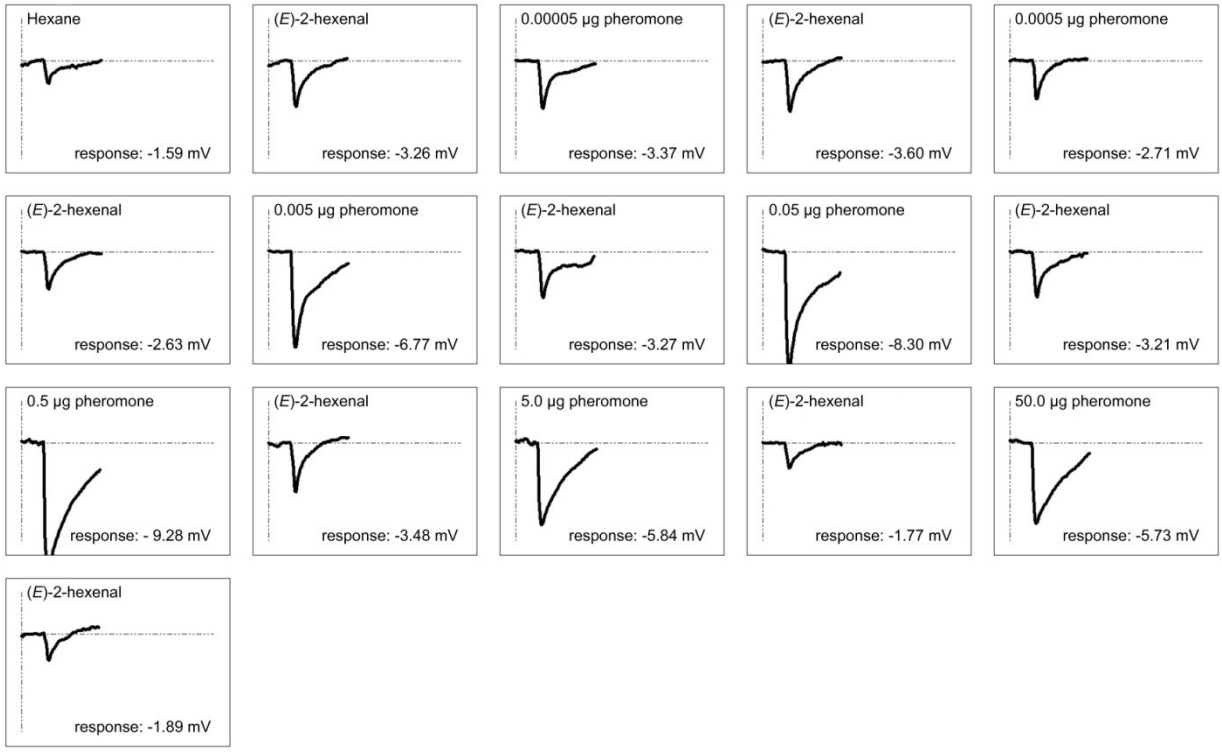
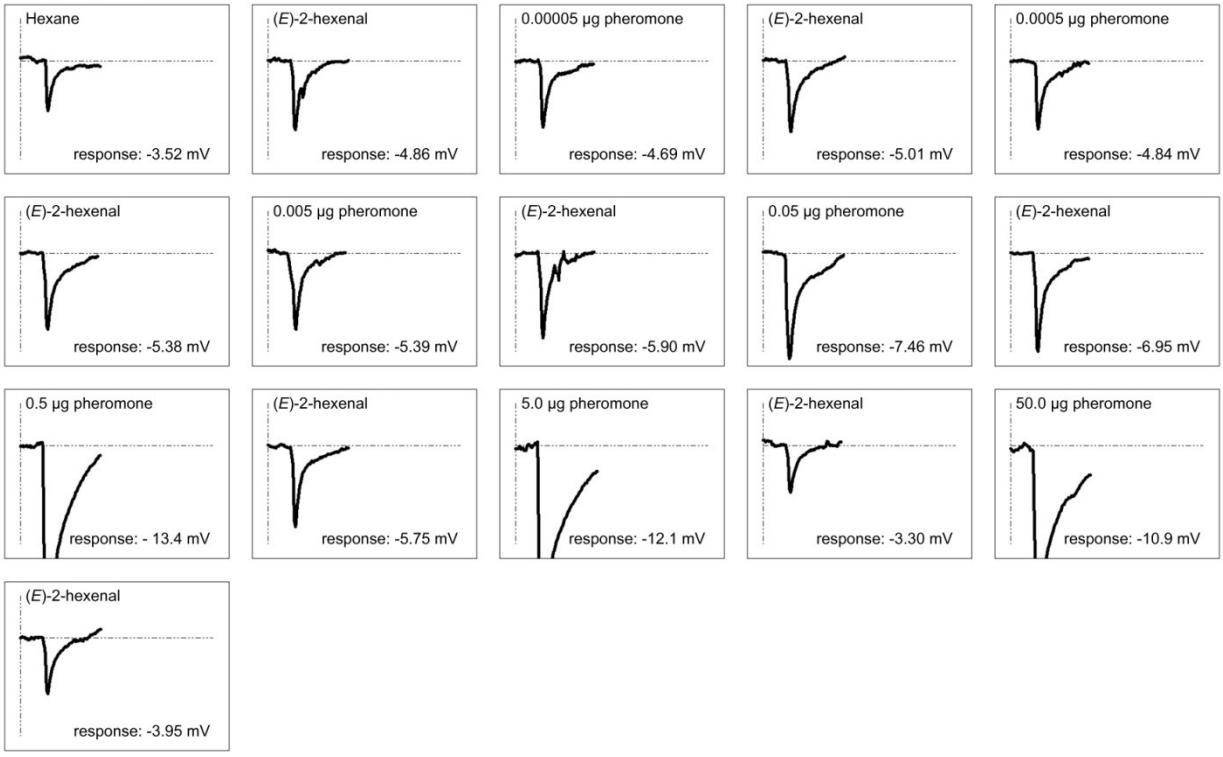
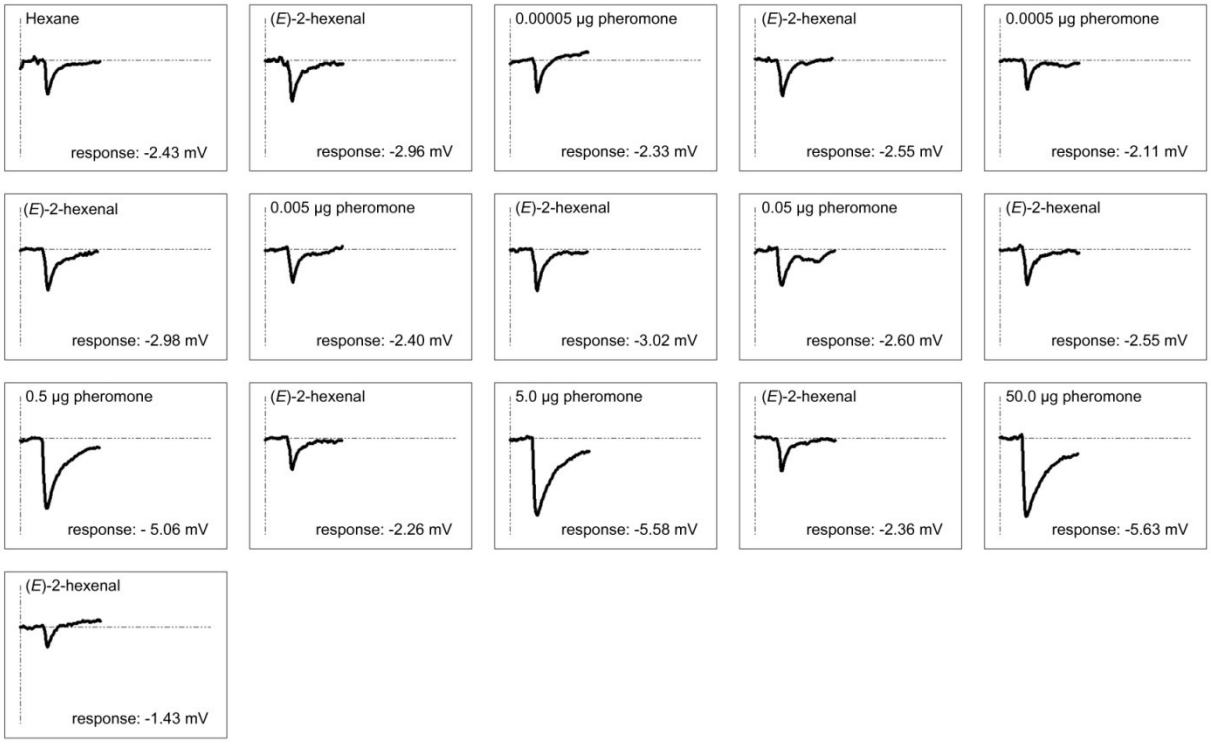


Appendix 4-1. Representative EAG traces for the series of stimuli presented to male *C. deauratella* antennae ($N = 10$) after a 1 hour pre-exposure to a blank (clean air control). The male antennae response shown here was from an individual tested on 7 July 2013.



Appendix 4-2. Representative EAG traces for the series of stimuli presented to male *C. deauratella* antennae ($N = 10$) after a 1 hour pre-exposure to a rope dispenser releasing the complete pheromone blend (10:1 *Z*7-12:OAc to *Z*5-12:OAc). The male antennae response shown here was from an individual tested on 7 July 2013.



Appendix 4-3. Representative EAG traces for the series of stimuli presented to male *C. deauratella* antennae ($N = 10$) after a 1 hour pre-exposure to a rope dispenser releasing the major pheromone component only (*Z*7-12:OAc). The male antennae response shown here was from an individual tested on 7 July 2013.

Chapter 5

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

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 reservoir-type rope, laminate flake, and aerosol-emitting dispensers. Hand-applied rope 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 rope 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 ropes, 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 light:dark

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 light:dark 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 cm^2) 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 g of active ingredients (AI) (10:1 *Z*7-12:OAc:*Z*5-12: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 ≥ 250 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 *Z*7-12:OAc:*Z*5-12: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. 5-1). 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. 5-1). Unitraps were placed throughout the plot at least 28 m from the nearest puffer and were positioned 35 cm above the soil surface (Fig. 5-1) (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. 5-1). 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. Plot nested within 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 χ^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 ($\chi^2 = 4.97$, df = 1, $P = 0.025$) with males emerging earlier in the emergence period than females (Fig. 5-2). 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. 5-3). 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. 5-3). 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/).

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. 5-4). 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. 5-5). 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. 5-5). 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. 5-6). 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 ($\chi^2 = 11.54$, df = 1, $P < 0.001$) (Fig. 5-6). There was no effect of pheromone treatment on seed yield ($\chi^2 = 0.0001$, df = 1, $P = 0.99$). 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

(Mori BA, unpublished). 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, MSTRS™) 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. 5-3). 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,b), 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 1996a, Burks and Brandl 2004). While other researchers apply a secondary treatment of rope 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,b). 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 ropes) 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*.

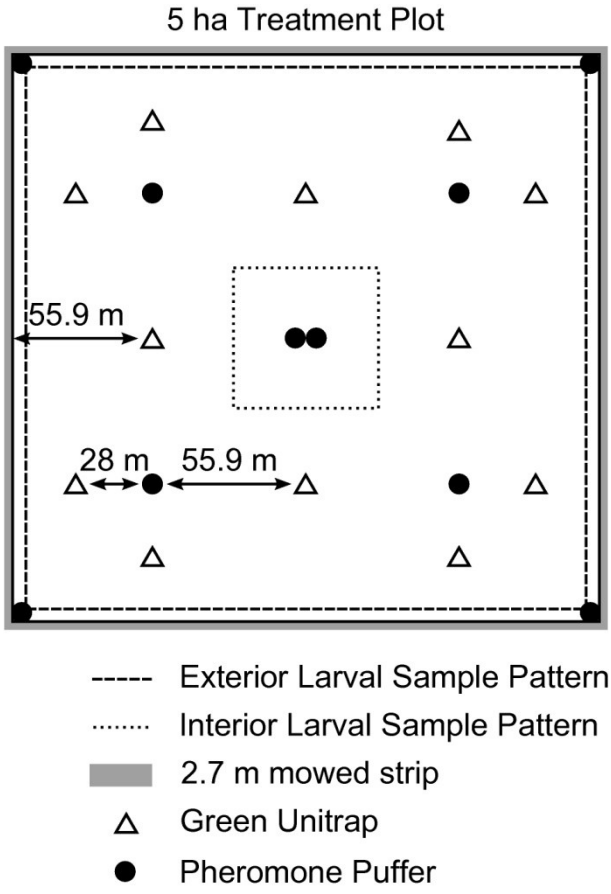


Figure 5-1. Large-plot pheromone puffer treatment layout indicating positions of pheromone puffers, unitraps, and larval sampling pattern. Control plots were laid out in the same way except no pheromone puffers were deployed. Plots were 5 ha (223.6 X 223.6 m).

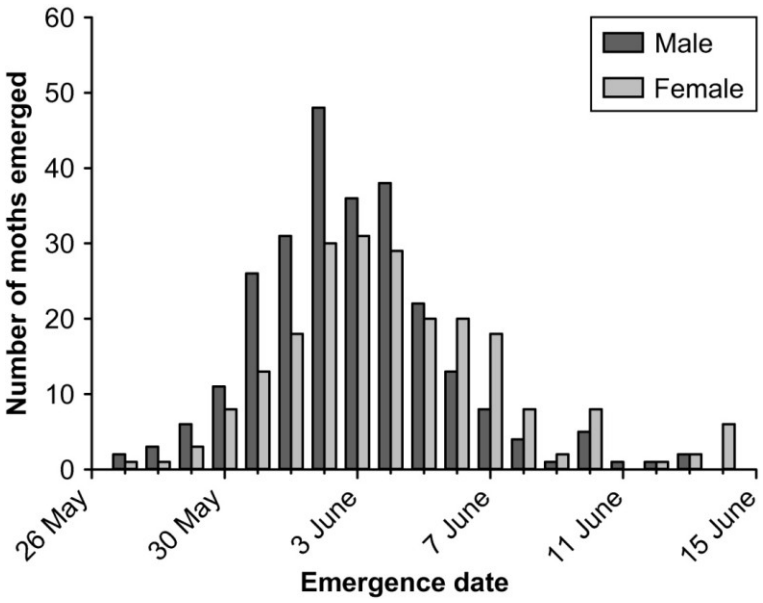


Figure 5-2. Emergence of *C. deauratella* males and females from field collected trash (leaf litter and stubble) in the laboratory. Bars indicate the total number of each sex that emerged each day. There was a significant sex by emergence day interaction ($P < 0.05$).

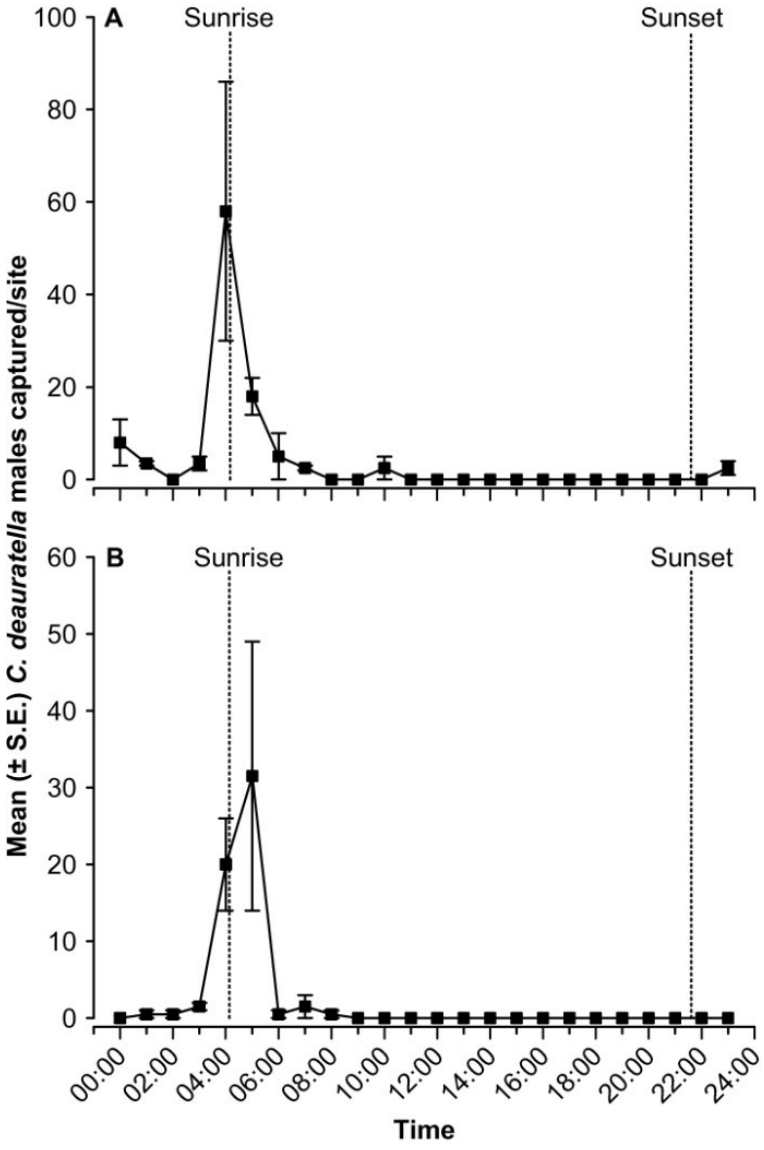


Figure 5-3. Periodicity of male *C. deauratella* over 24 h to pheromone-baited traps. A) Mean (± S.E.) number of male *C. deauratella* captured in green unitraps checked every hour on 29-30 June 2010 at two sites. B) Mean (± S.E.) number of male *C. deauratella* captured in wing traps checked every hour on 27-28 June 2011 at two sites. Vertical dashed lines represent sunrise and sunset.

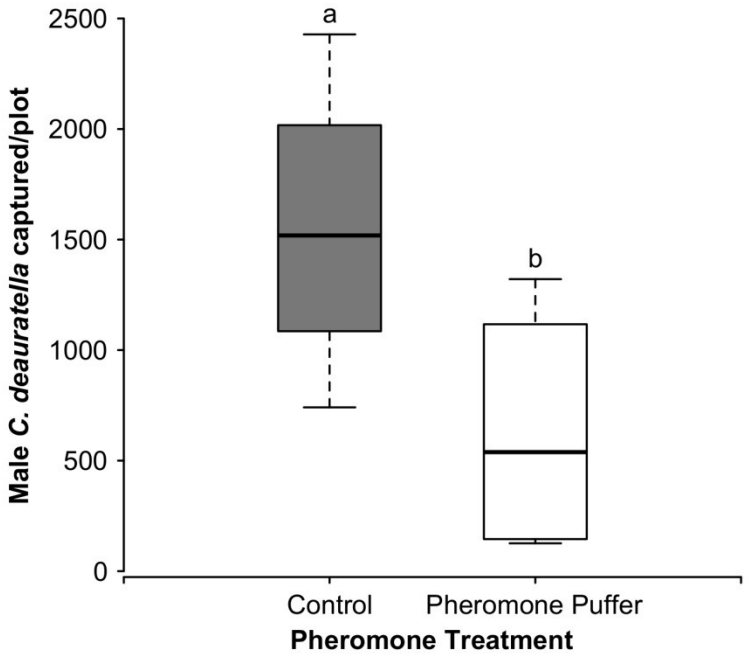


Figure 5-4. Box plot of the total number of male *C. deauratella* captured in control (grey) and pheromone-treated (white) plots in the proof-of-concept small-plot communication disruption trial. The bottom and top of the box represent the first and third quartiles, respectively, the midline indicates 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.

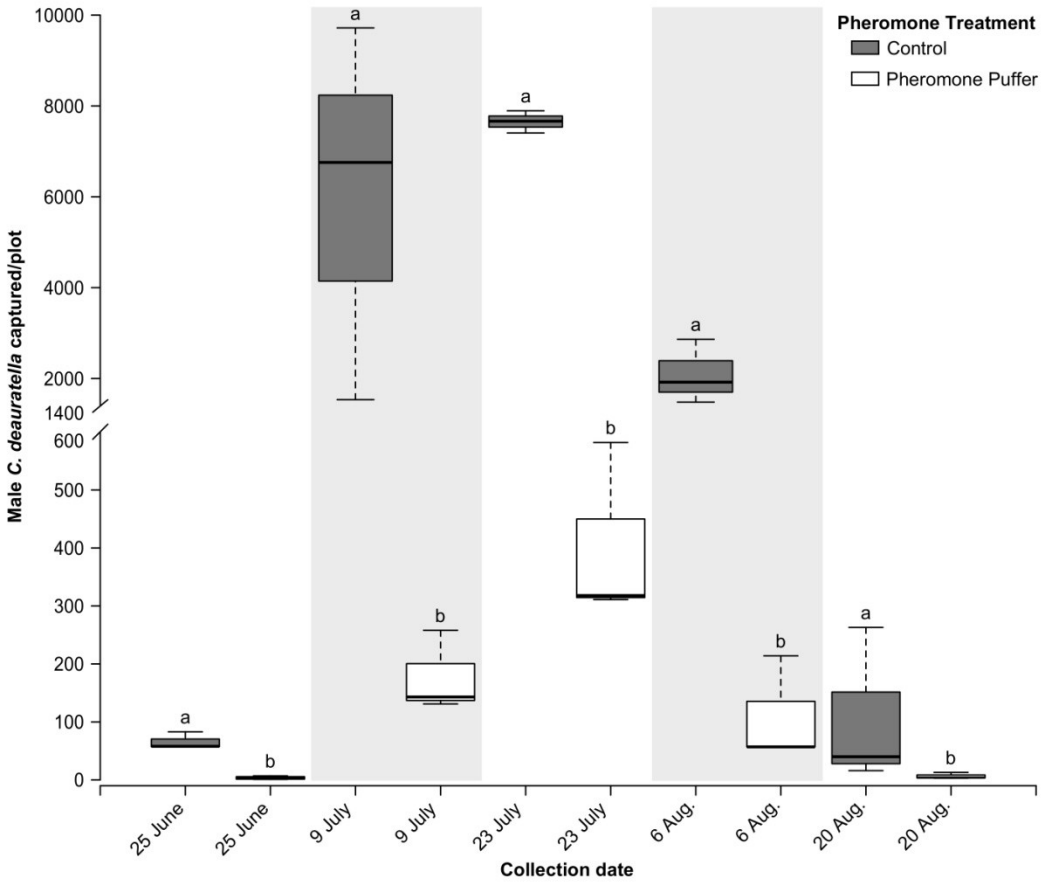


Figure 5-5. Box plot of the total number of male *C. deauratella* captured control (grey) and pheromone-treated (white) plots in the large-plot mating disruption study over the course of the season. The bottom and top of the box represent the first and third quartiles, respectively, the midline indicates 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. Grey shading behind the box plots is provided to aid in the visual interpretation of time periods.

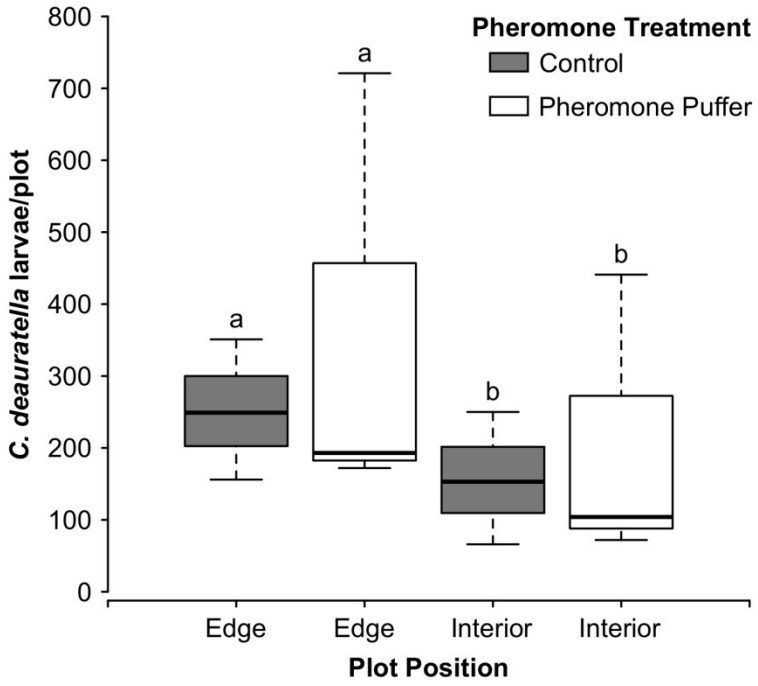


Figure 5-6. Box plot of the total number of *C. deauratella* larvae sampled at the edge and interior of control (grey) and pheromone-treated (white) plots in the large-plot mating disruption study. The bottom and top of the box represent the first and third quartiles, respectively, the midline indicates 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.

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Chapter 6

Mating disruption of *Coleophora deauratella* (Lepidoptera: Coleophoridae) using laminate flakes in red clover seed production fields

Introduction

Red clover, *Trifolium pratense* L. (Fabaceae), is an important temperate region forage and cover crop that 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 and Myllymäki, 1960, Ellis and 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 and Myllymäki 1960), however, in Canada *C. deauratella* infestations have led to >80% seed losses (Evenden et al. 2010, Ellis and Bjørnson 1996). Furthermore, *C. deauratella* was recently identified from the Willamette Valley in western Oregon (Mori BA, 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 and 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 and 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 mating disruption may have the potential to control *C. deauratella* as it targets the mobile adult stage by treatment of 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 meliferra* 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. 2006a). 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. 2006a). 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. 2006a). Mating disruption has been successfully developed for many lepidopteran field crop pests including *Sesamia nonagrioides* Lefèbvre (Noctuidae) (Albajes et al. 2002), *Cydia nigricana* 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. 2008a, 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 (*Z*7-12:OAc) to *Z*-5-dodecenyl acetate (*Z*5-12:OAc)) are used to test if pheromone-mediated 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 pheromone-treatment can cause mating disruption 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. (2006a) 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 formulations

Hercon Disrupt Micro-Flake® dispensers (Hercon Environmental, Emigsville, PA) (hereafter flakes) were formulated to contain 9.5 % active ingredients (complete pheromone blend: 10:1 *Z*7-12:OAc to *Z*5-12: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 AI/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 *Z*7-12:OAc and 10 µg *Z*5-12:OAc.

Study sites

All studies were conducted on red clover (*T. pratense* 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 cm^2) 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 and 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, directly on the crop, by hand evenly throughout the plot. No adhesive sticker additive was used to apply the flakes to the crop. 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 $\%\ inhibition\ =\ \left(\frac{C-T}{C}\right)\times\ 100\%$ 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 dominant 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 applied directly to the crop without an adhesive sticker additive. 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 ≥ 250 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. The lack of red clover fields in the same vicinity and with similar *C. deauratella* population densities, planting dates and phenology necessitated a study design with both control and treated plots within the same field. 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. 6-1). At each site, one plot was randomly designated the treatment plot and received 1400 g of flakes (280 g/ha) spread evenly, directly to the crop, by hand throughout the plot. No adhesive sticker additive was used to apply the flakes to the crop. 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 were checked for normality with visualization plots and Shapiro-Wilks tests. General linear mixed-effects models (GLMM) were used when the data were normally distributed. When the data were non-normal generalized-linear mixed-effects models specifying a Poisson error distribution were used (Package *lme4*, Bates et al. 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 repeated-measures 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 repeated measure. 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 *Z*7-12:OAc and *Z*5-12:OAc remaining on the flakes and time in the field (SigmaPlot 12.0, Systat Software, San Jose, CA).

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 ($\chi^2 = 51.3$, df = 4, $P < 0.001$) (Table 6-1). 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 6-1). 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 6-1).

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. 6-2). Trap capture in control plots in the first two-weeks of the experiment (mean ± SE, 1742.7 ± 782.8 moths/plot), which corresponded with peak *C. deauratella* flight (Mori BA, personal observation) 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 (χ^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. 6-3a; Table 6-2).

Response profiles of male *C. deauratella* captured and flake density (Fig. 6-3a-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. 6-3a), moth capture decreased asymptotically with the shape of an inverse function. On the Miller-Gut plot (Fig. 6-3b), 1/moth capture increased linearly with increasing flake density and on the Miller-de Lame plot (Fig. 6-3c), 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 (χ^2 = 15361, df = 1, P < 0.001) (Fig. 6-4). The reduction in trap capture decreased over the course of the season (Table 6-3). There was also a significant reduction in the number of larvae sampled in the pheromone-treated plots compared to the untreated controls (χ^2 = 12.5, df = 1, P < 0.001) (Table 6-4), but there was no significant effect of sample position (χ^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 (χ^2 = 3.1, df = 1, P = 0.079) (Table 6-4).

Experiment 5 – Field-aged pheromone flake release rate

The total percent AI and the percent of *Z*7-12:OAc and *Z*5-12: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; *Z*7-12:OAc: $\% AI = 8.17 \times e^{-0.012day}$, $F_{1,5}$ = 111.0, P = 0.0005, r^2 = 0.97; *Z*5-12:OAc: $\% AI = 0.86 \times e^{-0.014day}$, $F_{1,5}$ = 81.5, P = 0.0008, r^2 = 0.95) (Fig. 6-5). 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 6-3). The estimated release rate decreased as the season progressed (Table 6-3) 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 flake dispensers releasing the complete pheromone blend of *C. deauratella*. 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 1981, 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 1981). In large-plot trials in the current study, there was a significant disruption of male moth orientation to traps and subsequent larval densities were reduced in pheromone-treated plots. There was also 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 ± 0.3 % to a low of 38.3 ± 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 an application rate of 25.8 g AI/ha (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. 2008a). 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 pheromone-treated plots which has been attributed to immigration of mated females (Knight 1995, Gut and Brunner 1998) or lower pheromone concentration at the edge of pheromone-treated plots (Ogawa 1990, Karg and Sauer 1995). 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 aerosol dispensers 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. 6-3). The disruption profiles (Fig. 6-3a-c) match the theoretical predictions for competitive mechanisms (Miller et al. 2006a). The untransformed plot is concave and approaches zero asymptotically (Fig. 6-3a); the Miller-Gut plot increases linearly (Fig. 6-3b); and the Miller-de Lame plot decreases linearly (Fig. 6-3c). 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, however, if non-competitive mechanisms were invoked, males would not approach dispensers in pheromone-treated fields (Miller et al. 2006a,b). Competitive attraction is also considered a plausible explanation for the mechanism that disrupts *Cydia pomonella* L. (Lepidoptera: Tortricidae) when flakes were used (Stelinski et al. 2008b). Interestingly, mating disruption via competitive attraction is thought to be weak with high population densities (Baker and Heath 2005), yet it was moderately successful with high densities of *C. deauratella*. Further studies should

determine if non-competitive mechanisms, in addition to competitive attraction, occur on *C. deauratella* as non-competitive mechanisms are thought to be invoked when mating disruption is successful at high population densities (Miller et al. 2006a, Stelinski et al. 2008a).

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. 6-5), 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 of male trap capture inhibition was 96.8 ± 1.1%.The maximum male catch (C_{max}), given by the y-intercept in the Miller-de Lame plot (Fig. 6-3c) (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 = area (ha) over which one flake can theoretically reduce C_{max} by 50% (Miller et al. 2006a), is equal to the absolute value of the slope of the Miller-de Lame plot (Fig. 6-3c) 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 $D\bar{A}a = D_a \times D_D$ 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 $D_{\bar{A}a}$ for *C. deauratella* in this study is

495, which is over two-fold higher than any $D_{\bar{A}a}$ 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. molesta* 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 $D_{\bar{A}a}$ (194) are lower than the results of the current study. Recalculation of the maximum $D_{\bar{A}a}$ in the *G. molesta* study using the number of dispensers from the current study (76,204), results in a larger $D_{\bar{A}a}$ (541) than was calculated for *C. deauratella* in the current study. This corresponds with a higher level of mating disruption in the *G. molesta* study than was achieved for C. *deauratella* in this study (99.4% inhibition of males in *G. molesta* (Stelinski et al. 2005), 96.8% inhibition of *C. deauratella* observed in this study). Interestingly, the maximum male catch in untreated controls (C_{max}) was only 5.1 males/trap/night in the *G. molesta* 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 $D_{\bar{A}a}$ 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 $D_{\bar{A}a}$ 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, $D_{\bar{A}a}$ = 495) is more successful than that of *P. gossypiella* (88% inhibition, $D_{\bar{A}a}$ = 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 Experiment 3. If one flake can disrupt 50% of C_{max} over 65 m^2 (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. 6-3c, 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. 6-3a. 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 as there is a reduction in larval numbers and an increase in seed yield in large pheromone-treated plots. Furthermore, we determine that flakes can disrupt *C. deauratella* competitively based on 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.

Table 6-1. Mean male *C. deauratella* captured per trap in the point source attractiveness experiment.

Dispenser or Lure Type	Mean (± SE) male *C. deauratella* captured/trap
Blank	2.0 ± 0.8^{a}
One Pheromone Flake	248.2 ± 46.9^{b}
Five Pheromone Flakes	191.0 ± 25.1^{b}
Ten Pheromone Flakes	179.7 ± 32.3^{b}
Contech Grey Rubber Septa Lure	196.0 ± 46.7^{b}

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

Table 6-2. Flakes/ha and the disruption index for the various flake densities used across the eight week flake density experiment.

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

Table 6-3. Large-plot mating disruption trials disruption indices and estimated release rate over the duration of the season.

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

Table 6-4. Larval numbers and seed yield for the large-plot mating disruption trials**.**

Treatment	Mean (±S.E.) larvae/plot[¥]	Mean (±S.E.) seed yield (kg/ha)
Control	121.3 ± 14.8[a]	594.9 ± 126.3[a]
Pheromone Flakes	57.3 ± 12.7[b]	652.9 ± 159.3[a]

Means in the same column followed by a different letter indicate significant differences ($P < 0.05$). ¥ 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.

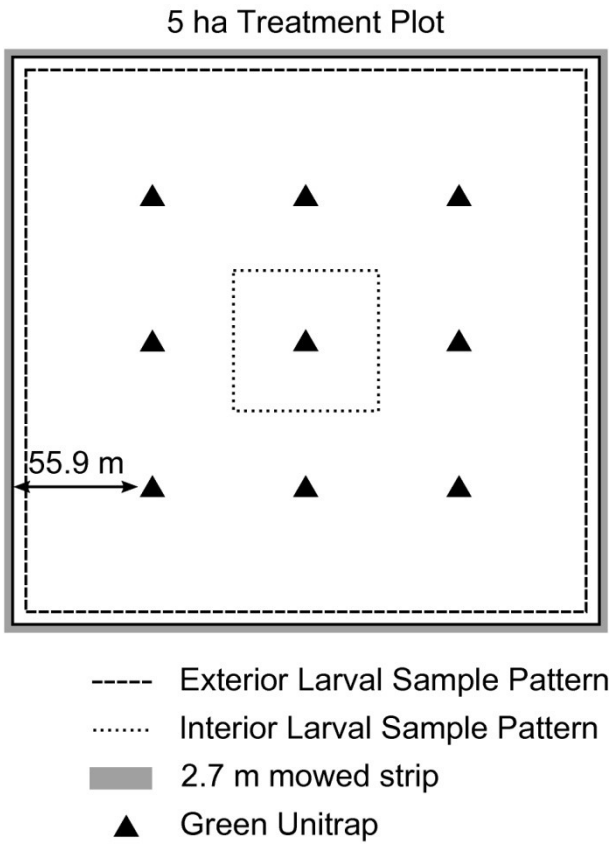

Figure 6-1. Large-plot pheromone disruption experimental design indicating position of pheromone traps, and larval sampling positions. Control plots were the exact same design, but received no pheromone flakes (0 g/ha). Plots are 5 ha in size (223.6 X 223.6 m).

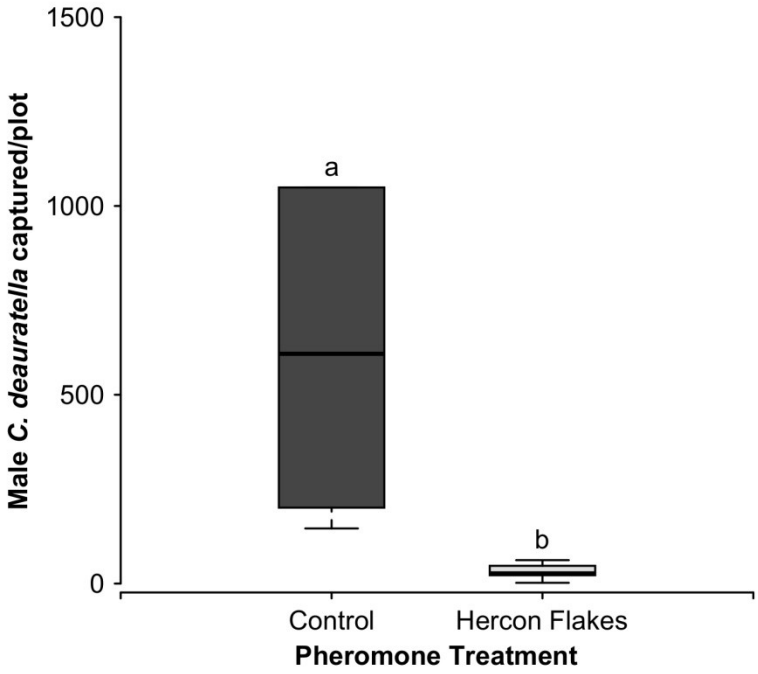

Figure 6-2. Box plot of the total number of male *C. deauratella* captured in assessment traps positioned in control (grey) and pheromone-treated (white) plots in the small-plot proof-of-concept experiment. The midline indicates the median and the bottom and top of the box represent the 25th and 75th 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.

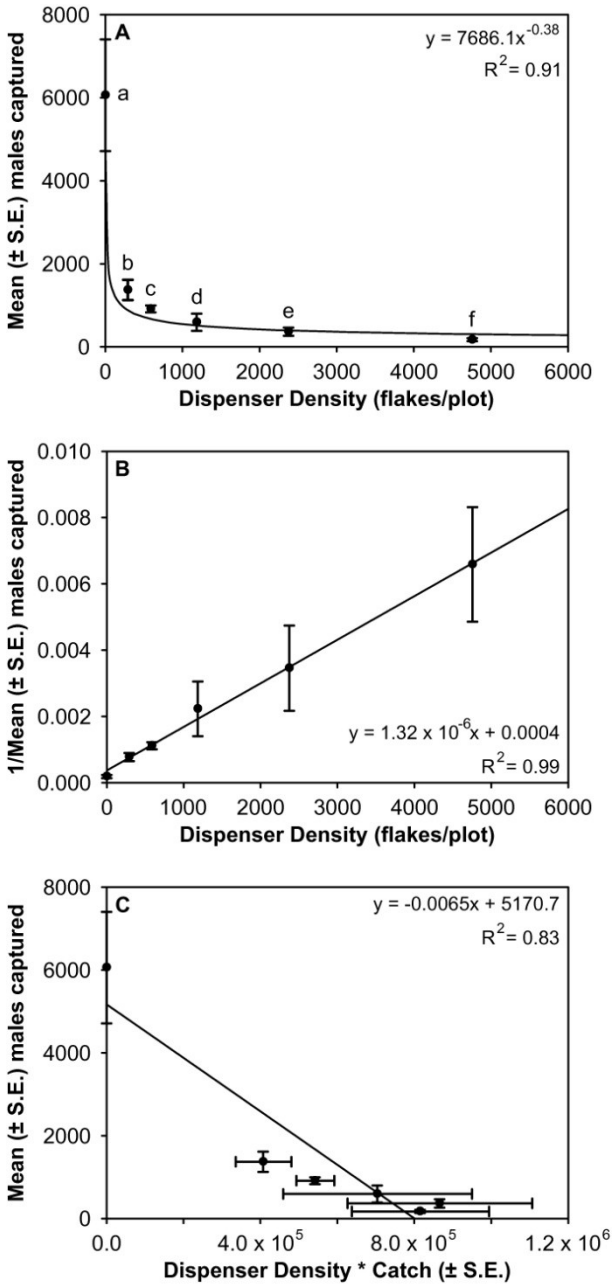

Figure 6-3. Disruption profiles (Miller et al. 2006a) for the flake density experiment. (A) Untransformed plot of male *C. deauratella* captured/plot by dispenser density. Letters by means indicated significant differences based on a generalized-linear mixed-effects model specifying a Poisson distribution 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.

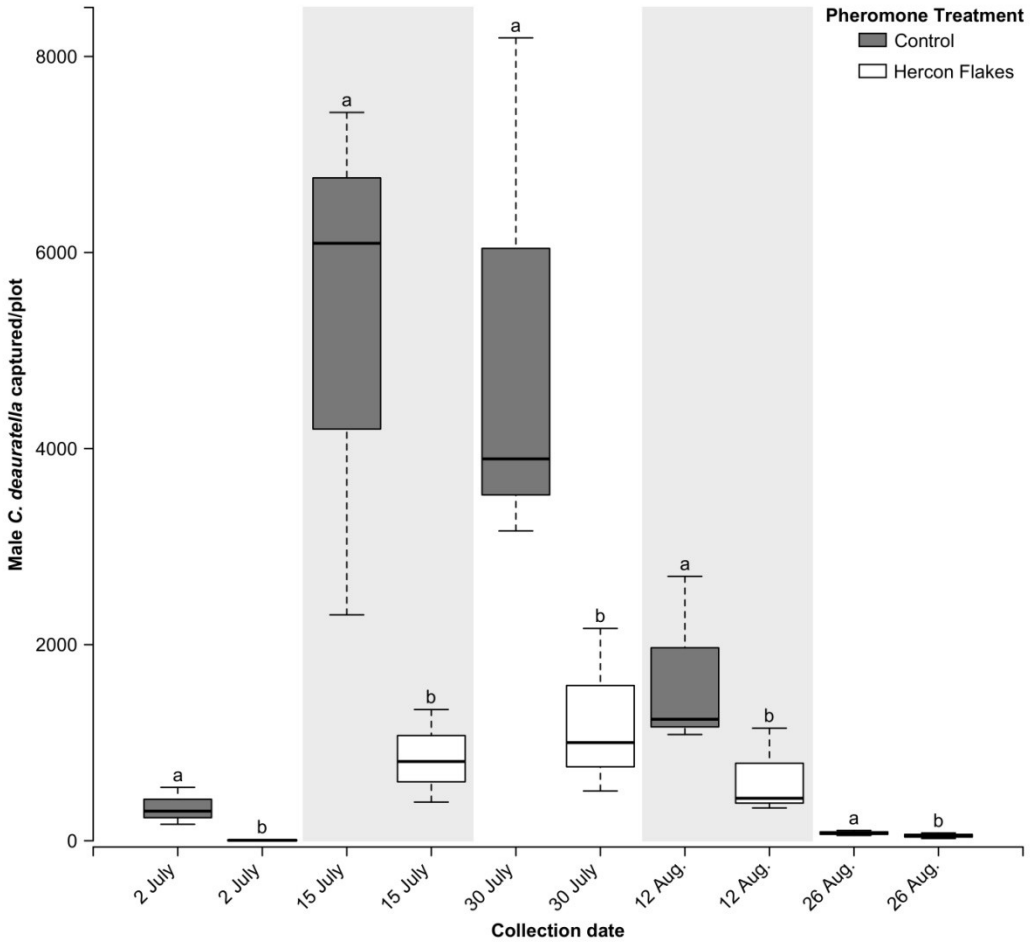

Figure 6-4. Box plot of the total number of male *C. deauratella* captured in assessment traps positioned in control (grey) and pheromone-treated (white) plots in the large-plot season long mating disruption experiment. The midline indicates the median and the bottom and top of the box represent the 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. Grey shading behind the box plots is provided to aid in the visual interpretation of time periods.

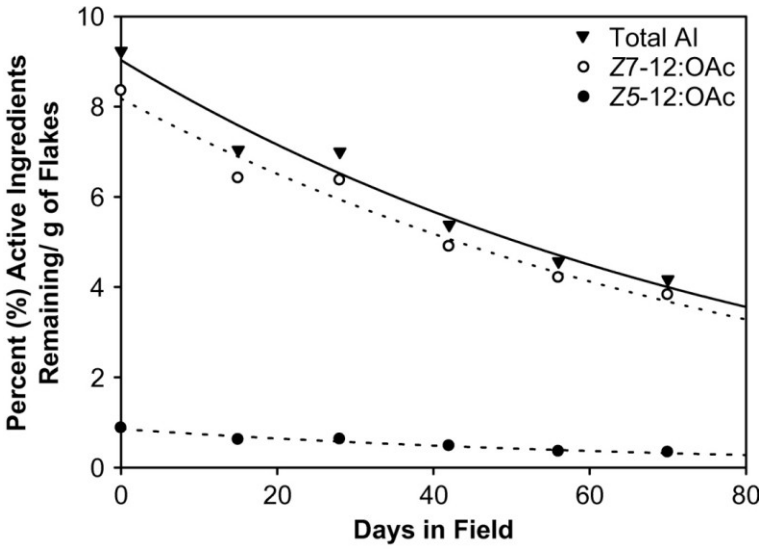

Figure 6-5. Percent active ingredients remaining in one gram of field-aged flakes at the end of the season. The individual pheromone components (*Z*7-12:OAc and *Z*5-12:OAc) and the total components are displayed. Total (black triangle): $\% AI = 9.03 \times e^{-0.012day}$, $r^2 = 0.96$; *Z*7-12:OAc (open circle): $\% AI = 8.17 \times e^{-0.012day}$, $r^2 = 0.97$; *Z*5-12:OAc (black circle): $\% AI = 0.86 \times e^{-0.014day}$, $r^2 = 0.95$).

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Chapter 7

Assessing the genetic diversity and population structure of *Coleophora deauratella* (Lepidoptera: Coleophoridae) in North America and Europe inferred from mitochondrial and microsatellite markers

Introduction

Biological invasions are becoming an ever present reality as global travel and trade continue to increase. In cases where invasive species are able to establish, they may have serious ecological and economic impacts (Sakai et al. 2001, Pimentel et al. 2005). Identifying the route of invasion and geographic origin of these invasive species can help determine details of the species biology and may help in control or eradication efforts (Roderick and Navajos 2003, Rugman-Jones et al. 2012).

Tracing the invasion of new species has been accomplished using several different molecular markers including randomly amplified polymorphic DNA (Kim and Sappington 2004), amplified fragment length polymorphisms (Grapputo et al. 2005), mitochondrial DNA (in particular, COI: Downie 2002, Scheffer and Grissell 2003, Laffin et al. 2005, Simonsen et al. 2008; 12S: Johnson et al. 2004; 16S: Navia et al. 2005), nuclear DNA (Davies et al. 1999, Navia et al. 2005), and microsatellite markers (Keever et al. 2013, Meixner et al. 2002, Valade et al. 2009). Several studies use a combination of markers, including mitochondrial DNA sequences and microsatellites, to give further resolution when tracking the geographic origin of invasive species (Meixner et al. 2002, Valade et al. 2009). Many invasive species are subjected to founder events in which a small subset of individuals from the original gene pool invade and establish in a new area (Sakai et al. 2001, Allendorf and Lundquist 2003). This founding event can result in a genetic bottleneck which may lead to decreased genetic diversity in the introduced range (Nei et

al. 1974, Dlugosch and Parker 2008) and may impact the fitness of individuals (Reed and Frankham 2003). However, in the case of multiple introductions, especially from genetically distinct source populations, an increase in genetic diversity may be observed in the introduced range compared to the source population (Allendorf and Lundquist 2003, Fitzpatrick et al. 2012). By using molecular markers, genetic relatedness and diversity can be used to infer the potential structure, origin and spread of an invasive species (Estoup et al. 2010, Grapputto et al. 2005, Ciosi et al. 2008).

Red clover is an important nitrogen-fixing crop grown widely in temperate regions of the world for seed, forage, honey production, and as a cover crop or green manure for soil improvement (Fairey 1981). In many parts of the world the yield of red clover seed can be severely impacted by larval feeding of the red clover casebearer, *Coleophora deauratella* Leinig and Zeller (Lepidoptera: Coleophoridae) (Markkula and Myllymäki 1960, Ellis and Bjørnson 1996, Evenden et al. 2010). *Coleophora deauratella* is native to Europe, eastern Siberia and the Middle East and was accidently introduced to North America over 50 years ago (Landry 1991). Due to misidentifications, the species was only reported in North America in 1991 (Landry 1991). Landry (1991) determined the earliest known *C. deauratella* record in North America was from Ithaca, NY in 1962 and subsequently identified specimens in collections from eastern Canada and the United States from the 1970's, 80's and early 90's. The introduced range of *C. deauratella* was thought to be restricted to eastern North America, but in 2006 a severe outbreak of this species began in the Peace River region of Alberta in western Canada (Evenden et al. 2010). Recent outbreaks of *C. deauratella* in parts of North America (Ellis and Bjørnson 1996, Evenden et al. 2010, Mori et al. 2014) make a compelling argument for a population genetics analysis to determine the invasion routes and the native source populations.

Coleophora deauratella is univoltine and each spring females lay eggs directly on the calyx of developing red clover florets. Upon hatching, larvae bore through the calyx or enter the tip of the floret and feed on the developing ovules (Landry 1991, Ellis and Bjørnson 1996). Larvae have four instars and tunnel between adjacent florets to feed on the developing seed (Landry 1991, Ellis and Bjørnson 1996). Fourth instars create a portable case which they can feed from and use for overwintering (Landry 1991, Ellis and Bjørnson 1996). Larvae are capable of destroying up to three seeds per day which can result in >80% seed loss over the growing season (Ellis and Bjørnson 1996, Evenden et al. 2010). The internal feeding nature of *C. deauratella* larvae make infestations difficult to control with insecticides and other control strategies are needed. An alternative biological control strategy may be implemented if the source population of *C. deauratella* in North America can be identified and sampled for possible control agents. Furthermore, identifying the invasion routes of *C. deauratella* is of importance as the species has not yet been detected in the Willamette Valley in western Oregon, the largest red clover seed production region (by area) in the world (Wong 2005).

Here, we develop and characterize four *C. deauratella* microsatellite markers for population genetic analyses. We then use these microsatellite markers, combined with sequence data from a portion of the mitochondrial Cytochrome Oxidase subunit-1 (COI) gene, to investigate the genetic diversity and structure of *C. deauratella* populations throughout North America and Europe. The aim of this study is to determine the source of North American populations and if single or multiple introductions occurred. Furthermore, we test for a genetic bottleneck upon introduction of *C. deauratella* into North America. The results of this study will provide an ecological and genetic understanding of *C. deauratella* populations which may improve monitoring and support the development of biological controls.

Materials and Methods

Sample collection and DNA extractions

Coleophora deauratella specimens were collected from 24 sites throughout Europe and North America during 2010-2013 (Fig. 7-1, Table 7-1). Moths were collected with green unitraps (Contech Enterprises, Delta, BC) baited with the complete pheromone blend (100:10 µg of *Z*-7-dodecenyl acetate to *Z*-5-dodecenyl acetate) (Evenden et al. 2010) loaded on a grey rubber septa lure (Contech Enterprises). Sweep nets were used to collect samples in Sherwood Park, AB and Hartola, FIN. Samples were preserved in 95% ethanol until they were shipped to the University of Alberta. At the University of Alberta, samples were sorted, their identification was confirmed based on morphology (Landry and Wright 1993) and their wings were removed as vouchers. Samples were stored in 95% ethanol at -20 °C until DNA extractions were conducted.

Total genomic DNA was extracted from the entire body of individual *C. deauratella* with a DNeasy® Blood and Tissue Kit with an RNase treatment following the manufacturer's instructions (Qiagen, USA) and eluted into 200 µl of AE buffer. An ethanol precipitation was used to further purify and concentrate the eluted DNA into 100 µl of DNase free water.

Mitochondrial DNA sequencing and analyses

A 615 bp (final edited product size) portion of the mitochondrial COI gene was amplified from *C. deauratella* using the universal lepidopteran primer pair LEP-F1, 5′-ATTCAACCAATCATAAAGATAT-3′ and LEP-R1, 5′-TAAACTTCTGGATGTCCAAAAA-3′ (Hebert et al. 2004). All PCR amplifications were performed in a 20 µl volume containing: ~50 ng template DNA, PCR buffer (10 mM Tris pH 8.8, 0.1 % Triton X-100, 50 mM KCl, 0.16 mg/mL BSA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM LEP-F1 and LEP-R1 primers, 1.6 U/µl

Taq DNA polymerase and autoclaved Millipore water up to 20 µl. The PCR cycling conditions were: 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 45 °C, and 2 min at 72 °C, and a final extension for 5 min at 72 °C. PCR amplifications were visualized on a 1.2% agarose gel to check for correct amplification and cleaned with 2 µl of ExoSAP (0.4 U Exonuclease I and 0.4 U Shrimp Alkaline Phosphatase) (New England Biolabs, Whitby, ON). Sequencing was performed using the BigDye™ v3.1 sequencing kit (Applied Biosystems Division, Life Technologies, Canada) and visualized on an Applied Biosystems 3730 capillary sequencer.

Two hundred and twenty five individuals from 21 populations were sequenced in both directions. Individual sequence contigs were edited using Lasergene® SeqMan software (DNASTAR, Inc., USA). Sequence alignment was conducted with ClustalW (Thompson et al. 1994) as implemented in Mesquite (Madison and Madison 2007) and all sequences were trimmed to 615 bp to remove missing data. Sequences of unique haplotypes are deposited in Genbank (Accession numbers: KJ830815-KJ830828).

Genetic differences among haplotypes were used to examine phylogeographic relationships by constructing a maximum parsimony haplotype network (Templeton et al. 1992) with a 95% connection limit in TCS 1.21 (Clement et al. 2000). Arlequin v.3.5 (Excoffier et al. 2005) was used to calculate haplotype and nucleotide diversity (Nei 1987) and Tajima's D (Tajima 1989). Negative values of Tajima's D indicate an excess of low frequency polymorphisms in the populations and imply a population expansion (Tajima 1989, Prijovic et al. 2014).

Microsatellite development

Coleophora deauratella DNA was extracted from thorax and leg tissue from two individuals separately using a DNeasy® Blood and Tissue Kit with an RNaseA treatment (Qiagen, USA). A genomic library was created and enriched for GT, CT, GATC, and GACA repeats following the methods of Glenn and Schable (2005). Genomic DNA was digested with *Alu*I, *Nhe*I, and *Xmn*I (New England Biolabs Inc., USA), ligated to SNX linkers and hybridized to biotinylated probes (GT_{12}, CT_{12}, $GACA_6$, $GATC_6$) (Operon Technologies, USA), and captured with streptavidin-coated magnetic beads (Dynabeads®; Dynal, USA). The SNX-forward linker was used as a primer to amplify and generate double strand repeat-enriched DNA. Repeat-enriched fragments were ligated via a TOPO TA Cloning® kit into a TOPO® vector and transformed into chemically competent One Shot® TOP10 *Escherichia coli* cells (Invitrogen Division, Life Technologies, Canada). Two hundred and forty-five putative insert bearing clones were amplified and run on a 1.2% agarose gel to confirm the presence of an insert. Ninety-five insert bearing clones were sequenced in both directions using T3 and T7 primers and a BigDye™ v3.1 sequencing kit (Applied Biosystems Division, Life Technologies, Canada) and visualized on an Applied Biosystems 3730 capillary sequencer. Putative microsatellite-bearing sequences were edited and aligned using Lasergene® SeqMan software (DNASTAR, Inc., USA) and checked for microsatellites visually.

Nineteen insert-bearing clones were of an appropriate length and sufficient quality to design forward and reverse primers using Primer3 v. 0.4.0 (Untergrasser et al. 2012). An M13(-21) (5'-TGT AAA ACG ACG GCC AGT-3') tail was added to the 5'-end of each forward primer (Schuelke 2000) and screened in eight individuals. Of the nineteen loci tested, four loci amplified reliably, but needed optimization, six loci amplified in only five individuals, three loci

were polymorphic but duplicated, four loci were monomorphic, and two loci failed to amplify. Due to the mixed results with M13-tailed primers, 5'-fluorescently labelled primers (Integrated DNA Technologies, USA) were obtained for the ten loci that had the most promise (four that amplified reliably and six that did not amplify in all individuals). Further screening of labelled primers was conducted on 15 individuals. Four loci were polymorphic and amplified reliably, whereas the other six loci did not amplify reliably in all individuals or were duplicated.

To test the attributes of the four polymorphic loci, we genotyped individuals from three populations (Harrington, Prince Edward Island (n = 15), Hines Creek, Alberta (n = 16), and Bad Bellingen, Germany (n = 16)) (Table 7-2). Each amplification reaction was conducted in a 15 µL volume and contained: approximately 25 ng template DNA, 1x PCR buffer (10 mM Tris pH 8.8, 0.1 % Triton X-100, 50 mM KCl, 0.16 mg/mL BSA), 2.4 mM $MgCl_2$, 0.2 mM dNTPs, 0.32 µM sequence-specific forward and reverse primers, and 0.06 U/µl *Taq* DNA polymerase. A touchdown PCR was conducted with the following conditions: 5 minutes at 94°C, 30 cycles of 30 s at 94°C, 45 s at the optimized annealing temperature decreasing 0.3°C per cycle (Table 7-1), 45 s at 72°C, and a final extension at 72°C for 10 minutes. Fragments were visualized on an Applied Biosystems 3730 capillary sequencer relative to the size standard GeneScan™-500-TAMRA® (Applied Biosystems) and genotyped with GeneMapper® v.4.0 (Applied Biosystems).

Arlequin v.3.5 (Excoffier et al. 2005) was used to estimate allelic diversity, calculate observed and expected heterozygosity, and test for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Evidence of null alleles was examined using Micro-Checker (van Oosterhout et al. 2004).

Microsatellite genotyping and analyses

Three hundred and seventy four *C. deauratella* from 24 populations were genotyped at all four loci developed above. To confirm that genotyping was consistent between runs, all individuals used for the initial microsatellite development were re-run and scored for the subsequent population genetic analysis. There was no difference in allele calling between the initial development run and the subsequent runs. Observed and expected heterozygosity and LD and HWE tests were calculated in Arlequin v.3.5 (Excoffier et al. 2005). The presence of null alleles was examined separately for each population using Micro-checker (van Oosterhout et al. 2004). Locus Cd.AT was removed from further analyses due to the presence of null alleles in 22 of the 24 populations.

A multilocus inbreeding index (F_{IS}), which estimates the deficit or excess of heterozygotes within each population, was calculated in Arlequin v.3.5 (Excoffier et al. 2005) with 10,000 permutations. Allelic richness adjusted to the minimum sample size of 10 was calculated with FSTAT v.2.9.3.2 (Goudet 1995).

To further explore the genetic variation between the sampled populations, the program Structure v.2.3.4 (Pritchard et al. 2000) was used to infer the most probable number of genetic groups (K) in the regions sampled based on the microsatellite data. This program uses a Bayesian approach to estimate the number of distinct genetic clusters by minimizing HWE and LD within groups. Structure was run using an admixture model and with sampling locations as priors (locprior). Setting sampling locations as priors in the model is useful for detecting weak genetic structure or when few loci are available (Hubisz et al. 2009). Initially, several test runs were conducted with various K values, burn-in periods, and iterations to determine the optimal settings

for reliable results. Reliable results were obtained with a burn-in period of 100,000, a total of 500,000 Markov chain Monte Carlo simulations, and with 10 iterations from $K = 1$ to 10. Structure Harvester v.0.6.93 (Earl and von Holt 2012) which implements the Evanno method (Evanno et al. 2005) was used to infer the optimal number of groups (K) within the data set. Results from Structure Harvester were input into CLUMPP v.1.1.2 (Jakobsson et al. 2009) which averaged each of the K replicates. The full search method was used for $K = 2$ and the Greedy method (1,000 repeats) was used for $K > 2$. Due to the presence of substructure within the data clusters produced by the most likely number of genetic groups ($K = 2$), the clusters were further divided to look for substructure. Each of the $K = 2$ clusters was then individually run in Structure with the same parameters.

Pair-wise F_{ST} values were estimated to assess differentiation between sampling localities (distance method, 1,023 permutations per comparison) based on COI haplotypes and microsatellites. Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was conducted to study the presence of genetic structure within and between continents, countries within Europe, and states/provinces within North America based on conventional F-statistics for COI data, and allele frequencies for microsatellite data. Both of the above analyses were performed in Arlequin v.3.5 (Excoffier et al. 2005). For all tests involving multiple comparisons (HWE, LD, and pair-wise F_{ST}) p-values were corrected with a false-discovery rate procedure (Benjamini and Hochberg 1995).

To determine if significant isolation-by-distance (Wright 1943) occurs with *C. deauratella* populations, matrices of standardized genetic distances [$F_{ST}/(1 - F_{ST})$] and the geographic distance between all sample localities were constructed based on COI and microsatellite data. Matrices were constructed for the entire data set, and sample populations

within Europe and North America separately. The degree of correlation between the standardized genetic distances and geographic distance was evaluated with a Mantel test over 9,999 randomizations (Mantel 1967) in GenAlx v.6.501 (Peakall and Smouse 2012).

Finally, non-parametric Spearman's rank correlations were used to determine if numbers of haplotypes or alleles were correlated with sample size. Wilcox rank sum tests were used to determine if the number of haplotypes or alleles, and allelic richness, observed and expected heterozygosity differed between populations grouped by continent (Europe vs. North America). All non-parametric tests were performed in R v.3.0.1 (R Core Team 2013).

Results

Mitochondrial COI analyses

A total of 14 haplotypes were observed across 21 populations sampled throughout North America and Europe (Fig. 7-1, Table 7-3). Four haplotypes occurred in North America; one was unique to the continent and the other three were also observed in Europe. Thirteen haplotypes occurred in Europe, with ten unique to the continent (Fig. 7-1, Table 7-3). Interestingly, the dominant haplotype observed across North America (A) was not the dominant haplotype observed across Europe (B). Finland had the most haplotypes (7), whereas several sites in North America contained only one haplotype (Fig. 7-1, Table 7-3). Six haplotypes (I, J, K, L, M, N) were represented by single individuals (Table 7-3, Figs. 1, 2). The COI haplotype network consists of two main haplotypes (A, B) with several additional haplotypes branching in a star-like pattern off of each (Fig. 7-2). The largest number of nucleotide differences observed between haplotypes was five, with most haplotypes differing from the two main haplotypes by only a single nucleotide (Fig. 7-2). Significantly more haplotypes were observed in Europe

compared to North America (W = 70.5, $p = 0.003$) and haplotype number was not correlated with sample size ($r_s = 0.22$, $p = 0.34$). Globally, Tajima's D was negative (D = -1.57) and significantly differed from zero ($p = 0.018$).

Pair-wise F_{ST} comparisons ranged from 0 to 0.86 and were greatest between North American and European populations, however only 34 of 210 comparisons were significant after correction for multiple comparisons (Table 7-4). There was significant structure between populations in Europe and North America with the exception of the Riehen, Switzerland population which did not differ significantly from any North America populations (Table 7-4). All comparisons between North American populations were not significant after correction for multiple comparisons and reveal little genetic structure within North America. Within Europe, Finland was significantly and highly differentiated from both German sites and Delémont, Switzerland. Finland was also moderately differentiated from Sjöbo, Sweden although it was not significant (Table 7-4).

Analysis of molecular variance revealed that a large portion of the variation (47.09%) was accounted for by differences among continents (Europe vs. North America) and among individuals within populations (46.79%) (Table 7-5). When populations were grouped by countries within Europe most of the variation was accounted for among individuals within populations (82.26%). Among North American populations grouped by state/province no significant structure was found (Table 7-5). There was a significant positive relationship with isolation-by-distance globally ($r^2 = 0.29$, $p < 0.001$); however, no significant isolation by distance occurs ($p > 0.05$) when populations in Europe and North America were analyzed separately.

Microsatellite development

Microsatellite loci that were polymorphic and amplified reliably were difficult to obain. The success rate from positive clones to final useable loci was < 2%. Of the four loci that were developed, the number of alleles ranged from 6 to 41 in initial testing with an average of 13.75 alleles per locus (Table 7-6). In initial testing of the 4 loci, locus Cd.AT was found to significantly deviate from Hardy-Weinberg equilibrium in the three test populations and locus Cd.H in the Harrington, PEI population (Table 7-6) after correction for multiple comparisons. There was an excess of homozygotes observed at locus Cd.AT in all three populations (Table 7-6). Null alleles were confirmed at locus Cd.AT in all tested populations with Micro-Checker, and for locus Cd.H in the Harrington, PEI population. No loci were in linkage disequilibrium after correction for multiple comparisons.

Microsatellite analyses

Microsatellite loci were genotyped in all individuals in each population with a high degree of success (Appendix 7-2 to 7-4). Loci were in HWE in all populations with the exception of Locus Cd.H in Fairview, Kinburn, and Harrington populations, and Locus Cd.L in the Ottawa population (Appendix 7-2, 7-3). All loci were in linkage equilibrium with the exception of Loci Cd.H and Cd.Q in the Eaglesham and Guy populations. Across all populations, the number of alleles per locus ranged from 8 to 46 with a mean of 25. The inbreeding coefficients (F_{IS}) in only two populations were significantly different than zero (Table 7-7) indicating that random mating occurs within most populations. Allelic richness and the number of alleles per population were significantly higher in Europe compared to North America (alleles per population: W = 101, p = 0.0018; allelic richness: W = 101, p = 0.0019) (Fig. 7-3) and number of alleles was not correlated

with sample size (r_s = 0.06, p = 0.77). There was no significant difference in observed heterozygosity (W = 75.5, p = 0.16), however, expected heterozygosity was significantly higher in Europe than in North America (W = 92, p = 0.012) (Fig. 7-3).

Pair-wise F_{ST} comparisons were generally lower than those based on COI sequences, but more comparisons were significant (193 out of 276) (Table 7-4). There was weak (0-0.05) to moderate (0.5-0.14) differentiation between the Oregon population and all other populations. Agassiz, BC was moderately differentiated from Harrington, PEI and all Alberta and Saskatchewan populations, but only weakly differentiated from European populations. Within Alberta, Hines Creek was moderately differentiated from all other populations with the exception of Sherwood Park. Love, SK was moderately differentiated from all other populations except for Carrot River, SK, Rosebank, MB and Riehen, CHE. Kinburn, ON populations were moderately differentiated from Eaglesham, AB and Love, SK, but had weak differentiation with all other Canadian populations. Ottawa, ON, was also weakly differentiated from most Canadian populations except Guy, Hines Creek, and Whitemud, AB, and Love, SK and Harrington, PEI where no differentiation was observed. Harrington, PEI was moderately differentiated from Sherwood Park, AB, but generally both PEI populations had low differentiation with most Alberta sites. Most European populations were moderately differentiated from North American populations, however, within Europe there was low differentiation between all populations (Table 7-4).

Analysis of molecular variance revealed most of the variation was within populations, but small significant variation occurred between continents and among populations within continents (Table 7-5). There was no variation attributed to countries within Europe when populations were grouped by country. When North American populations were grouped by state/province most of

the variation was attributed to within populations, however small, but significant variation occurred between state/province and among populations within state/province (Table 7-5). Similar isolation-by-distance results were obtained with microsatellites as with COI sequence data. Globally, there was weak but significantly positive isolation-by-distance ($r^2 = 0.04$, $p = 0.009$), but there was no significant relationships when populations within Europe or North America were examined separately ($p > 0.05$).

When considering the global population, the most likely number of genetic clusters (K) inferred from Structure was $K = 2$ (Fig. 7-4a). Most European samples were grouped into a single cluster (cluster 1, grey), with admixture occurring in most North American populations. Alberta populations were grouped into a single cluster (cluster 2, white) with the exception of Hines Creek. Due to evidence of substructure the two clusters were considered individually.

Cluster 1 showed further subdivision at $K = 3$ (Fig. 7-3b). All samples from BC, Oregon, Finland, Sweden, Switzerland and Gündlingen, DEU grouped together (black). Harrington, PEI and Hines Creek, AB grouped together (grey). Bad Bellingen, DEU samples were admixed with the European (black) and Alberta and PEI populations (grey) (Fig. 7-3b). Carrot River and Love, SK grouped together in a separate cluster (white). Carman, MB and both Ontario samples were admixed with primarily European (black) and Saskatchewan (white) clusters (Fig. 7-3b). Rosebank, MB samples resided in all three clusters (Fig. 7-3b). Cluster 2 composed of predominately Alberta samples (plus two samples from Carrot River, SK, and Harrington, PEI, and one Hines Creek, AB sample) showed no evidence of further substructure.

Discussion

Mitochondrial DNA (COI) sequence data and microsatellite markers were used to explore genetic diversity and differentiation of *C. deauratella* populations throughout North America and Europe in an attempt to determine the structure and source of populations introduced to North America. Both types of genetic data revealed significant structure between the native and invasive ranges, but within the native range structure was limited. It appears that a limited number of introductions occurred in North America and results gathered here suggest that *C. deauratella* may have originated from several European populations; however, the exact source cannot be inferred.

Low mitochondrial haplotype diversity was observed worldwide with most haplotypes differing from each other by only a single mutation (Fig. 7-2). Fourteen haplotypes were observed across Europe and North America. Of the four haplotypes observed in North America only one was unique, the remaining three haplotypes were shared with European populations (Table 7-2). The unique North American haplotype (E) was found in six individuals at two sites. There are three possible explanations for the unique North American haplotype: i) the haplotype may have arisen *de novo* within North America at both sites; ii) it may have arisen *de novo* in one site and subsequently spread to the other site; or iii) it may suggest that there was inadequate sampling in the native range and it may be a remnant from the true source population of the introduced individuals (Muirhead et al. 2008). In Europe, 13 haplotypes were observed across the six sampled populations, with 10 unique to the continent. Finland had the largest number of haplotypes observed for any single population and also shared the greatest number of haplotypes (3) with North American populations, suggesting it may be a source of the introduced *C. deauratella*.

Founding events can result in reduced genetic variation and, increasingly, are being recognized by a higher loss in allele number and richness compared to heterozygosity (Allendorf 1986, Dlugosch and Parker 2008). Allelic diversity is lost faster than heterozygosity after a bottleneck because of loss of rare alleles that may have little impact on heterozygosity, but have a large impact on allelic diversity (Allendorf 1986). Genetic diversity within North America is reduced compared to Europe which suggests a founder's effect leading to a genetic bottleneck. The low number of haplotypes in North America is evidence that a reduced subset of the genetic diversity was captured in introduced populations compared to that found in Europe. This also corresponds with a decrease in the number of alleles and allelic richness and a slight decrease (although not significant) in observed heterozygosity in North American populations compared to Europe (Fig. 7-3). Furthermore, genetic bottlenecks often follow a population expansion which is supported by the negative Tajima's D observed here (Tajima 1989, Valade et al. 2009, Prijovic et al. 2014).

F_{ST} and AMOVA results indicate that most genetic differentiation occurs between populations on the two continents, however, F_{ST} comparisons based on microsatellite markers also found weak to moderate genetic differentiation among most populations within each continent (Table 7-4). Microsatellites, with an increased number of polymorphic loci compared to mitochondrial DNA, may be more sensitive to the remnants of historical gene flow that can shape contemporary population genetic structure (Hutchison and Templeton 1999, Ramstad et al. 2004). Thus, the structure observed in North America is most likely a remnant from the original source populations. The isolation-by-distance analysis also demonstrated that the majority of genetic structure exists between the two continents. When populations from both the introduced and native range were included in the analysis, isolation-by-distance was significant. This broad

scale isolation, supported by multiple analyses, suggests there may be differential outcomes of genetic drift and selection occurring in each continent (Keever et al. 2013). However, within each continent there was no significant isolation-by-distance. The lack of isolation-by-distance combined with weak genetic differentiation within continents suggests that *C. deauratella* has moved widely, across long distances in each region.

The Structure analysis found the most support for only two genetic clusters across the sampled populations in North America and Europe. European populations were grouped into cluster 1, whereas Alberta populations (except Hines Creek) grouped in cluster 2. All other North American populations were an admixture of both clusters (Fig. 7-4). The two clusters within North America may have resulted from separate introductions or a single introduction of individuals from both clusters. The evidence of admixture of the two genetic clusters in Europe suggests there may be unsampled populations of *C. deauratella* that could be the origin of North American populations. There was evidence of substructure within the whole data set, so each cluster was analyzed individually. When cluster 1 (grey, Fig. 7-4a), composed of both North American and European populations, was observed for substructure, three genetic clusters were best supported (Fig. 7-4b). Three subclusters were found in North America, while one is dominant in Europe which indicates there are higher levels of population structure within North America, with lower levels of diversity, compared to the native European range. This is further supported by the AMOVA results in which regions (states/provinces) in North America explained a significant and high amount of genetic variation, whereas in Europe there was no significant variation explained by region (countries) (Table 7-4). If post-introduction expansion occurred sporadically in North America as evidence suggests, separate founder events may have occurred. Following each of these events, genetic drift can influence the structure of each

population separately (Nei and Tajima 1981, Neit et al. 1975) and thus lead to the observed subclusters.

The evidence presented here using both mitochondrial and microsatellite markers suggest that a limited number of introductions of *C. deauratella* occurred in North America. Multiple introductions, especially from highly divergent source populations, can lead to increased genetic diversity in introduced ranges of invasive species compared to that of the source population (Allendorf and Lundquist 2003, Kolbe et al. 2004, Lockwood et al. 2005, Lawson Handley et al. 2007, Dlugosch and Parker 2008). If multiple introductions occurred, we would expect to see an increase in diversity either evident as additional mitochondrial haplotypes or allelic diversity within North America. A single introduction composed of individuals from both genetic clusters with a variety of mitochondrial haplotypes could lead to the present distribution of *C. deauratella*. Given that the genetic diversity within North America is highest in Kinburn, ON, when both haplotype and allelic diversity are considered (Table 7-3, 7-7), it is probable that southern Ontario or the adjacent states may be the likely site of introduction of *C. deauratella* in North America. Historical collection and outbreak information on *C. deauratella* also adds some support to this origin. The first North American record is from Ithaca, NY in 1962, and the first documented outbreak occurred in southern Ontario in 1989 (Landry 1991, Ellis and Bjørnson 1996). Furthermore, this region is home to a vast array of transportation hubs for both the United States and Canada, which may have resulted in the accidental distribution of *C. deauratella* throughout North America.

Alberta populations represent a unique cluster compared to the rest of North America with the exception of Hines Creek. Hines Creek is not a prominent clover seed production region or major transportation hub compared to other sample areas; it is possible that the populations in

this area were from a separate introduction compared to those in the rest of Alberta. Records from the Canadian National Collection of Insects, Arachnids, and Nematodes (Ottawa, ON) indicate *C. deauratella* has been present in northern Alberta since 2001, however, damage was not documented until 2006 (Evenden et al. 2010, Mori et al. 2014). This lag time between the first recorded specimen and the outbreak is a common feature of invasions. Lag time may allow for evolutionary change to take place in which adaptation to the new habitat occurs or invasive life history characteristics could evolve (Sakai et al. 2001). During this lag period, Alberta experienced an extreme drought; water stress on red clover can reduce flower production (Oliva et al. 1994) and weaken plant defenses (Mattson and Haack 1987). The weakened plant defenses could have allowed *C. deauratella* to readily establish, and combined with reduced flower production seed yields were drastically reduced (Evenden et al. 2010, Mori et al. 2014). Outbreaks in other insects homogenize genetic structure (Chapuis et al. 2008) and lead to unique genetic clusters compared to other nearby endemic populations (Boisson et al. 2012), both of which are observed in Alberta. Moreover, Alberta populations have a reduced number of alleles and richness compared to other North American populations suggesting a subsequent population bottleneck occurred in that region.

The drought of the early 2000's was so severe that farmers in western Canada had to purchase hay to feed their livestock, particularly eastern Canada (Plamondon 2004). Hay may act as a vector of *C. deauratella* as we are aware of one instance in PEI of a farmer obtaining hay infested with *C. deauratella* larvae (K. Hillier, Pers. Comm.). Clover is a component of hay in many regions and it is highly probable that movement of hay throughout Canada led to the movement of *C. deauratella*. The unique shared haplotype shared only between Alberta and Ontario may be evidence of this movement (Fig. 7-1).

Successful invasions can be attributed to the enemy release hypothesis, in which invasive species experience a decrease in regulation by natural enemies upon entry into a novel environment which can result in rapid population increase (Keane and Crawley 2002). The successful invasion and establishment of *C. deauratella* in North America may be explained by enemy release as few natural enemies are present (Ellis and Bjørnson 1996, Mori et al. 2014). Generally, there is a time lag for predator and parasitoid establishment to increase on introduced herbivorous insects (Crooks 2005) and eventually some native natural enemies may switch hosts to exploit the invasive species (Mason et al. 2014). Thus the number of natural enemies an invasive species has in its introduced range may be used as a proxy for the amount of time the invader has been present. In previous work, we found only a single parasitoid from hundreds of larval cases (Mori et al. 2014). Given the high population numbers that can be present in some fields (1 larvae/5 flowers) (Mori et al. 2014), it is unlikely that natural enemies currently play a large role in reducing *C. deauratella* numbers in Alberta, although several different species have been found to attack *C. deauratella* in Ontario (Ellis and Bjørnson 1996). This further supports our hypothesis that North American populations resulted from an initial introduction in southern Ontario or the adjacent states, as more natural enemies of *C. deauratella* are found there. More recently, the enemy release hypothesis has been adapted to include resource availability (Blumenthal 2006). Prior to the introduction of *C. deauratella* into Alberta, there were very few problems with insect pests in red clover. Red clover seed production fields were often harvested for two years before rotation which supplied a large resource for multiple years for *C. deauratella* population growth with limited competition and natural enemies.

Here, we developed four polymorphic microsatellite markers which are notoriously difficult to develop for Lepidoptera (Zhang 2004). We also had difficulty isolating microsatellite

markers from *C. deauratella* as the success rate from positive clones to usable markers was < 2%. We used these microsatellites in combination with mitochondrial COI sequence data to explore the population structure of *C. deauratella* populations in North America and Europe. A founder effect, evident by reduced genetic diversity, occurred within North American populations. Based on both mitochondrial and microsatellite markers, a limited number of introductions from Finland or an unsampled European population may have been the source of *C. deauratella* populations in North America and most likely were introduced to southern Ontario or the adjacent states. The largest red clover seed production region in the world is located in western Oregon where *C. deauratella* had not previously been found prior to this study. We can confirm *C. deauratella* is present Oregon; however, this population does not cluster with Alberta populations indicating it is likely not a result of spread from the current outbreak in Alberta. Currently, there are limited control measures available for *C. deauratella* in red clover seed production regions in Canada. Although, the current research does not specifically identify the source populations of North American *C. deauratella* populations, in the future the markers developed and information provided here may help identify source population locations in the native range which may help to identify possible biological control agents.

Table 7-1. Information for *Coleophora deauratella* population samples including continent, sampling region (province/state within North America; country within Europe), site number, site name, year the samples were collected and the number of samples (n) used for both mitochondrial COI (mtDNA) and microsatellite analyses. GPS coordinates are provided in Appendix 7-1.

Continent	Region	Region Abbreviation	Site Number	Site Name	Sample Year	Sample Number (n)	
						mtDNA	Microsatellites
North America	Oregon	OR	1	St. Louis	2013	11	16
	British Columbia	BC	2	Agassiz	2012	5	16
	Alberta	AB	3	Beaverlodge	2010	0	11
			4	Eaglesham	2010	9	16
			5	Fairview	2010	11	15
			6	Guy	2010	9	16
			7	Hines Creek	2010	14	16
			8	Rycroft	2010	0	16
			9	Sherwood Park	2013	7	16
			10	Whitemud	2010	12	16
	Saskatchewan	SK	11	Carrot River	2012	11	16
			12	Love	2012	14	16
	Manitoba	MB	13	Carman	2012	3	16
			14	Rosebank	2012	0	16
	Ontario	ON	15	Kinburn	2012	14	16
			16	Ottawa	2012	15	15
	Prince Edward Island	PEI	17	Harrington	2012	15	15
			18	Mermaid	2012	16	16
Europe	Finland	FIN	19	Hartola	2013	13	16
	Germany	DEU	20	Bad Bellingen	2013	12	16
			21	Gündlingen	2013	8	16
	Switzerland	CHE	22	Delémont	2013	9	16
			23	Riehen	2013	7	15
	Sweden	SWE	24	Sjöbo	2013	13	15

Table 7-2. Characteristics and optimal PCR conditions for four polymorphic microsatellite loci developed for *Coleophora deauratella* from three populations throughout the world. Expected allele length refers to the product size generated from the cloned microsatellite loci.

Locus	Primer Sequence (5' → 3')	Repeat Motif	Expected Allele Length (bp)	Annealing Temperature (°C)	Size Range (bp)
Cd.AT	F: TET-GCCTGTGTTTTTGAGGCATT R: TATTTTGAGGGCCGAAAGAA	$(GAT)_2AT(GAT)_2$	318	60-51	282-372
Cd.H	F: TET-AATCCTTTATCTGAACTTCGTAATCT R: ATAACGCCAATCCGCTTC	$(GT)_{13}AT(GT)_9$	249	56-47	205-295
Cd.L	F: HEX-TGGACTCGCTTTTTGTTTACC R: TCATGTGTCTGCCAAATTCAA	$(GT)_{10}$	182	60-51	156-188
Cd.Q	F: HEX-ACCGCGACCTCGTGAGTA R: GAGTCCATTTTTGACATCTTGG	$(CT)_3TT(CT)_4AT(CT)_8$... $(CT)_1TT(CT)_3$... $(CT)_5$	212	60-51	200-246

TET and HEX refer to fluorescent dyes (IDT) labelling the 5'-end of the forward primer.

Table 7-3. Distribution of haplotypes and haplotype and nucleotide diversity by population. The symbol ± is followed by the standard deviation of each estimate.

Site Number	Distribution of Haplotypes (number)	Haplotype Diversity	Nucleotide Diversity
1	A	0.000 ± 0.000	0.0000 ± 0.0000
2	A	0.000 ± 0.000	0.0000 ± 0.0000
4	A	0.000 ± 0.000	0.0000 ± 0.0000
5	A	0.000 ± 0.000	0.0000 ± 0.0000
6	A (6), B (2), E (1)	0.556 ± 0.165	0.0012 ± 0.0011
7	A (12), B (2)	0.264 ± 0.136	0.0004 ± 0.0006
9	A (6), B (1)	0.286 ± 0.196	0.0005 ± 0.0006
10	A (11), B (1)	0.167 ± 0.134	0.0003 ± 0.0004
11	A (10), B (1)	0.182 ± 0.144	0.0003 ± 0.0005
12	A	0.000 ± 0.000	0.0000 ± 0.0000
13	A	0.000 ± 0.000	0.0000 ± 0.0000
15	A (9), B (2), E (2)	0.513 ± 0.144	0.0012 ± 0.0011
16	A (13), B (1), C (1)	0.257 ± 0.142	0.0004 ± 0.0006
17	A (13), B (2)	0.248 ± 0.131	0.0004 ± 0.0005
18	A (14)	0.000 ± 0.000	0.0000 ± 0.0000
19	A (2), B (2), K (1), C (5),I (1), L (1), M (1)	0.846 ± 0.085	0.0026 ± 0.0019
20	A (1), B (9), G (1), H (1)	0.455 ± 0.170	0.0008 ± 0.0008
21	A (6), G (1), H (1)	0.464 ± 0.200	0.0008 ± 0.0009
22	B (10), F (1), J (1)	0.417 ± 0.191	0.0007 ± 0.0008
23	A (4), B (2), F (1)	0.667 ± 0.160	0.0014 ± 0.0013
24	B (9), D (3), N (1)	0.500 ± 0.136	0.0019 ± 0.0015

Table 7-4a. Pair-wise F_{ST} estimates based on mitochondrial COI sequences (above diagonal) and microsatellite data (below diagonal) for populations 1-13.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13
1		0.00	-	0.00	0.00	0.22	0.05	-	0.07	0.00	0.00	0.00	0.00
2	**0.04**		-	0.00	0.00	0.09	0.00	-	0.00	0.00	0.00	0.00	0.00
3	**0.08**	**0.02**		-	-	-	-	-	-	-	-	-	-
4	**0.10**	**0.05**	0.00		0.00	0.19	0.03	-	0.04	0.00	0.00	0.00	0.00
5	**0.04**	**0.05**	0.01	0.02		0.22	0.05	-	0.07	0.00	0.00	0.00	0.00
6	**0.07**	**0.07**	0.01	**0.04**	0.00		0.02	-	0.00	0.08	0.06	0.26	0.00
7	**0.06**	**0.08**	**0.05**	**0.07**	**0.04**	**0.04**		-	0.00	0.00	0.00	0.08	0.00
8	**0.07**	**0.06**	0.00	0.00	0.00	0.01	**0.05**		-	-	-	-	-
9	**0.03**	**0.06**	**0.04**	0.03	0.00	0.01	**0.04**	0.00		0.00	0.00	0.11	0.00
10	**0.08**	**0.06**	0.00	0.01	0.01	0.00	**0.06**	**0.00**	0.01		0.00	0.01	0.00
11	**0.06**	**0.06**	**0.04**	**0.06**	**0.03**	**0.04**	**0.06**	**0.03**	0.02	**0.03**		0.02	0.00
12	**0.08**	**0.07**	**0.06**	**0.09**	**0.07**	**0.09**	**0.08**	**0.08**	**0.07**	**0.07**	0.00		0.00
13	**0.03**	**0.03**	0.02	**0.06**	0.01	0.01	**0.03**	**0.04**	0.02	**0.04**	**0.03**	**0.05**	
14	**0.06**	**0.03**	0.03	**0.06**	**0.04**	**0.03**	**0.05**	**0.05**	**0.04**	**0.04**	0.02	**0.04**	0.00
15	**0.04**	**0.03**	0.01	**0.06**	0.02	0.02	0.02	**0.03**	**0.03**	**0.03**	0.02	**0.05**	0.00
16	**0.02**	0.00	0.01	**0.04**	**0.03**	**0.05**	**0.05**	**0.04**	**0.04**	**0.05**	**0.04**	**0.06**	0.01
17	**0.10**	**0.06**	0.02	**0.04**	**0.04**	0.02	0.02	**0.03**	**0.05**	**0.03**	**0.05**	**0.07**	0.02
18	**0.03**	0.02	0.00	**0.04**	0.01	0.01	**0.04**	0.02	0.02	0.02	0.02	**0.05**	0.01
19	**0.07**	**0.03**	0.01	**0.06**	**0.05**	**0.05**	**0.06**	**0.07**	**0.07**	**0.04**	**0.06**	**0.07**	**0.03**
20	**0.04**	**0.03**	0.05	**0.08**	**0.05**	**0.07**	**0.05**	**0.07**	**0.06**	**0.08**	**0.03**	**0.05**	**0.03**
21	**0.09**	**0.04**	0.01	**0.06**	**0.07**	**0.06**	**0.08**	**0.07**	**0.09**	**0.05**	**0.06**	**0.06**	**0.05**
22	**0.03**	**0.02**	**0.03**	**0.06**	**0.04**	**0.05**	**0.04**	**0.05**	**0.04**	**0.05**	**0.03**	**0.05**	0.02
23	**0.02**	**0.03**	0.02	**0.07**	**0.04**	**0.04**	**0.03**	**0.05**	**0.04**	**0.05**	**0.04**	**0.04**	0.01
24	**0.08**	0.02	0.00	**0.04**	**0.05**	**0.05**	**0.07**	**0.06**	**0.07**	**0.04**	**0.05**	**0.07**	**0.03**

F_{ST} estimates in **bold** are significantly different from zero after a false-discovery rate correction procedure. The population numbers correspond with populations in Table 7-1.

Table 7-4b. Pair-wise F_{ST} estimates based on mitochondrial COI sequences (above diagonal) and microsatellite data (below diagonal) for populations 14-24.

Population	14	15	16	17	18	19	20	21	22	23	24
1	-	0.17	0.00	0.04	0.00	0.23	**0.76**	**0.83**	**0.84**	0.34	**0.44**
2	-	0.07	0.00	0.00	0.00	0.12	0.69	0.75	**0.77**	0.18	0.33
3	-	-	-	-	-	-	-	-	-	-	-
4	-	0.15	0.00	0.02	0.00	0.20	0.74	**0.81**	**0.82**	0.30	**0.41**
5	-	0.17	0.00	0.04	0.00	0.23	**0.76**	**0.83**	**0.84**	0.34	0.44
6	-	0.00	0.07	0.03	0.26	0.00	0.34	0.40	0.42	0.08	0.10
7	-	0.03	0.00	0.00	0.08	0.16	**0.61**	**0.68**	**0.69**	0.11	0.31
8	-	-	-	-	-	-	-	-	-	-	-
9	-	0.00	0.00	0.00	0.11	0.09	0.57	0.64	0.65	0.03	0.24
10	-	0.07	0.00	0.00	0.01	0.18	**0.67**	**0.74**	**0.75**	0.19	0.36
11	-	0.06	0.00	0.00	0.02	0.16	**0.66**	**0.72**	0.73	0.16	0.34
12	-	0.20	0.00	0.06	0.00	**0.26**	**0.78**	**0.85**	0.86	0.39	**0.48**
13	-	0.00	0.00	0.00	0.00	0.03	0.64	0.71	0.73	0.07	0.26
14		-	-	-	-	-	-	-	-	-	-
15	0.01		0.06	0.04	0.20	0.00	0.36	0.42	**0.43**	0.04	0.15
16	**0.03**	0.01		0.00	0.00	0.16	**0.66**	**0.72**	**0.72**	0.20	0.38
17	**0.05**	0.02	**0.05**		0.06	0.17	**0.63**	**0.69**	**0.70**	0.13	0.33
18	**0.03**	0.00	0.02	**0.03**		**0.26**	**0.78**	**0.85**	**0.86**	0.39	0.48
19	**0.06**	**0.03**	0.02	**0.04**	0.01		0.27	0.28	0.29	0.04	0.13
20	**0.04**	0.02	0.01	**0.05**	**0.03**	**0.04**		0.00	0.00	0.24	0.07
21	**0.07**	**0.04**	**0.03**	**0.04**	**0.03**	0.00	**0.04**		0.00	0.30	0.10
22	0.01	0.02	0.01	**0.06**	**0.02**	**0.04**	0.01	**0.04**		0.30	0.11
23	**0.03**	0.01	0.01	**0.03**	0.00	0.01	0.01	0.01	0.01		0.04
24	**0.05**	**0.03**	0.02	**0.03**	0.01	0.00	**0.04**	0.00	**0.03**	0.02	

F_{ST} estimates in **bold** are significantly different from zero after a false-discovery rate correction procedure. The population numbers correspond with populations in Table 7-1.

Table 7-5. Analysis of molecular variance results on mitochondrial COI (mtDNA) and microsatellite data.

		mtDNA			Microsatellites		
			Variation			Variation	
Group	Source of Variation	d.f.	(%)	*p*-value	d.f.	(%)	*p*-value
Europe and North America	Among groups	1	47.09	< 0.0001	1	1.67	< 0.0001
	Among populations	19	6.13	< 0.0001	22	3.15	< 0.0001
	Within populations	204	46.79	< 0.0001	724	95.18	< 0.0001
Countries within Europe	Among groups	3	12.78	n.s.	3	-0.20	n.s.
	Among populations	2	4.96	< 0.05	2	2.04	< 0.05
	Within populations	56	82.26	< 0.0001	182	98.16	< 0.01
Provinces/states within North America	Among groups	6	-2.25	n.s.	6	2.55	< 0.001
	Among populations	8	6.29	n.s.	11	1.59	< 0.001
	Within populations	148	95.97	n.s.	542	95.86	< 0.0001

n.s, not significant

Table 7-6. Summary statistics for tests of four polymorphic microsatellite loci developed for *Coleophora deauratella* on three populations throughout the world.

		Hines Creek, AB			Harrington, PEI			Bad Bellingen, GER		
Locus	N_a	n	H_o	H_e	n	H_o	H_e	n	H_o	H_e
Cd.AT	10	15	0.13*	0.52	15	0.27*	0.74	16	0.31*	0.87
Cd.H	24	16	0.81	0.89	15	0.73*	0.92	16	0.81	0.93
Cd.L	6	16	0.38	0.48	15	0.80	0.71	15	0.40	0.53
Cd.Q	15	16	0.81	0.84	15	0.67	0.79	16	0.69	0.89

Number of alleles per loci (N_a), number of loci amplified (n), observed heterozygosity (H_o) and expected heterozygosity (H_e).

*indicates deviation from Hardy-Weinberg equilibrium ($P < 0.05$) after a false-discovery rate correction procedure.

Table 7-7. Summary statistics (mean ± S.D.) of three microsatellite loci across 24 populations from North America and Europe (see appendix 7-2 to 7-4 for data at each locus).

Population	1	2	3	4	5	6	7	8
N_a	9.00 ± 5.00	9.00 ± 3.61	7.67 ± 2.52	8.33 ± 2.08	6.33 ± 2.08	8.00 ± 2.65	8.67 ± 3.21	8.67 ± 4.73
N_s	7.48 ± 4.32	7.78 ± 2.79	7.54 ± 2.60	6.85 ± 1.36	5.80 ± 1.93	6.84 ± 2.31	7.15 ± 2.37	6.96 ± 2.96
Freq. P_{AL}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02
H_o	0.69 ± 0.44	0.85 ± 0.16	0.85 ± 0.19	0.60 ± 0.04	0.66 ± 0.18	0.64 ± 0.23	0.67 ± 0.25	0.73 ± 0.16
H_e	0.66 ± 0.38	0.80 ± 0.12	0.83 ± 0.10	0.74 ± 0.05	0.70 ± 0.22	0.73 ± 0.15	0.73 ± 0.22	0.71 ± 0.17
F_{IS}	-0.07	-0.10	-0.06	**0.18**	0.04	0.09	0.10	-0.03
Population	9	10	11	12	13	14	15	16
N_a	8.67 ± 5.69	8.33 ± 3.51	9.00 ± 6.24	8.33 ± 4.51	9.33 ± 4.51	8.67 ± 5.13	11.67 ± 6.51	9.67 ± 5.51
N_s	7.05 ± 4.40	6.85 ± 2.76	7.73 ± 5.16	7.01 ± 3.90	7.86 ± 3.66	7.23 ± 3.77	9.36 ± 4.43	8.21 ± 4.09
Freq. P_{AL}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.02	0.01 ± 0.02
H_o	0.60 ± 0.31	0.73 ± 0.16	0.64 ± 0.19	0.71 ± 0.24	0.81 ± 0.27	0.71 ± 0.24	0.79 ± 0.22	0.73 ± 0.29
H_e	0.66 ± 0.30	0.76 ± 0.12	0.75 ± 0.24	0.76 ± 0.20	0.76 ± 0.22	0.75 ± 0.23	0.81 ± 0.20	0.78 ± 0.19
F_{IS}	0.09	0.04	0.11	0.07	-0.07	0.06	0.03	0.07
Population	17	18	19	20	21	22	23	24
N_a	9.33 ± 4.16	11.00 ± 7.94	11.67 ± 6.03	10.00 ± 5.00	12.33 ± 5.51	10.67 ± 5.13	11.67 ± 6.81	10.33 ± 5.13
N_s	7.93 ± 3.16	8.81 ± 5.43	9.37 ± 4.44	8.20 ± 3.77	9.87 ± 3.80	8.67 ± 4.14	9.82 ± 5.57	8.75 ± 3.69
Freq. P_{AL}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.02	0.02 ± 0.04	0.01 ± 0.02	0.03 ± 0.03
H_o	0.73 ± 0.07	0.75 ± 0.16	0.85 ± 0.25	0.63 ± 0.21	0.88 ± 0.13	0.73 ± 0.28	0.76 ± 0.16	0.78 ± 0.10
H_e	0.81 ± 0.11	0.81 ± 0.18	0.85 ± 0.12	0.78 ± 0.22	0.88 ± 0.08	0.77 ± 0.26	0.80 ± 0.26	0.87 ± 0.08
F_{IS}	0.09	0.06	0.00	**0.18**	0.01	0.05	-0.02	0.11

N_a, number of alleles; N_s, allelic richness based on smallest sample size of 10; Freq. P_{AL}, frequency of private alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient. **Bold** F_{IS} values indicate they are significantly different from zero ($p < 0.05$). Population numbers correspond to populations in Table 7-1.

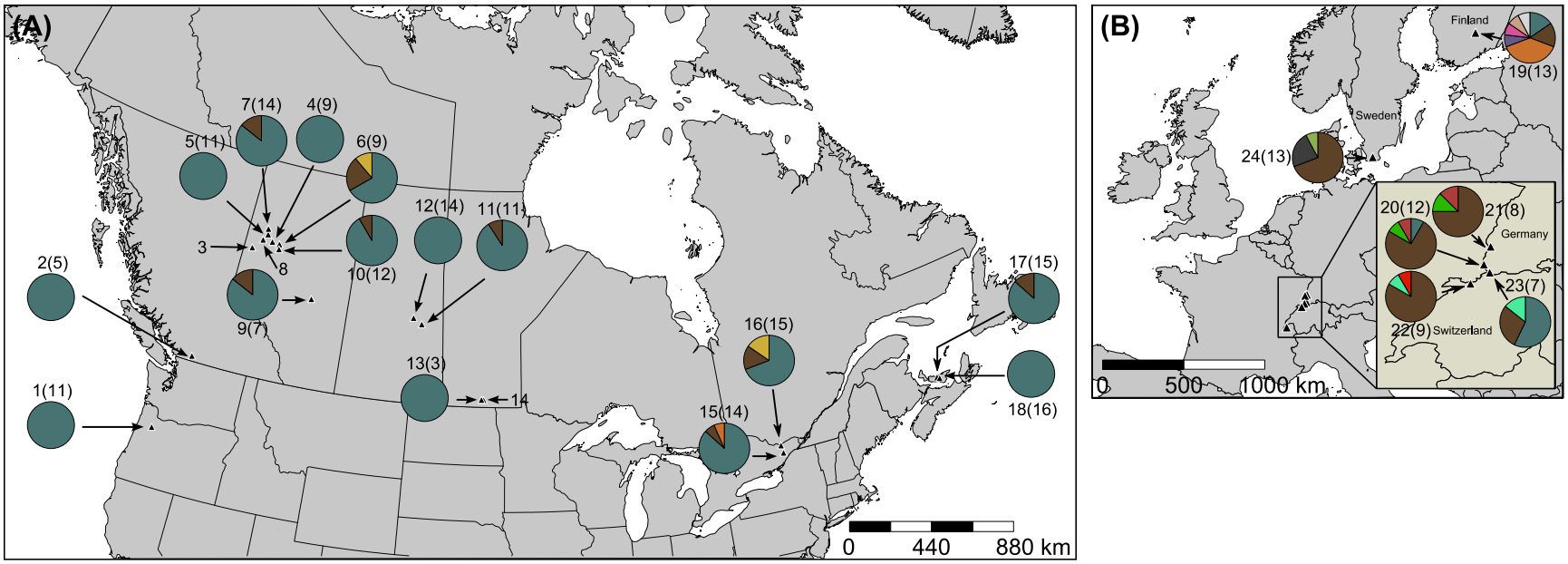

Figure 7-1. Geographic location of all 24 sampled populations and distribution of the 14 haplotypes among the 21 sites used for mitochondrial COI analyses. Each triangle represents a sampling location; triangles without a corresponding pie chart were not used for the mitochondrial COI analyses. Numbers beside pie charts are the site number (corresponding to Table 7-1) and the sample size (in brackets). Each colour in the pie charts corresponds to a different haplotype.

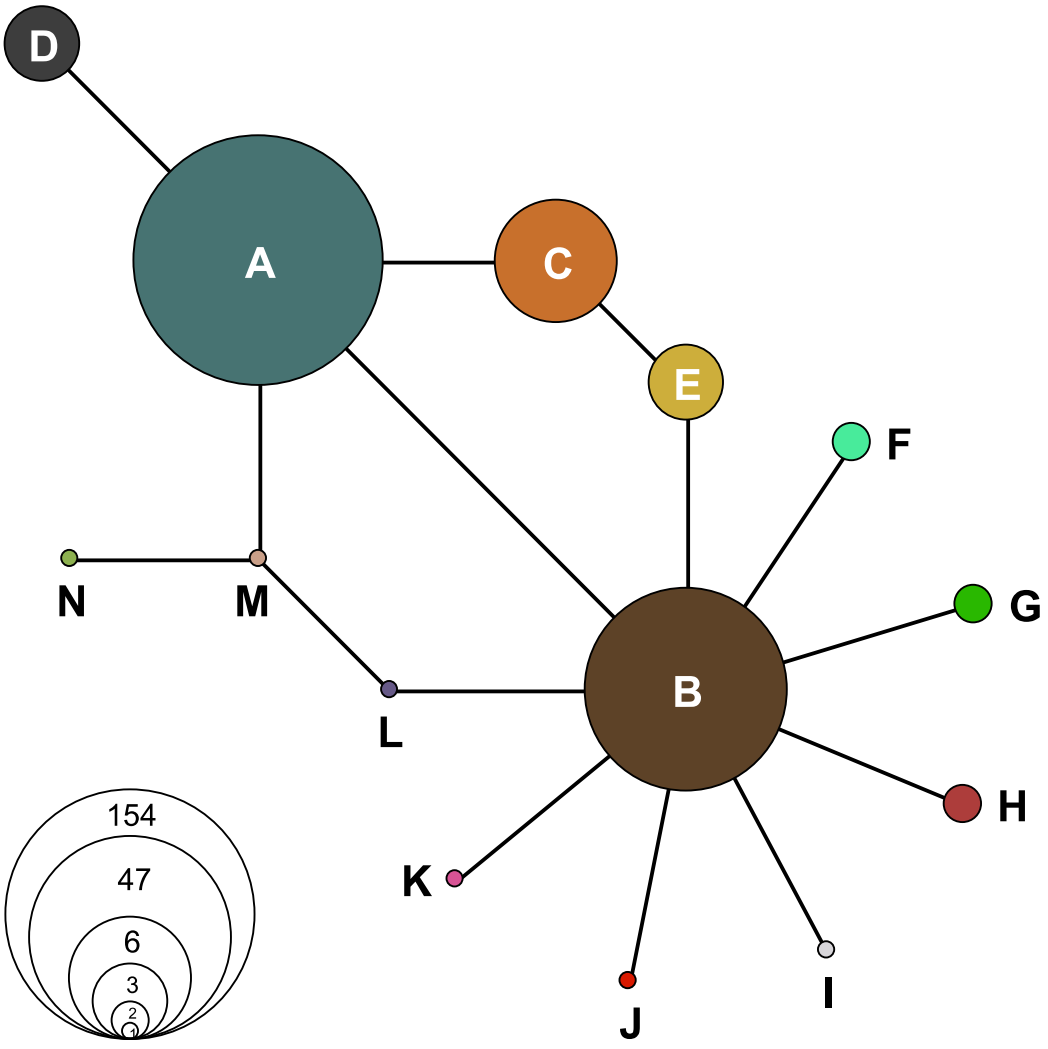

Figure 7-2. Mitochondrial COI DNA haplotype network for the 14 identified haplotypes with corresponding letters. Size of the nodes is proportional to the number of individuals with each haplotype. Each connection represents a single mutational step.

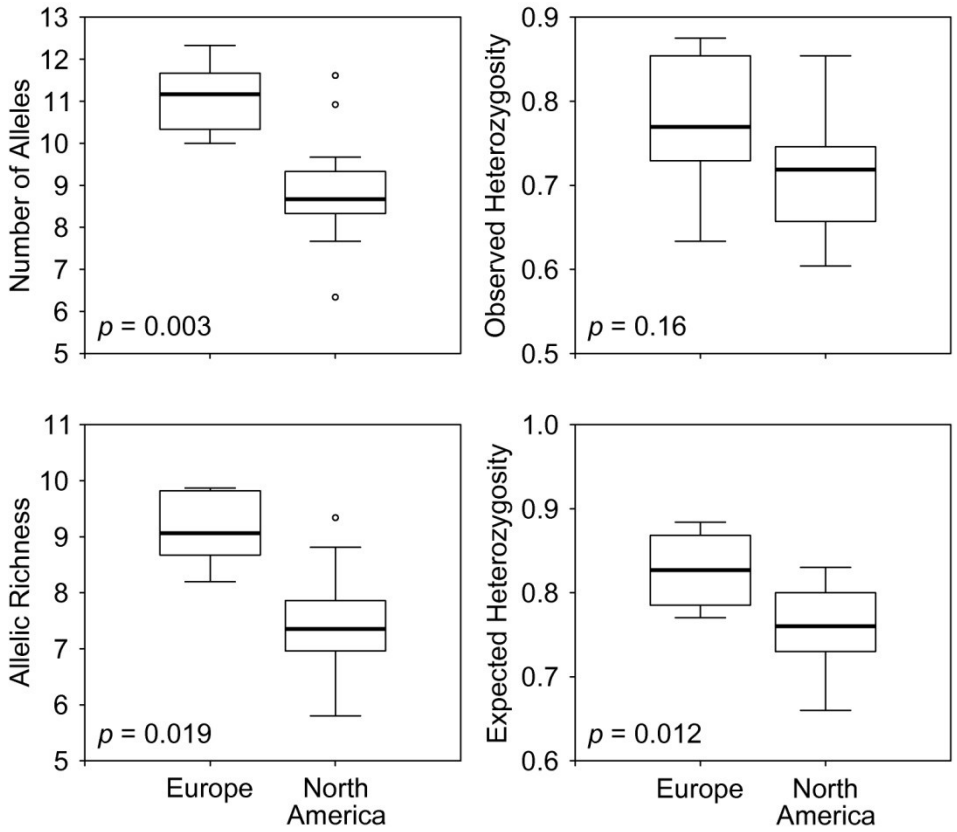

Figure 7-3. Box and whisker plots of genetic diversity comparisons based on microsatellite markers between European and North American samples. The first and third quartiles are represented by the top and bottom of the box, respectively. The midline indicates the median and the vertical lines extending from the box (whiskers) represent the maximum and minimum values. *P*-values are from non-parametric Wilcox rank sum tests.

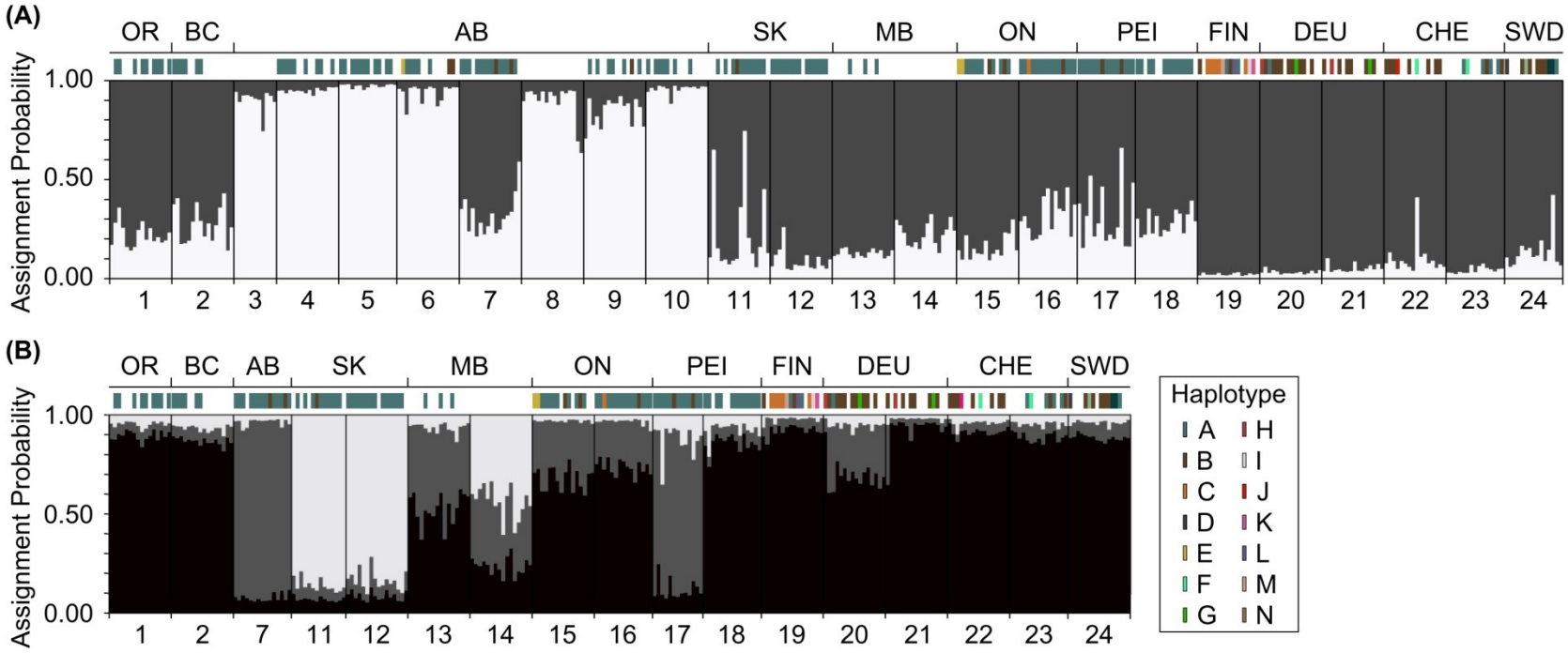

Figure 7-4. Bayesian assignment probabilities base on microsatellite data as inferred by Structure. Each individual is represented by a single column divided in to *K* genetic clusters. A) Assignment of the all individual samples (n = 374) from the whole data set to *K* = 2 genetic clusters. B) Assignment of individuals from cluster 1 (n = 263) to *K* = 3 genetic clusters. Small bars above each cluster plot correspond with mitochondrial DNA haplotypes. Numbers below the plots refer to individual populations from Table 7-1. Sampling regions are abbreviated above the plots: OR = Oregon, BC = British Columbia, AB = Alberta, SK = Saskatchewan, MB = Manitoba, ON = Ontario, PEI = Prince Edward Island, FIN = Finland, DEU = Germany, CHE = Switzerland, SWE = Sweden.

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Appendix 7-1. GPS coordinates for all population samples

Site Number	Site Name	Coordinates (Decimal Degrees)
1	St. Louis	45.1226, -122.9423*
2	Agassiz	49.2425, -121.7633
3	Beaverlodge	55.1938, -119.3988
4	Eaglesham	55.6940, -117.8940
5	Fairview	55.9996, -118.5325
6	Guy	55.6650, -117.2757
7	Hines Creek	56.2483, -118.5014
8	Rycroft	55.6843, -118.6983
9	Sherwood Park	53.5119, -113.1227
10	Whitemud	55.4291, -117.2214
11	Carrot River	53.2156, -103.5144
12	Love	53.4855, -104.2104
13	Carman	49.3695, -98.0386
14	Rosebank	49.3695, -98.1114
15	Kinburn	45.0038, -75.6508
16	Ottawa	45.3814, -75.7067
17	Harrington	46.3402, -63.1591
18	Mermaid	46.2576, -63.0140
19	Hartola	61.5802, 26.0140
20	Bad Bellingen	47.7139, 7.5378
21	Gündlingen	48.0017, 7.6463
22	Delémont	47.3734, 7.3267
23	Riehen	47.5716, 7.6208
24	Sjöbo	55.5938, 13.6305

***** GPS coordinates are approximate

Appendix 7-2. Locus H summary statistics.

Population	1	2	3	4	5	6	7	8
N_a	14	12	10	10	8	10	11	14
N_s	11.76	10.36	10.00	8.01	7.33	8.66	9.19	10.21
H_o	1.00	1.00	1.00	0.56	0.57*	0.80	0.81	0.75
H_e	0.94	0.91	0.89	0.74	0.86	0.88	0.89	0.82
F_{IS}	-0.07	-0.10	-0.13	0.25	0.34	0.09	0.09	0.09
% Missing	6	6	9	0	6	6	0	0
P_{AL}	0	0	0	0	0	0	0	1
Freq. P_{AL}	0	0	0	0	0	0	0	0.031
Population	9	10	11	12	13	14	15	16
N_a	15	12	16	13	14	13	18	15
N_s	11.95	9.75	13.50	11.04	11.54	10.18	13.25	12.11
H_o	0.94	0.75	0.86	0.88	0.94	0.88	0.81*	0.93
H_e	0.93	0.90	0.96	0.91	0.93	0.89	0.94	0.93
F_{IS}	-0.01	0.17	0.11	0.04	-0.01	0.02	0.14	0.00
% Missing	0	0	12	0	0	0	0	0
P_{AL}	0	0	0	0	0	0	1	0
Freq. P_{AL}	0	0	0	0	0	0	0.031	0
Population	17	18	19	20	21	22	23	24
N_a	14	20	18	15	18	15	17	16
N_s	11.50	14.89	13.97	11.74	13.81	12.19	14.45	12.78
H_o	0.73*	0.88	1.00	0.81	1.00	0.75	0.85	0.87
H_e	0.92	0.97	0.96	0.93	0.96	0.94	0.97	0.94
F_{IS}	0.21	0.10	-0.04	0.13	-0.04	0.21	0.13	0.09
% Missing	0	0	0	0	0	0	13	0
P_{AL}	0	0	0	0	1	1	0	2
Freq. P_{AL}	0	0	0	0	0.031	0.063	0	0.066

N_a, number of alleles; N_s, allelic richness based on smallest sample size of 10; P_{AL}, number of private alleles; Freq. P_{AL}, frequency of private alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient. * indicates populations significantly deviate from HWE ($p < 0.05$) after a false-discovery rate correction procedure. % Missing is the percent missing data in each population. Population numbers correspond to populations in Table 7-1.

Appendix 7-3. Locus L summary statistics.

Population	1	2	3	4	5	6	7	8
N_a	4	5	5	6	4	5	5	5
N_s	3.12	4.82	4.82	5.35	3.63	4.24	4.56	4.43
H_o	0.19	0.69	0.64	0.63	0.53	0.38	0.38	0.56
H_e	0.24	0.68	0.71	0.68	0.45	0.58	0.48	0.51
F_{IS}	0.21	-0.02	0.11	0.09	-0.20	0.36	0.22	-0.10
% Missing	0	0	0	0	0	0	0	0
P_{AL}	0	0	0	0	0	0	0	0
Freq. P_{AL}	0	0	0	0	0	0	0	0
Population	9	10	11	12	13	14	15	16
N_a	4	5	4	4	5	3	5	4
N_s	3.45	4.25	3.58	3.25	4.22	2.99	4.53	3.96
H_o	0.31	0.56	0.50	0.44	0.50	0.44	0.56	0.40*
H_e	0.34	0.65	0.50	0.53	0.51	0.49	0.58	0.57
F_{IS}	0.07	0.14	0.00	0.17	0.02	0.11	0.04	0.31
% Missing	0	0	0	0	0	0	0	0
P_{AL}	0	0	0	0	0	0	0	0
Freq. P_{AL}	0	0	0	0	0	0	0	0
Population	17	18	19	20	21	22	23	24
N_a	6	5	6	5	7	5	4	6
N_s	5.50	4.45	5.10	4.23	6.22	4.11	3.64	5.55
H_o	0.80	0.56	0.56	0.40	0.75	0.44	0.57	0.67
H_e	0.71	0.61	0.72	0.53	0.79	0.47	0.51	0.78
F_{IS}	-0.14	0.08	0.22	0.25	0.06	0.07	-0.14	0.15
% Missing	0	0	0	6	0	0	6	0
P_{AL}	0	0	0	0	2	0	0	0
Freq. P_{AL}	0	0	0	0	0.063	0	0	0

N_a, number of alleles; N_s, allelic richness based on smallest sample size of 10; P_{AL}, number of private alleles; Freq. P_{AL}, frequency of private alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient. * indicates populations significantly deviate from HWE ($p < 0.05$) after a false-discovery rate correction procedure. % Missing is the percent missing data in each population. Population numbers correspond to populations in Table 7-1.

Appendix 7-4. Locus Q summary statistics.

Population	1	2	3	4	5	6	7	8
N_a	9	10	8	9	7	9	10	7
N_s	7.55	8.16	7.81	7.19	6.45	7.63	7.71	6.22
H_o	0.88	0.88	0.91	0.63	0.87	0.73	0.81	0.88
H_e	0.81	0.81	0.87	0.79	0.81	0.74	0.84	0.78
F_{IS}	-0.08	-0.09	-0.04	0.21	-0.07	0.01	0.03	-0.12
% Missing	0	0	0	0	0	6	0	0
P_{AL}	0	0	0	0	0	0	0	0
Freq. P_{AL}	0	0	0	0	0	0	0	0
Population	9	10	11	12	13	14	15	16
N_a	7	8	7	8	9	10	12	10
N_s	5.74	6.56	6.10	6.74	7.82	8.51	10.28	8.56
H_o	0.56	0.88	0.56	0.81	1.00	0.81	1.00	0.87
H_e	0.72	0.74	0.80	0.84	0.85	0.87	0.92	0.85
F_{IS}	0.23	-0.20	0.31	0.03	-0.19	0.07	-0.10	-0.03
% Missing	0	0	0	0	0	0	0	0
P_{AL}	0	0	0	0	0	0	0	1
Freq. P_{AL}	0	0	0	0	0	0	0	0.033
Population	17	18	19	20	21	22	23	24
N_a	8	8	11	10	12	12	14	9
N_s	6.79	7.10	9.05	8.63	9.57	9.71	11.36	7.91
H_o	0.67	0.80	1.00	0.69	0.88	1.00	0.87	0.80
H_e	0.79	0.84	0.88	0.89	0.90	0.90	0.93	0.88
F_{IS}	0.16	0.05	-0.14	0.24	0.03	-0.11	0.07	0.09
% Missing	0	6	0	0	0	0	0	0
P_{AL}	0	0	0	0	1	0	1	1
Freq. P_{AL}	0	0	0	0	0.031	0	0.033	0.033

N_a, number of alleles; N_s, allelic richness based on smallest sample size of 10; P_{AL}, number of private alleles; Freq. P_{AL}, frequency of private alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient. No populations significantly deviate from HWE ($p < 0.05$) after a false-discovery rate correction procedure. % Missing is the percent missing data in each population. Population numbers correspond to populations in Table 7-1.

Chapter 8

General Conclusion

Coleophora deauratella Leinig and Zeller (Lepidoptera: Coleophoridae) is an invasive pest of red clover grown for seed production in North America. Larval feeding by *C. deauratella* can drastically reduce seed yields and currently there are no control measures available (Ellis and Bjørnson 1996, Evenden et al. 2010). Like many moths, *C. deauratella* relies on a female-produced sex pheromone for mate finding (Evenden et al. 2010) which can be exploited for integrated pest management (IPM). Two main uses of pheromone in IPM programs are for pheromone-based monitoring and mating disruption. In this thesis, I investigate the use of pheromone to both monitor and manage *C. deauratella* populations in the Peace Region of Alberta, Canada. My goal was to create a pheromone-monitoring system which can be used to determine the relationships between pheromone-baited trap capture, larval numbers, and damage. I also assess the potential of pheromone-mediated mating disruption to control *C. deauratella* in red clover seed production fields and explore the mechanisms by which mating disruption acts to interfere with mating behaviour. Using reservoir-type rope dispensers, I specifically test the hypothesis that communication disruption can be achieved by treatment with complete and partial pheromone formulations although the mechanisms by which this occurs will likely differ between formulations. I also test mathematical predictions of competitive attraction as a mechanism of mating disruption with laminate flake dispensers that release the complete pheromone blend. My final goal was to use pheromone-baited traps positioned throughout the native and introduced range of *C. deauratella* to detect its presence and test the subsequent samples in a phylogeographic analyses to determine the origin of the invasive populations. Ultimately, this study will add to our understanding of the biology and management of *C.*

deauratella in its invasive range and more generally to the mechanisms of mating disruption, while providing useful monitoring and management tools for producers and crop consultants.

Pheromone-based monitoring

The female-produced sex pheromone of *C. deauratella* was recently identified, and the ratio of components and dosages necessary for attraction of males to pheromone-baited traps was quantified (Evenden et al. 2010). However, in order to create a reliable pheromone-based monitoring system the factors that predictably affect trap capture need to be assessed (Mayer and Mitchell 1999, Francis et al. 2007, Reddy et al. 2011). Although several characteristics of the trap design and lure type were studied, the main factors that affect trap capture are trap type and the height of the trap in the crop (Chapter 2). Male moth response to pheromone-baited traps can be influenced by the design of trap which is known to alter plume structure (Knight and Fisher 2006), and the trap colour which may be perceived by the insect (Mitchell et al. 1989, Suckling et al. 2005). More *C. deauratella* males were captured in non-saturating unitraps compared with saturating sticky traps (Chapter 2); however, trap colour had minimal impact on moth capture. Trap colour did significantly affect the capture of non-target *Bombus*. Higher numbers of *Bombus* spp. were captured in yellow and white unitraps compared to green (Chapter 2). Thus, in order to minimize the impact on *Bombus* spp. and other non-target organisms, I recommend using green unitraps to monitor *C. deauratella.*

Trap height also influences the capture of insects in pheromone-baited traps. More *Grapholita molesta* Busck (Lepidoptera: Tortricidae) were captured in traps placed in the lower compared to the upper tree canopy within an orchard (Kovanci et al. 2006), whereas *Stenoma catenifer* Walsingham (Lepidoptera: Elachistidae) capture was not affected by trap height, therefore a convenient height for placement and monitoring of 1.75 m in the avocado canopy was

recommended (Hoddle et al. 2011). Traps placed 35 cm above the soil surface, within the clover canopy, captured significantly more moths than those applied at ground level or 1 m above the ground (Chapter 2). Male *C. deauratella* most often fly just above the canopy in red clover fields which may increase their chance of encountering a calling female or may avoid natural enemies. Based on the results of Chapter 2, the recommended trapping system for *C. deauratella* is a green unitrap deployed 5 m from the field edge and 35 cm above the soil. These parameters will lead to consistent results between field sites and are simple for producers and crop consultants to follow and monitor.

Determining the characteristics that affect pheromone-baited trap capture of *C. deauratella* allows for the creation of a pheromone-based monitoring program which can be used to determine the overall flight phenology of the insect and relationships between the number of males captured, larval abundance, damage, and seed yield. Often these relationships are the first step to the establishment of an economic threshold for insect pests (Turnock 1987, Cullen and Zalom 2005). Trap capture of male *C. deauratella* was significantly and positively related to larval abundance and the proportion of seed damage at sampled sites (Chapter 3), which indicates pheromone-baited traps can be used to assess population densities. This relationship was linear even at the moderate to high pest densities sampled in the current study which contrasts with other studies on *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Miluch et al. 2013) and *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae) (Jones et al. 2009) in which high population densities led to a plateau or decline in trap capture.

The proportion of seed damaged in each field also corresponded with trap capture in two out of three years (Chapter 3). The seed damage found across sites in Alberta (0.016-0.043) was lower than the proportion of seed damage in Ontario (0.064) where seed loss was estimated at

80% (Ellis and Bjørnson 1996). In Alberta, seed loss can exceed 99% which indicates that there may be other mitigating factors besides *C. deauratella* pest pressure contributing to the reduction in yield of the seed crop compared to that found in Ontario (Evenden et al. 2010). This is further supported by the fact that in all three years of this study, larval numbers (~1/4.63 larvae/inflorescence) were never as high as those in Ontario in 1989-90 (1.0-2.4 larvae/inflorescence) (Ellis and Bjørnson 1996). Unfortunately, relationships between seed yield and trap capture could not be determined as the data was intermittent over the years. Ultimately, it would be beneficial to conduct further experiments to determine if seed yield is also related to trap catch data.

Phenological models based on $DD_{11.7}$ consistently described the median male *C. deauratella* flight period better than the ordinal date model (Chapter 3). The model found that 258.3 $DD_{11.7}$ from January 1 are needed for median flight to occur. Several other studies have also found degree days to be a more accurate prediction of flight period than ordinal dates (Potter and Timmons 1983, Riedl et al. 1976, Rice et al. 1984, Hoffman and Dennehy 1989). Degree days are based on physiological time and can vary with temperature, which has been used to describe poikilotherm development for years (Davidson 1944). Whereas, ordinal date is standard and does not vary with climate conditions and therefore will not describe poikilotherm development as well. By being able to predict the male flight period, sampling effort for larvae may be improved and in the future phenological models could compare the flight period with oviposition and larval development within the crop to help time control tactics.

Pheromone-mediated mating disruption

Although a pheromone-monitoring program is a useful tool to predict population densities and damage, a non-insecticidal control tactic for *C. deauratella* in its extended range is needed. Red clover seed production is highly dependent on honey bees (*Apis meliferra* L.) and bumble bees (*Bombus* spp.) for pollination (Forester and Hadfield 1954, Holm 1966) so any management strategy must take into consideration the impact on these non-target organisms. Pheromone-mediated mating disruption is an alternative, species-specific, control method which acts by release of synthetic sex pheromone into the cropping environment to prevent or delay mating (Baker and Heath 2005, Witzgall et al. 2010). Mating disruption may work especially well against *C. deauratell*a as larvae of this univoltine species overwinter as mature fourth instars, and pupation occurs in the spring. Thus, the adult stage can be targeted early in the spring before larval feeding damage occurs. Further, this study is the first to examine the potential for mating disruption in a forage seed crop, and will add to the relatively few mating disruption studies conducted on field crop pests.

Based on field studies, my work has shown that mating disruption can be used to prevent males from locating pheromone-baited traps (Chapter 4, 5, 6) and can suppress *C. deauratella* populations (Chapter 6). This is the first mating disruption study on a coleophorid moth, but several others within the superfamily Gelechioidea are also controlled with mating disruption (Cardé et al. 1998, Vacas et al. 2011, McCormick et al. 2012). Rope, aerosol, and laminate flake dispensers, that release the complete two-component pheromone formulation, significantly reduced the number of *C. deauratella* males captured in pheromone-baited assessment traps positioned in pheromone treated small-plots (0.25 ha) compared to those in untreated control plots (Chapter 4, 5, 6), indicating that sexual communication is disrupted by pheromone

treatment. Rope dispensers (1000/ha) provided the highest level of communication disruption (99.6%) of the dispensers tested in small plot studies compared to 93.6% disruption achieved by treatment with laminate flakes (280 g flakes/ha) and 60.7% disruption provided by aerosol dispensers (4/ha). The release rate of each dispenser type varied. Rope dispensers released the highest amount of pheromone per day (4.19 g AI/ha/day), followed by aerosol dispensers (1.82 g AI/ha/day) and finally, laminate flake dispensers (0.28 g AI/ha/day). It appears that the design of the small-plot experiments had a greater impact on the level of communication disruption compared to the amount of pheromone released as the poorest disruption was observed with aerosol dispensers that released the second highest overall amount of pheromone. Significant communication disruption was achieved in small plots even at high moth population densities (> 100 moths captured/night in untreated controls) which further suggests that this species is amenable to control by pheromone-based mating disruption.

Wind disperses pheromone throughout the pheromone-treated cropping environment (Gut et al. 2004). The single large plume released from the one aerosol dispenser positioned in the centre of the small plot was probably not distributed evenly by the wind throughout the plot and as a result communication was disrupted by only 60% in small plots treated with aerosol dispensers. The plots were relatively small (50 m X 50 m) and wind can deplete pheromone up to 15 m into the plot (Milli et a. 1997) which may have led to pockets of clean air at the edge of each plot which allowed males to find the assessment traps located only 12.5 m from the edge. Other studies have used aerosol dispensers placed around the edge of plots or in large plots to overcome these edge effects (Shorey and Gerber 1996, Burks and Brandl 2004). In comparison, relatively even coverage of pheromone would have been achieved with both the rope and flake dispensers tested here.

There are several issues regarding assessment of mating disruption in small-plots including immigration of mated females and attraction of males. Immigration of mated females is a primary concern as females may mate outside of pheromone-treated areas, and then enter the treated areas to oviposit which can lead to edge effects with higher damage at the field margins (Cardé and Minks 1995). If attractive formulations are used, males may be attracted into small-plots which inadvertently increase population densities and mating disruption may appear to fail (Witzgall et al. 1999). Mating disruption treatments applied to large plots over wide areas can mitigate these concerns by reducing the likelihood of immigrating mated females or the attraction of males which can confound the effects of the pheromone treatment. Treatment of large areas with pheromone dispensers for mating disruption has been successful for several species including *G. molesta* (Vickers et al. 1985, Il'chev et al. 2002), *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) (Cardé and Minks 1995), and *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Thomson et al. 2001). Thus, even though aerosol dispensers only demonstrated a moderate level of disruption in small plots compared to the other formulations tested, they were also tested in large-plot studies (Chapter 5).

There was a significant reduction in pheromone-baited *C. deauratella* trap capture in large (5 ha) plots treated with aerosol dispensers compared to untreated control plots; however, there was no significant reduction in larval numbers or increase in seed yield as a result of pheromone treatment (Chapter 5). Disruption of pheromone-based communication does not always result in a reduction of larvae in the next generation or a significant decline in crop damage. Other attempts of mating disruption of lepidopteran pests in a variety of cropping systems have illustrated communication disruption but no resultant reduction in crop damage including *Choristoneura rosaceana* Harris (Tortricidae) (Lawson et al. 1996) and *Anarsia*

lineatella Zeller (Gelechiidae) (Baker and Heath 2005). Failure of mating disruption was most likely a result of immigration of mated females in the current study. Trap capture in pheromone-baited traps was still reduced in traps positioned towards the edge of the plot in pheromone-treated plots compared to that in untreated control plots, indicating pheromone was still present towards the edge of the large plot treated with 10 aerosol dispensers. There were higher numbers of larvae at the edge of both control plots and large plots treated with aerosol dispensers. Plots were surrounded by clover from which females could immigrate into the study plots. If pheromone concentration was reduced at the edge and caused an edge effect, a lower number of larvae would be expected in the centre of the treated plots compared to untreated control plots, but this was not observed (Chapter 5). The dispersal capability of *C. deauratella* is unknown, and it seems plausible that moths are capable of flying ~111 m to the centre of the plot, therefore to overcome the possible effects of immigration, mating disruption should be applied area-wide (Ogawa 1990) or at least to a whole field (~64.7 ha).

In contrast to the large-plot study using aerosol dispensers, treatment of large (5 ha) plots with laminate flake dispensers resulted in a significant reduction of male *C. deauratella* capture in pheromone-baited assessment traps and a significant reduction of larvae sampled in these same plots. Pheromone treatment with laminate flakes also increased seed yield (Chapter 6). Season-long trap capture was suppressed by ~72% in treated plots compared to untreated controls, however, suppression declined as the season progressed from a high of 98.6% in June to 38.8% in August. In order to maintain a high level of communication disruption (>90%), the release rate of the flakes should be increased or another application of flakes should be applied at mid-season. Samples of field-aged flakes still contained a significant amount of pheromone equivalent to 11.7 g AI/ha at the end of the season which suggests the flake formulation should

be modified to maximize pheromone release as any unreleased pheromone at the end of the season is wasted. Unlike the large-plot study with aerosol dispensers (Chapter 5), the number of larvae at both the interior and edge of laminate-flake treated plots was reduced compared to larvae sampled in the untreated control plots (Chapter 6). This indicates that mating was disrupted in the treated plots and immigration of mated females does not appear to be a factor in the large plots. The even distribution of flakes throughout the plot most likely prevented pockets of untreated air around the plot edges, and thus no edge effects were observed (Ogawa 1990, Karg and Sauer 1995). Although larval numbers were significantly reduced in plots treated with laminate flakes, seed yield was only marginally increased by 58.0 kg/ha, unfortunately this increase in seed yield would not offset the cost of applying the flakes. Mating disruption of *Lymantria dispar* L. (Lepidoptera: Eberidae) using laminate flakes is successful when flakes are loaded with 17.9 % disparlure (Tcheslavskaia et al. 2005), this is over 8 % higher than the active ingredients loaded into flakes for *C. deauratella* in this study. It appears that an increase in the number of applications or an increase in the release rate per flake is needed to further reduce larval numbers and have an economically significant effect on seed yield.

In the large-plot experiments, the aerosol dispensers released ~672 mg AI/ha/day compared to the season-long average of 205.2 mg AI/ha/day for the laminate flake dispensers (Chapter 5, 6). The higher release rate of the aerosol dispensers coincided with a higher percent disruption (93.7 %) compared to the laminate flakes (~72 %). The results of these experiments suggest that season-long communication disruption of *C. deauratella* of > 90 % can occur when the release rate of pheromone is ~672 mg AI/ha/day and in the future the release rate of each dispenser type should be modified to optimize mating disruption. Furthermore, the number of point sources releasing pheromone was vastly different between aerosol (2 dispensers/ha) and

laminate flake dispensers (38 102 dispensers/ha) in large-plot experiments. The even distribution of laminate flakes throughout the plot led to a reduction in larval numbers and slight increase in seed yield which suggests a more even distribution of dispensers is preferable to the sparser and clumped distribution of the aerosol dispensers (Chapter 4).

Mechanisms of mating disruption on *C. deauratella*

Mating disruption has the potential to suppress *C. deauratella* populations (Chapter 5), however, characteristics of the pheromone formulation and dispenser type can result in different mechanisms which act to disrupt mating even on the same species (Evenden et al. 1999, Reinke et al. 2014). When the complete pheromone blend was released from rope, aerosol or flake dispensers a combination of competitive and non-competitive mechanisms likely occurred (Chapter 4, 5, 6). However, when the major component alone was used, disruption was most likely achieved by one or more non-competitive mechanisms (Chapter 3). Minks and Cardé (1988) hypothesized that synthetic pheromone blends that most closely resemble the natural pheromone blend of the target species should be the most effective mating disruptants as more mechanisms will be invoked compared to similar treatment with suboptimal incomplete or off-ratio blends. However, several studies have refuted this hypothesis, as mating disruption can be just as successful with incomplete blends or off-ratios (Evenden et al. 1999, Fitzpatrick et al. 2004, McCormick et al. 2012). The current study also refutes the Minks and Cardé (1988) hypothesis, and found that a partial blend can be a better disruptant than the complete blend in small-plot trials (Chapter 4). Treatment with the partial blend in rope dispensers reduced male *C. deauratella* pheromone-baited trap capture significantly more than dispensers releasing the complete blend (Chapter 4). Further, *C. deauratella* males were attracted to dispensers releasing the complete blend in pheromone-treated plots, indicating false-trail following or competitive

attraction as a mechanism when the full blend is used. Few moths oriented to rope dispensers releasing the major component. Previous studies (Evenden et al. 2010) also confirmed that male *C. deauratella* are not attracted to the point of source contact to the major component alone. Attraction of moths to high releasing pheromone dispensers is not uncommon as several other species are capable of orienting and landing near these dispenser types (Cardé et al. 1998, Reinke et al. 2014, Stelinski et al. 2004a) and in *C. deauratella* attraction may be attributed to a shift in the pheromone response threshold in the pheromone-treated environment as a result of adaptation or habituation. Further, dispensers releasing the complete blend at the edge of plots attracted more moths than those in the interior of the same plot (Chapter 4) which indicates that camouflage, adaptation or habituation may occur in the interior of the plots.

Electroantennograms conducted after individual *C. deauratella* males were exposed to high doses of the complete blend or major component alone released from rope dispensers for one hour illustrated that antennal adaptation occurs to low doses of pheromone stimuli (Chapter 4). However, this result may be misleading as moths were pre-exposed to high doses of pheromone they would not normally encounter in the field. Antennal response of pre-exposed males to high pheromone stimuli is stronger than that of untreated control moths indicating a classic threshold elevation (Mafra-Neto and Baker 1996). Interestingly, the shift in the response threshold is different for moths exposed to the complete blend and major component, with moths pre-exposed to the complete blend having a stronger response to pheromone stimuli than those exposed to the major component alone. This most likely is a result of differential adaption of the pheromone receptors tuned to the major and minor components. Antennae pre-exposed to the major component alone experience less of a shift in response threshold because the minor component receptors are not adapted (Chapter 4). The results of the study indicate the

mechanisms of mating disruption vary when different pheromone formulations are used against *C. deauratella.* When the complete blend is used for disruption, false-trail following can initially occur which can then subsequently expose moths to high levels of pheromone that may result in adaptation or habituation (not tested). Whereas, when the major component alone is released, adaptation or habituation (not tested) are the main mechanisms (Chapter 4).

A combination of attraction and habituation has been hypothesized as the mechanisms to explain how aerosol dispensers disrupt mating (Baker and Heath 2005). The high concentration of pheromone released from aerosol dispensers is thought to attract males far down wind of the dispenser, as males fly upwind in the plume they may become habituated to the pheromone (Baker and Heath 2005). In this thesis, the mechanisms of mating disruption of aerosol dispensers were not studied with a specific experiment but an incidental observation of moth scales similar to those of *C. deauratella* located on the nozzle port of the aerosol dispensers indicates that males were attracted to the dispensers and false-trail following could occur (Chapter 5). This is supported by recent evidence that aerosol dispensers disrupt *C. pomonella* through competitive attraction (McGhee et al. 2014) and adds overall support to the theory that aerosol dispensers disrupt mating through competitive attraction (Baker and Heath 2005).

Mechanisms associated with female-equivalent dispensers (i.e. flakes, wax droplets) are hypothesized to act via competitive attraction and empirical evidence supports this hypothesis in a number of moth species (Stelinski et al. 2005, Stelinski et al. 2008a, Reinke et al. 2014, Rodriguez-Saona et al. 2014). Studies to test the theoretical models of Miller et al. (2006a) and determine the mechanisms that mediate mating disruption when laminate flakes are used against *C. deauratella* were conducted in Chapter 6. The results of a small-plot study in which the density of flake dispensers was varied were comparable to the mathematical formulae and

graphical disruption profiles used to predict competitive or non-competitive mechanisms in mating disruption-treated studies (Miller et al. 2006a). The results of the graphical disruption profiles in the current study support a competitive attraction mechanism which is further supported by observations of male *C. deauratella* attraction to laminate flakes. Theory from Miller et al. (2006a) was subsequently used to determine the dispenser activity (area over which one dispenser can reduce the maximum trap capture by half) and the dispenser application activity (potency of a given pheromone formulation applied to one ha) for laminate flakes on *C. deauratella*. The dispenser application activity is two-fold higher than any previously reported values (Miller et al. 2006b) and may be partially driven by the large number of laminate flakes applied to each field which would create thousands of pheromone point sources that compete with calling females. Furthermore, using the equations of Miller et al. (2006a), I calculated the theoretical percent disruption using the maximum number of flakes used in this study which gave a value of 97.9% disruption, which is only slightly higher than the actual value obtained in the field with the maximum number of flakes (96.8%). This adds to a growing body of literature (Miller et al. 2006b, McGhee et al. 2014, Reinke et al. 2014, Rodriguez-Sanoa et al. 2010) that evaluate mechanisms using the theory presented in Miller et al. (2006) and illustrate that competitive attraction is occurring in plots treated with laminate flake dispensers to disrupt *C. deauratella*.

Across all *C. deauratella* mating disruption studies population densities were high and most often mating disruption failure is attributed to high population densities (Gut et al. 2004, Baker and Heath 2005). However, this doesn't seem to be the case for *C. deauratella*. Often non-competitive mechanisms, which are density independent, are thought to be invoked with successful disruption at high population densities (Miller et al. 2006a, Stelinski et al. 2008b).

Competitive attraction can disrupt *C. deauratella* orientation to traps (Chapter 4, 6); however, it may lead to non-competitive mechanisms being invoked. Moths flying towards dispensers may expose themselves to high levels of synthetic pheromone which may lead to desensitization (adaptation or habituation) which can prevent males from being able to locate calling females. The results of the EAG study (Chapter 4) also suggest that subsequent exposure to high doses of pheromone can increase the response threshold of *C. deauratella* males, so that they would no longer react to the minute quantities of pheromone released by a calling female. Thus, competitive attraction leading to subsequent adaptation or habituation may explain why mating disruption was successful against *C. deauratella* even at high population densities.

Tracing the invasion of *C. deauratella* with pheromone traps and population genetics

In order to identify the source of *C. deauratella* populations and determine possible routes of invasions in North America, I used pheromone-baited traps to collect specimens throughout North America and Europe (Chapter 7). This study is the first to isolate microsatellite markers for species within Coleophoridae and one of only a handful developed for the superfamily Gelechioidea (Torres-Leguizamon et al. 2009, Liu et al. 2006, Bettaibi et al. 2013). In general, microsatellites have been difficult to develop for Lepidoptera (Zhang 2004, Ji and Zhang 2004) and this is also the case for *C. deauratella*. The success rate from positive insert-bearing clones to useable markers was < 2% for *C. deauratella* which is similar to the low cloning efficiency observed for *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (2.5%) (Ji et al. 2003). Furthermore, the success rate of obtaining microsatellites in many Lepidoptera species is no more than five loci per genomic library in 80% of the cases (Ji and Zhang 2004) and I was only able to develop four usable microsatellite markers. The difficulty in the development of microsatellites for Lepidoptera has been attributed to multiple copies of the

microsatellites in the genome, and microsatellites with similar or identical flanking regions (Zhang 2004, Meglécz et al. 2004, Meglécz et al. 2007). Even after microsatellites have been developed, they are often associated with a high proportion of null alleles (Meglécz et al. 2007) in the Lepidoptera. Each of these problems was also associated with the development of microsatellite markers for *C. deauratella* (Chapter 7). Nevertheless, microsatellites once developed can be useful for population genetic studies (Valade et al. 2009, Boisson et al. 2012) and may contribute to studies of closely related species through cross-species amplification (Lumley et al. 2009).

Both microsatellite markers and mitochondrial DNA sequence data indicate that within continents there was only weak population structure. Weak structure may result from long-range transport of *C. deauratella* which can introduce new individuals into the gene pool in different areas and lead to a mixed genetic structure. I also found evidence of a founder effect by which the genetic diversity was reduced in North America compared to Europe (Chapter 7). Reduced genetic diversity (genetic bottleneck) may reduce the fitness of populations (Reed and Frankham 2003), but that does not appear to be the case with *C. deauratella* as it has successfully colonized a large area of North America. Populations in North America are composed of two main genetic clusters, one cluster primarily of Alberta populations, and the other composed of all other North American and European populations (Chapter 7). The unique cluster found in Alberta populations may be the result of a second introduction into North America, or a subsequent founding event from another North American population with a limited number of individuals. Evidence provides more support for the latter theory, as there is reduced genetic diversity within Alberta compared to the rest of the North American populations which is indicative of a founder effect. As well, population outbreaks, such as the one that has occurred in Alberta since 2005,

have been shown to homogenize genetic structure (Chapuis et al. 2008) and can lead to unique genetic clusters compared to nearby endemic populations (Boisson et al. 2012).

Through this study, I identified specimens of *C. deauratella* from western Oregon, the largest forage seed production region in the world (Wong 2005). Previously, producers in western Oregon were unaware of the presence of *C. deauratella* (N. Anderson, pers. comm.). This early detection will allow Oregon producers to monitor the situation and determine if *C. deauratella* is causing damage in their crops. The populations present in Oregon, are not closely related to those found in the outbreak area of Alberta, and suggest that the Oregon populations are remnants from a different introduction. The origin of North American populations could not be specifically pin-pointed, most likely based on the limited populations sampled in the native range; however, the study identifies a possible area of introduction in North America as southern Ontario or the adjacent United States (Chapter 7). Further sampling in the native range of *C. deauratella* combined with analyses based on the microsatellites developed here may in the future identify the origin of North American populations and allow for biological control agents to be explored.

Future directions

Coleophora deauratella is still a significant pest of red clover seed production fields in Alberta, and has the potential to develop into a problematic pest in Oregon. The results presented here contribute to the knowledge of the biology of *C. deauratella* and provide a useful pheromone-monitoring tool (Chapter 2, 3, 4) that can predict larval density. Furthermore, the potential to suppress populations of *C. deauratella* through mating disruption has been demonstrated (Chapter 4, 5, 6). Mating disruption studies combined with EAG and theoretical

predictions (Chapter 4, 5, 6) allowed for the mechanisms of mating disruption to be further explored and understood.

There are opportunities to further examine the biology of *C. deauratella* which could have major implications on its control. Currently, it is not known how fecund females are or how many times they mate. Dissections of females were performed (data not presented); however, it was extremely difficult to determine the number of times they had mated given their small size and lack of a true spermatophore. One possible way to overcome this would be to stain female preparations for sperm to determine if they had mated, but this would not be able to determine how many times they mate. It is also not known how far *C. deauratella* can disperse. Dispersal ability will have implications for mating disruption as the farther moths can travel the more likely they would be to enter plots from untreated areas (Baker and Heath 2005). *Coleophora deauratella* dispersal ability could also add to the information found on their invasion routes, if they are capable of long distance dispersal, then the theory of human-mediated transport which led to weak genetic structure (Chapter 7) may be incorrect.

Relationships between pheromone-baited trap capture and larval abundance and damage are useful prediction tools (Chapter 3), but ultimately it should be determined if there is a relationship with seed yield. Yield is the ultimate goal of any producer, and an early indication of potential yield may allow producers more management options to change their strategy for the field (i.e. may leave for green manure rather than harvest for seed). Furthermore, phenology models should be developed which take into consideration oviposition and larval stage. Although currently insecticides are not used to control *C. deauratella*, a phenological model which could detect when the majority of egg laying occurs may be used to time sprays against eggs, before larvae have the chance to hatch and enter the flower.

This is the first study that has used pheromone-mediated mating disruption to control insect pests in a forage seed crop. Most mating disruption studies have been developed for high-value crops (i.e. fruits, vegetables, nuts, cotton) and the cost of dispenser and application technique will need to be lowered in order to use mating disruption in clover seed production. The laminate flake dispensers show the most promise for suppression; however, the plastic residue of the flakes remaining in the field is undesirable. In the future biodegradable flakes should be investigated, the release rate of which has been determined (results not shown). As well, flakes releasing the major component alone should be tested as this would also reduce their cost.

This study adds to the growing knowledge on the mechanisms by which mate finding is disrupted in pheromone-treated crops. This study is the second, after Lapointe et al.2009, to show that a partial pheromone blend is more successful at communication disruption than the complete blend (Chapter 4). However, this may be a result of the study design. This was a small-plot study and given that the complete blend is attractive whereas the partial blend is not, the complete blend may have attracted moths from outside the plot and inadvertently increased the male population density. To overcome this effect and determine if the partial blend is truly more successful, large-plot studies are needed. Large-plots would help to mitigate the effect of immigration (Gut et al. 2004) and determine if this is a true effect.

The shift in the response threshold after moths were exposed to high doses of pheromone from rope dispensers in Chapter 4 is different for moths exposed to the complete blend and major component. Moths pre-exposed to the complete blend have a higher response to the pheromone stimuli than moths exposed to the major component alone. This most likely is a result of differential adaption of the pheromone receptors. Receptors specific to the major and minor

components adapt when exposed to the complete blend, while receptors specific to only the major component would adapt when pre-exposed to the major component alone. Thus, when the complete pheromone blend is used as the EAG stimulus, antennae from males pre-exposed to the major component alone show less of a shift in response threshold because the minor component receptors are not adapted (Chapter 4). This observation could be further tested with single sensillum recordings to determine the adaptation of each receptor type to the complete blend and major and minor components alone and could be enhanced by stimulation of the antennae with major and minor components separately. This could also determine whether any cross-adaptation occurs in individual receptor types (D'Errico et al. 2013). Further studies could also be performed in the field to determine if sensory system imbalance is a mechanism when the major component alone is released from dispensers. Field trials with dispensers releasing the major component alone evaluated with pheromone traps baited with lures that vary the concentration and ratio of the two components in the complete blend (Judd et al. 1995) could help to determine if this mechanism occurs.

Crop breeding for *C. deauratella* resistance is another potential area of future research. In my investigations I was able to trap thousands of male *C. deauratella* in alsike clover (*Trifolium hybridum* L.) fields; however, I was unable to find any developing larvae even though alsike clover is a potential food plant (Landry 1991). It is possible that alsike flowers are unable to support much *C. deauratella* larval development as their morphology is quite different than red clover flowers. Another possible explanation is that alsike clover ovules have glandular trichomes on their surface (Retallack and Martin Willison 1988) that may prevent *C. deauratella* larval feeding, whereas red clover ovules have no trichomes. Altaswede is the dominant variety of red clover grown in the Peace region of Alberta, and it is considered a "hairless" variety in

which very few trichomes are present (Nowsad et al. 1953). Trichomes can interfere with oviposition of a variety of species including *Diatraeae saccharalis* (F.) (Lepidoptera: Pyralidae) (Sosa 1988) and *Maruca testulalis* (Lepidoptera: Pyralidae) (Oghiakhe et al. 1992) and if a red clover variety was bred to have trichomes on the sepals or calyx of the florets *C. deauratella* oviposition may be deterred.

The knowledge that *C. deauratella* may be transported across long distances by humans shows that producers should be on the lookout for insect pests. There is still a possibility to identify the source populations of North American specimens based on a population genetics approach and the microsatellites developed here. More populations from the native range particularly Scandinavia, mainland Europe (including the Mediterranean region) and the Middle East would be ideal. More samples from the United States would also further determine the population structure in North America and confirm if southern Ontario or the adjacent United States was the primary site of introduction (Chapter 7). Furthermore, other researchers are actively pursuing biological control agents for *C. deauratella* so any information on the origin may help to locate *C. deauratella* populations that can be examined for potential biological control agents.

There is also a possibility that the microsatellites developed here may be used for cross-species amplification (Lumley et al. 2009). In particular, another Coleophorid, the larch casebearer, *Coleophora laricella* Hübner (Lepidoptera: Coleophoridae), was accidently introduced from Europe into North America and has become a significant pest of larch (*Larix* spp. (Pinaceae)) (Torgersen 2001). Microsatellites developed here may help determine the population structure of *C. laricella* which may have implications on control methods used in North America.

Final thoughts

I believe this study demonstrates the utility of pheromones in IPM in a novel setting (forage seed production) and demonstrates the potential for sustainable control. I identified and developed a pheromone-monitoring tool which is now commercially available for producers and crop consultants to use to estimate population densities within their field (Chapter 2, 3). A pheromone-monitoring tool capable of predicting population densities would allow for earlier decisions to be made as to whether to keep the crop for seed, hay or green manure. This is the first study to demonstrate the use of mating disruption in a forage seed crop and is successful even under high population densities which often causes mating disruption to fail (Baker and Heath 2005) (Chapter 4, 5, 6). This study also demonstrates that the partial pheromone blend can be more successful at communication disruption than the complete blend and refutes the hypothesis of Minks and Cardé (1988) that the natural blend is the most efficacious and supports the findings of Evenden et al. (1999) that the most attractive blend is not necessarily the most effective mating disruptant. I confirm that multiple mechanisms cause mating disruption of *C. deauratella* (Chapter 4, 5, 6), and it appears that non-competitive mechanisms may be of utmost importance as the high population densities observed in this study are generally not controlled when competitive mechanisms act alone. Finally, this is the first study to develop microsatellite markers for a Coleophorid and they may be useful for other closely related species. The putative area of introduction was identified in North America as being southern Ontario or the adjacent United States and it appears only a few introductions occurred in the introduced range (Chapter 7). This study adds to the literature on successful invasions following a founder effect which results in a genetic bottleneck and in the future may help to identify areas to be searched for biological control agents (Chapter 7). Overall, the results of the study indicate that mating

disruption has the potential to suppress *C. deauratella* populations and in the future an IPM program that combines mating disruption and biological control may be successful at helping reduce *C. deauratella* populations.

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