University of Alberta

Plasticity in response to semiochemicals as part of a reproductive diapause syndrome in a long-lived moth, *Caloptilia fraxinella* (Lepidoptera: Gracillariidae)

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Ecology

Department of Biological Sciences

Spring 2014
Edmonton, Alberta

Abstract

In Lepidoptera that exhibit a delay in mating, response to semiochemicals associated with mating may be plastic, to optimize timing of reproductive events with appropriate environmental conditions. This thesis examines male response to female sex pheromone and male and female response to host plant volatiles in a long-lived adult moth, *Caloptilia fraxinella* (Lepidoptera: Gracillariidae). Adult *C. fraxinella* undergo a nine month period of reproductive inactivity from eclosion in July until the following spring when adults emerge from overwintering locations in a reproductively active state. Male response to female sex pheromone is highest when moths are reproductively active. In Chapters 2 and 3, I investigate exogenous and endogenous mechanisms that impact male pheromone response plasticity. When males are in reproductive diapause, long day/warm conditions terminate, while short day and cool conditions or a natural declining photoperiod maintain diapause. The juvenile hormone analogues (JHA) methoprene and pyriproxyfen similarly enhance male antennal and behavioural response to pheromone in the fall. In the most recent experiments, JHA treatment impacts pheromone response earlier in the summer, indicating a possible physiological change in the tested population of moths. Chapter 4 tests whether males and females exhibit plasticity in response to host plant volatiles that depends on physiological state. Male behavioural response to pheromone is not enhanced by the presence of an ash seedling. Male and female antennal response to individual ash tree volatiles is plastic, and response to the volatiles is highest when moths are reproductively active. JHA treatment enhances male and female

antennal responses to host volatiles during reproductive diapause, and impacts males and females differently. Chapter 5 confirms that males are in a state of reproductive diapause in the summer and fall with shorter sex accessory glands compared to reproductively active males in the spring. JHA treatment enhances the protein content of male sex accessory glands in the fall. The main proteins present in male sex accessory glands are identified. The plasticity of response to semiochemicals documented here in *C. fraxinella* depends on physiological state, and is mediated by environmental conditions and JH in males, and JH and nutrition in females.

Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Maya Evenden for her endless support, encouragement and interest in my project. Dr. Evenden always encouraged me to research and try new experimental techniques, and to develop a greater curiosity and understanding of my own project. Drs. B. A. Keddie and C. Sturdy were also part of my supervisory committee for the last six years, and Dr. R. Kaufman for five years before his retirement, and they were integral to the completion of this project. I am grateful for the support and advice provided to me by my committee, and I have benefited from their time and commitment to my project. I would also like to thank Dr. K. Tierney for being my arms-length examiner and Dr. H. Fadamiro for agreeing to be my external examiner.

I would like to thank the wonderful Evenden lab, including past and present students Caroline Whitehouse, Boyd Mori, Tyler Wist, Christina Elliott, Jessica Kwon, Chris Miluch, Marius Aurelian, Brad Jones, Artem Abdukakharov, Ellis Chan, Jeremiah Bolstad, Ronald Batallas, Chaminda De Silva and Amanda St. Onge. I have valued my time with them, and appreciate all of their help, advice and the company they provided while conducting experiments. My experience doing this project was enriched both professionally and personally from being in the lab with everyone. Thank you especially to my hard-working summer students, including Matt Ferguson, Elizabeth Hodges and Brady Chase who helped with dissections, moth collections and running experiments. Thank you to Tyler Wist for his observations and photographs of *Caloptilia fraxinella* and Caroline Whitehouse and Boyd Mori for statistical help.

Collecting *Caloptilia fraxinella* can be a time-consuming task, and I could not have done it alone. I thank Dr. M. Evenden and members of the Evenden lab, Hannah and Nyssa Dokken, Andrew Lechelt, Dr. H. Proctor, Dr. B. A. Keddie, Dr. B. Heming and Dr. J. Roland for aiding in the collection of *Caloptilia fraxinella*.

I thank Dr. C. Davis and C. Anders for their invaluable help with protein extraction and analysis, and Dr. B. A. Keddie for advice and help with protein analysis. Thank you to Dr. Wilbert Ronald and Jeffries Nurseries of Portage la Prairie, Manitoba for donating the ash seedlings.

I would also like to thank my family, for their endless love and support. My parents, Donna and Ken Lemmen encouraged me to go back to school and enter the field of Entomology, and I could not have accomplished this without their encouragement. I am eternally grateful to my husband, Andrew Lechelt for standing by me through my degree, for bringing suppers and coffee to me during late nights in the lab, and for working hard to make our home happy and supportive. Thank you also to our son Henry and our doggies Jimmy and Lillian for bringing fun and playtime to every day.

I am very grateful for the funding for this project that was provided by the Alberta Crop Industry Development Fund, the University of Alberta and an NSERC-PGS-D3 scholarship to J.L.

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List of Abbreviations

BA – Biogenic Amine

BCA – Bicinchoninic

BSA – Bovine Serum Albumin

JH – Juvenile Hormone

JHA – Juvenile Hormone Analogue

ORN – Olfactory Receptor Neuron

SAG – Sex Accessory Gland

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Chapter 1

General Introduction

In the Lepidoptera, females signal with sex pheromone to attract males. The male response to the signal can be energetically costly and increase the risk of predation to males (Cardé and Haynes 2004). Due to this cost, mate-detection and location behaviours in Lepidoptera should be limited to times when females are highly receptive to mating (Anton et al. 2007). The Lepidoptera can be characterized into four main groups based on reproductive strategies and characteristics (Ramaswamy et al. 2007). The first group includes species in which female reproductive development is controlled by ecdysteroids and females eclose with a full complement of eggs and mate immediately upon eclosion. In the second group, females eclose with the majority of eggs mature and both ecdysteroids and juvenile hormone (JH) control egg development. Only JH controls egg development in females in the third group, which mate several hours after eclosion. Females in the fourth group initiate egg development in the adult stage under the control of JH. Adults in this group typically mate at least several days after eclosion and adult females require a food source to develop their full complement of eggs. Lepidoptera in this fourth group have an extended adult life stage during which there is opportunity for plasticity in the detection of semiochemicals that dictate mating behaviour.

In Lepidoptera with a prolonged adult stage that experience a delay in mating due to migration or reproductive diapause, signalling systems used for

mate finding and oviposition should be plastic and most acute at reproductive maturity and under suitable environmental conditions for mating and offspring production and development (Anton et al. 2007). In Lepidoptera that undergo a period of reproductive inactivity as an adult, males are typically unresponsive to female sex pheromone until they are physiologically capable of mating under conditions that are appropriate for mating to occur (Anton et al. 2007). Reduced pheromone responsiveness in newly-eclosed or reproductively inactive male Lepidoptera has been documented in several species of noctuid moths, including *Trichoplusia ni* (Lepidoptera: Noctuidae), *Heliothis zea* (Lepidoptera: Noctuidae) and *H. virescens* (Lepidoptera: Noctuidae) (Shorey et al. 1968), the migratory species *Agrotis ipsilon* (Lepidoptera: Noctuidae) (Gadenne et al. 1993) and *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) (Turgeon et al. 1983), and the long-lived moth *Caloptilia fraxinella* (Lepidoptera: Gracillariidae) (Evenden and Gries 2008). Female sensitivity to oviposition cues in the Lepidoptera can also be plastic, and females demonstrate increased responsiveness to host plant oviposition cues after mating in *T. ni* (Landolt 1989), *Manduca sexta* (Lepidoptera: Sphingidae) (Mechaber 2002) and *Lobesia botrana* (Lepidoptera: Tortricidae) (Masante-Roca et al. 2007).

Induction of mate finding and oviposition behaviour after a period of reproductive inactivity requires physiological changes in moths in response to both endogenous and exogenous stimuli. In Lepidoptera studied to date, maintenance and termination of reproductive diapause and induction of pheromone responsiveness is under the regulation of exogenous factors such as

temperature and photoperiod that trigger endogenous regulating factors including hormones and biogenic amines (Anton et al. 2007; Goehring and Oberhauser 2002).

Reproductive Diapause

Diapause is a genetically programmed state of arrested development that occurs at a specific developmental stage depending on the species (Denlinger et al. 2005; Hahn and Denlinger 2007). Diapause is characterized by a halt in development or reproduction (Denlinger 2002; Macrae 2005) during which insects exhibit lowered metabolic rates (Denlinger 2002; Denlinger et al. 2005) with little or no feeding (Hahn and Denlinger 2007). Species may undergo an obligatory diapause, during which individuals enter diapause at the same developmental stage in every generation regardless of environmental conditions. Diapause can also be facultative, in which case environmental conditions experienced by individuals determine whether they will enter diapause (Denlinger 2002; Hahn and Denlinger 2007). Diapause is a dynamic state, and insects undergo several stages during diapause that can include diapause induction, preparation, initiation, maintenance, termination and post-diapause quiescence (Kostal 2006). The number and length of diapause stages that an insect experiences depends on species-specific genetics and also on environmental conditions (Kostal 2006). Induction of facultative diapause relies mainly on environmental cues, especially temperature and photoperiod (Danks 1991; Denlinger 2002). Diapause enables insects to avoid unfavourable environmental conditions, and to resume development or reproduction once conditions become favourable (Danks 1991).

Reproductive diapause in insects occurs during the adult stage and results in a complete cessation of reproduction. Reproductive diapause halts the development of reproductive organs and prevents reproductive behaviours during unfavourable environmental conditions, such as winter, or at inappropriate times, such as during or prior to migration. Reproductive diapause can serve to time adult reproduction with favourable environmental conditions for mating and offspring development (Denlinger et al. 2005; Macrae 2010; Pener 1992). Symptoms of reproductive diapause include arrestment of oocyte development and lack of oviposition in females, and reduced sexual organs and unresponsiveness to sexual cues in males (Denlinger et al. 2005; Pener 1992). Mating can occur prior to the onset of reproductive diapause, in which case males usually die after mating and only females enter reproductive diapause (Denlinger et al. 2005). In species in which both males and females enter diapause, mating usually occurs after the termination of reproductive diapause (Denlinger et al. 2005). Lack of male response to sexual cues, such as female sex pheromone in Lepidoptera, and failure of males to mate successfully with receptive females are the most reliable indicators of male reproductive status in insects that delay reproduction (Denlinger et al. 2005). Therefore, male response to pheromone can be used as a measure of reproductive maturity. A variety of factors are involved in the maintenance or termination of reproductive diapause and modulation of pheromone response in insects that delay reproduction. Environmental cues signal the onset and termination of reproductive diapause in insects, with

hormones including JH and ecdysteroids acting as internal messengers to regulate the appropriate reproductive development (Denlinger et al, 2005).

Endogenous Factors in the Regulation of Reproductive Diapause

Juvenile hormone is a well-studied and important gonadotropic hormone in many adult Lepidoptera (Ramaswamy et al. 1997). It is a sesquiterpenoid that is produced in and secreted by the *corpora allata* in insect brains, (Ramaswamy et al. 1997), and includes several natural JH homologues produced by the *corpora allata* in different orders of insects (Jindra et al. 2013). It is the main hormone responsible for terminating reproductive diapause in insects (Denlinger et al. 2005; Kopper et al. 2001; Shiga et al. 2003), and it also regulates sex pheromone response (Anton et al.2007; Cusson et al. 1993; Denlinger 2002; Duportets et al. 1996; Duportets et al. 1998; Gadenne et al. 1993; Ramaswamy et al. 1997) and sex accessory gland development (Gillott 1996; Gillott and Gaines 1992) in male Lepidoptera with delayed reproduction. Monarch butterflies, *Danaus plexippus* (Lepidoptera: Nymphalidae) undergo a reproductive diapause with low levels of endogenous JH prior to migration that is terminated by JH treatment (Barker and Herman 1976). *Agrotis ipsilon* is a migratory species in which both sexes are sexually immature at the time of adult eclosion (Anton et al. 2007; Swier et al. 1976). Newly-eclosed males and females have low JH titres, and underdeveloped sexual organs (Duportets et al. 1998; Gadenne et al. 1993). Moths mature over a period of about five days as endogenous JH titre increases, during which time females become able to produce sex pheromone, male response to the pheromone increases, and male sex accessory glands enlarge and increase in protein content

(Duportets et al. 1998; Gadenne et al. 1993). A similar phenomenon occurs in another migratory species, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae), in which females delay pheromone calling for several days, and male response to pheromone is low upon emergence, and increases over a period of five days with the production of JH acids (Cusson et al. 1993; Turgeon et al. 1983).

Exogenous Factors in the Regulation of Reproductive Diapause

Many insects experience a facultative adult reproductive diapause that depends on environmental conditions and seasonality, mediated by JH (de Loof and de Wilde 1970; Matsuo et al. 1997; Morita and Numata 1997; Okuda and Chinzei 1988; Sakurai et al. 1986; Teal et al. 2000). Temperature and photoperiod trigger induction and termination of reproductive diapause and the timing of reproductive maturity and pheromone communication in several Lepidoptera species that delay reproduction (McNeil et al. 1995; Ramaswamy et al. 1997). In Lepidoptera that experience a reproductive diapause, short day conditions typically induce reproductive diapause, while long day conditions either terminate reproductive diapause or prevent individuals from entering diapause (Barker and Herman, 1976; Fujita et al., 2009; Pieloor and Seymour, 2001; Pullin, 1986). *Agrotis ipsilon* delays reproduction until after migration, and short day conditions delay the age of first calling in females and mating in males and females compared with moths held under long day conditions (Gadenne 1993; Gemeno and Haynes 2001). Reproductive diapause is maintained with short day conditions and terminated with long day conditions in adult *Polygonia c-aureum* butterflies (Fujita et al. 2009). Monarch butterflies, *D. plexippus*, experience a reproductive

diapause before migration that is also impacted by temperature and photoperiod and terminated with JH application (Barker and Herman 1976; Goehring and Oberhauser 2002; Herman 1981). Monarch butterflies exposed to decreasing day lengths that mimic natural conditions during the period of natural reproductive diapause, are more likely to remain in reproductive diapause post-eclosion compared to individuals held under long day or short day conditions (Goehring and Oberhauser 2002).

Pheromone Communication in Moths

Reproduction is arguably the most important aspect of an insect's lifecycle, and timing of reproduction is essential to successful mating, oviposition and offspring development. In Lepidoptera, mate finding is accomplished by male detection and response to female-produced, species-specific pheromones. Pheromone-specific olfactory receptor neurons are housed in olfactory sensillae located on male antennae (Anton et al., 2007; Galizia and Szyszka 2008). Pheromone molecules enter the sensilla *via* pores, and are bound to pheromone-binding proteins in the sensillar lymph (Kaissling 2009). The pheromone-binding protein solubilizes and protects the pheromone molecule and transports it to the receptor neuron where it binds to olfactory receptors (Kaissling 2009). An electrical signal is generated and sent down the olfactory receptor neuron via the antennal nerve into the antennal lobe, which is the primary olfactory centre in the insect brain (Galizia and Szyszka 2008). Male moths have several pheromone-specific glomeruli that make up the macroglomerular complex in the antennal lobe (Anton et al. 2007). In pheromone perception, this is called a labelled-line system, since

pheromone-specific olfactory receptor neurons only detect pheromone, and stimulate a specific region in the macroglomerular complex, therefore, a stimulus in the macroglomerular complex unambiguously indicates the presence of pheromone (Galizia and Szyszka 2008). From the antennal lobe, the signal is either enhanced or inhibited by local interneurons, and the output is sent *via* projection neurons to the mushroom bodies, which are the higher brain centres, where stereotypical pheromone-response behaviour is generated (Galizia and Szyszka 2008).

Plasticity of Pheromone Processing

In long-lived moths that experience a delay in mating, pheromone response can be plastic to ensure that male response to pheromone is most acute during female receptivity (Anton et al. 2007). Pheromone response plasticity can be impacted by moth age (Dumont and McNeil 1992; Turgeon et al. 1983), hormonal state (Anton et al. 2007; Gadenne et al. 1993), and biogenic amines (Linn and Roelofs 1986; Linn et al. 1992; Linn et al. 1996; Pophof 2000; Pophof 2002).

Along with its role in terminating reproductive diapause, JH directly impacts male response to pheromone in species that exhibit pheromone response plasticity. In the migratory species *A. ipsilon* (Gadenne et al. 1993) and *P. unipuncta* (Turgeon et al. 1983), newly-eclosed males are not responsive to female sex pheromone, but become responsive after a period of about five days. This observed age-dependent plasticity in pheromone response is mediated by JH

in both cases. Treatment with a JH analogue (JHA) induces response in newly-eclosed immature male *A. ipsilon* in wind tunnel assays (Gadenne et al. 1993). In male *A. ipsilon*, electroantennogram (EAG) recordings show that the antennae of newly-eclosed sexually immature males are equally responsive to pheromone as JHA-treated and sexually mature males (Gadenne et al. 1993). Rather than impacting the peripheral nervous system in *A. ipsilon*, JH impacts the central nervous system. Pheromone-specific antennal lobe neurons are more sensitive to pheromone when sexually immature males are injected with JH compared with control males (Anton and Gadenne 1999). Currently, the mode of action of JH in the central nervous system is not known. Juvenile hormone could act directly on JH receptors in the central nervous system, although none have been described yet, or JH could act indirectly on the antennal lobe neurons *via* another neural substance (Anton and Gadenne 1999; Gadenne and Anton 2000).

Biogenic amines, such as octopamine and serotonin, act as neurotransmitters, neuromodulators or neurohormones in insects, and are produced throughout an insect's nervous system (Blenau and Baumann 2001). Biogenic amines have also been specifically implicated in the regulation of sex pheromone communication in some moths. Treatment with octopamine or serotonin can increase male moth behavioural (Linn and Roelofs 1986; Linn et al. 1992; Linn et al. 1996; Pophof 2000) and electrophysiological (Pophof 2000; Pophof 2002) responses to female sex pheromone. Octopamine receptors are located near the base of pheromone-specific sensillae in *Bombyx mori* (Lepidoptera: Bombycidae) and *Heliothis virescens* (Lepidoptera: Noctuidae)

(Nickisch-Rosenegk et al. 1996). Octopamine likely acts directly at these receptor sites to impact male antennal response to pheromone. In male *Antheraea polyphemus* (Lepidoptera: Saturniidae) (Pophof 2000) and *Mamestra brassicae* (Lepidoptera: Noctuidae) (Grosmaitre et al. 2001), octopamine acts on receptors in the olfactory receptor neurons to increase male sensitivity to pheromone. Octopamine and serotonin both impact male response to pheromone in *M. brassicae* (Grosmaitre et al. 2001) and *Lymantria dispar* (Lepidoptera: Lymantriidae) (Linn et al. 1992). In *A. ipsilon* both octopamine and JH are necessary to elicit a behavioural response of males to female sex pheromone (Jarriault et al. 2009).

Host Volatile Cues Used by Lepidoptera

Host volatiles are chemicals released from plants and exploited by phytophagous insects for host and mate location, and oviposition (Anton at al. 2007). Female moths are generally more responsive to host volatiles than males (Das et al. 2007), since females have more olfactory receptor neurons tuned to plant volatiles than males (De Bruyne and Baker 2008; King et al. 2000). Female response to host plant volatiles can be plastic and should be most sensitive during periods of active host search and oviposition (Martel et al. 2009; Masante-Roca et al. 2007; Mechaber et al. 2002; Yan et al. 1999). In *Lobesia botrana* (Lepidoptera: Tortricidae), mating induces females to become responsive to host volatiles (Masante-Roca et al. 2007). Male response to host volatiles can also be plastic, and the addition of host volatiles to female sex pheromone in *L. botrana* increases male responsiveness compared to the attractiveness of pheromone alone in a wind

tunnel (von Arx et al. 2012). Electroantennogram recordings of *Manduca sexta* (Lepidoptera: Sphingidae) moths reveal that both males and females respond to host plant volatiles, however, females have a higher response and lower threshold of response than males (Fraser et al. 2003). In *Spodoptera littoralis* (Lepidoptera: Noctuidae), mating status and age impact female EAG response to plant and sex pheromone signals (Martel et al. 2009). To date, there are no studies on the response of male or female moths to host volatiles during an extended period of reproductive inactivity.

Life History of *Caloptilia fraxinella*

The ash leaf cone roller moth, *Caloptilia fraxinella* (Ely) (Lepidoptera: Gracillariidae), is a pest of ash trees (*Fraxinus* spp.) across the Canadian Prairie Provinces (Pohl et al. 2004). It was first reported in Edmonton, Alberta in 1999 on green ash (*Fraxinus pennsylvanica* Marsh. Var. *subintegerrima* (Vahl) Fern.) and black ash (*F. nigra* Marsh), and is also found on Manchurian ash (*F. mandshurica* Rupr.) and white ash (*F. americana* L.) (Pohl et al. 2004). It is a Nearctic species known from the eastern United States and from Québec and Ontario in Canada, and may be native but undetected inside the native range of ash host trees in Canada in southern Saskatchewan and Manitoba (Pohl et al. 2004).

Adult moths are small, with a wingspan of approximately 12.0 mm (Pohl et al. 2004), and have white, grey, black and orange scales covering forewings (Pohl et al. 2004). Larvae are approximately 7.3 mm at maturity and are cream

coloured (Pohl et al. 2004). Aesthetic damage to ash trees is caused by the larvae, which mine the leaflets in early instars, and roll leaflets into a cone shape around themselves in later instars in preparation for pupation.

Adult *C. fraxinella* overwinter in reproductive diapause (Fig. 1-1), and emerge from overwintering locations in the spring in April and May. Adults mate in the spring away from the ash host before males and females fly to ash trees where females lay eggs on the upper surface of newly-flushed ash leaflets (Evenden 2009). Larvae hatch after approximately one week (Pohl et al. 2004) and early instars mine the leaflet, and later instars roll the leaflet into a cone shape and pupate inside (Pohl et al. 2004). Adults eclose in July and spend a summer aestivation and overwintering period in a state of reproductive diapause (Evenden et al. 2007).

Pheromone response by male *C. fraxinella* varies with physiological state, and male response is highest in the spring when moths are reproductively active (Evenden and Gries 2008; Lemmen and Evenden 2009). Treatment with a JHA, methoprene, enhances male behavioural and EAG response to pheromone when males are in a later stage of reproductive diapause in the fall, but not when males are in an earlier stage in the summer (Lemmen and Evenden 2009). Juvenile hormone impacts male *C. fraxinella* pheromone response at the peripheral nervous system level during reproductive diapause (Lemmen and Evenden 2009). This is different from what has previously been found in another male moth species with delayed reproduction, *A. ipsilon*, in which there is no difference in male antennal response to pheromone between reproductively active and inactive

males (Gadenne et al. 1993). The physiology of both female (Evenden et al. 2007) and male (Lemmen and Evenden 2009) *C. fraxinella* changes throughout the period of reproductive diapause. In general, both sexes are more susceptible to termination of reproductive diapause with JHA treatment in the fall compared with the summer. The long adult life of *C. fraxinella*, in which almost nine months is spent in reproductive diapause, offers a unique opportunity to study male response to pheromone and male and female response to host plant volatiles throughout the period of reproductive diapause and reproductive activity.

Thesis Overview

In this study, I examine the plasticity of antennal and behavioural responses of male and female *C. fraxinella* to chemical signals. Using wind tunnel and EAG bioassays, in Chapters 2 and 3 I aim to identify mechanisms behind male pheromone response plasticity and in Chapter 4 to determine whether a similar plasticity exists in males and females in response to host plant volatiles. In Chapter 2, I test the effectiveness of two different JHAs, methoprene and pyriproxyfen, and three biogenic amines, octopamine, dopamine and serotonin for enhancing pheromone response of male *C. fraxinella*. I also test the number and timing of JHA applications required to achieve the highest pheromone responsiveness in males while moths are in reproductive diapause. The effect of biogenic amines alone and in conjunction with JHA treatment is tested on male pheromone response while males are reproductively active or in reproductive diapause. In Chapter 3, I test the impact of temperature and photoperiod on the state of reproductive diapause of male *C. fraxinella*, and determine the role of

environmental cues on the maintenance and termination of reproductive diapause. In Chapter 4, I examine whether male and female response to ash host volatiles is plastic and depends on physiological state, in a manner similar to male pheromone response. In Chapter 5, I aim to determine if male *C. fraxinella* pheromone response plasticity is part of a larger reproductive diapause phenomenon in this insect. To test this, I measure the length of male sex accessory glands at different stages of reproductive activity and diapause, use a BCA protein assay to determine the total protein concentration of male sex accessory glands, and finally use SDS-PAGE to determine the sizes and relative amounts of specific proteins in male sex accessory glands at different stages of reproductive activity and diapause. With these observations and analysis, I aim to provide a more complete understanding of the olfactory plasticity and reproductive diapause that exists in the long-lived moth *C. fraxinella*.

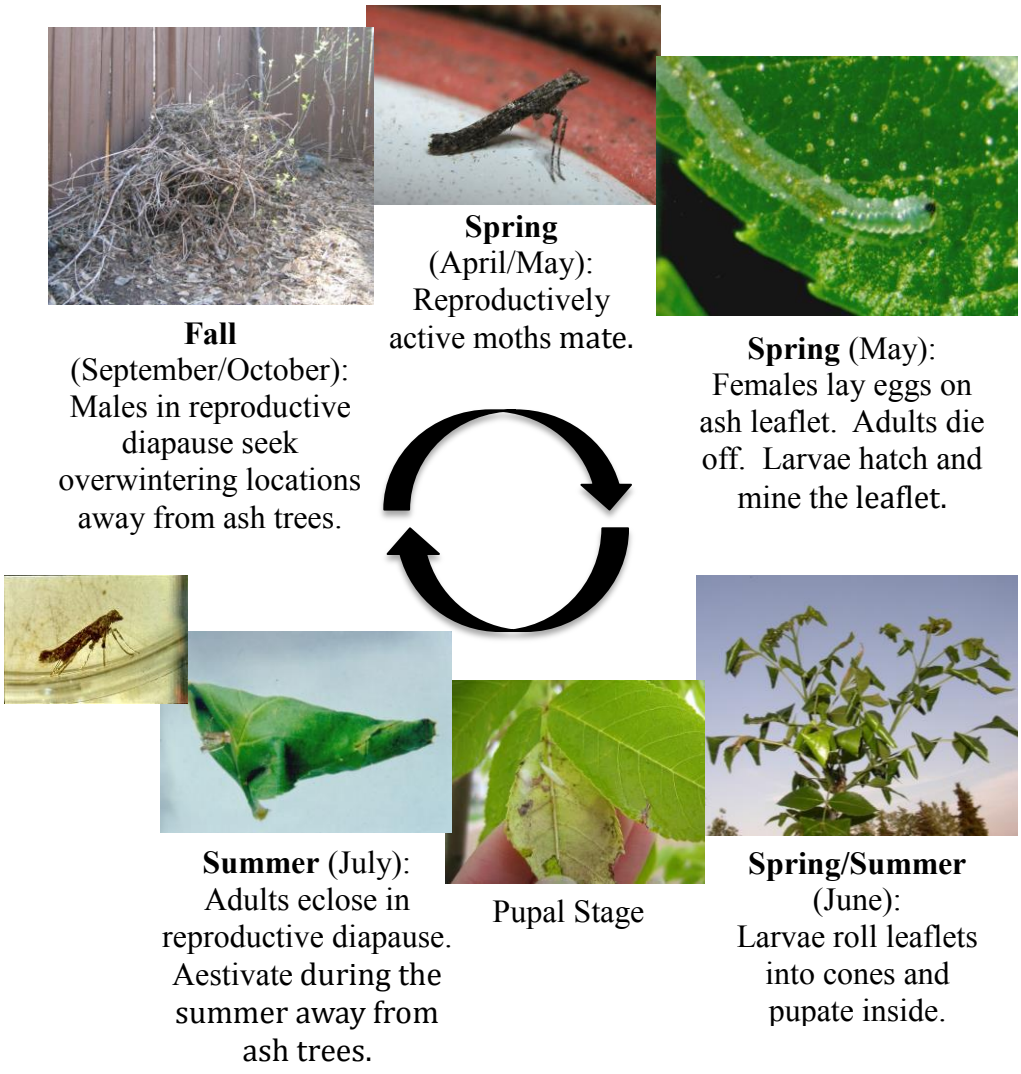

Figure 1-1. Lifecycle of *Caloptilia fraxinella*. Photos courtesy of T. Wist, M. Evenden and J. Lemmen.

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Chapter 2

The roles of juvenile hormone and biogenic amines on pheromone response plasticity in male *Caloptilia fraxinella* (Lepidoptera: Gracillariidae)

Introduction

Long-lived insects may experience a period of reproductive inactivity as adults during migration or diapause. In these species, signalling systems used for finding mates and oviposition locations should be plastic and most acute at reproductive maturity and under suitable environmental conditions for mating and offspring development (Anton et al. 2007). In insects studied to date, induction of sexual communication with pheromones after reproductive inactivity is under the regulation of exogenous factors such as temperature and photoperiod that trigger endogenous regulating factors including hormones (Goehring and Oberhauser 2002; Anton et al. 2007).

Juvenile hormone (JH) is a sesquiterpenoid hormone that regulates insect growth and development at all life stages, and is well studied as an important gonadotropic hormone in many Lepidoptera (Ramaswamy et al. 1997, Minakuchi and Riddiford 2006). Juvenile hormone regulates male sex pheromone response and sex accessory gland development in lepidopterans with delayed reproduction (Cusson et al. 1993; Duportets et al. 1996; Ramaswamy et al. 1997; Duportets et al. 1998; Denlinger 2002; Anton et al. 2007). Monarch butterflies, *Danaus plexippus* (Lepidoptera: Nymphalidae), experience a reproductive diapause before migration that is terminated with JH application (Barker and Herman 1976).

Newly-eclosed male *Agrotis ipsilon* (Lepidoptera: Noctuidae) do not respond to female sex pheromone, however treatment with a JH analogue (JHA) induces response in wind tunnel assays (Gadenne et al. 1993). There is no difference in male *A. ipsilon* peripheral nervous system response to pheromone from newly-eclosed, sexually mature, or JHA-treated reproductively immature males (Gadenne et al. 1993), but JHA application affects the sensitivity of central olfactory neurons to pheromone (Gadenne et al. 1993, Anton and Gadenne 1999; Gadenne and Anton 2000). The *corpora allata*, the site of synthesis and secretion of JHs, of adult male *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) produce JH acids, which correlates with male responsiveness to sex pheromone (Cusson et al. 1993; Cusson et al. 1994). Treatment with the JHA methoprene enhances male *Caloptilia fraxinella* (Lepidoptera: Gracillariidae) antennal and behavioural response to pheromone in the latter part of reproductive diapause in the fall, but not earlier in the summer (Lemmen and Evenden 2009).

Biogenic amines (BAs), such as octopamine, dopamine and serotonin can act as chemical messengers in the forms of neurotransmitters, neuromodulators or neurohormones (Blenau and Baumann 2001). Biogenic amines are synthesized from amino acids and have many roles in controlling various functions of the nervous system in insects (Blenau and Baumann 2001). In moths, BAs have been implicated in the regulation of sex pheromone communication. Treatment with BAs can increase male moth behavioural (Linn and Roelofs 1986; Linn et al. 1992; Linn et al. 1996; Pophof 2000) and electrophysiological (Pophof 2000; Pophof 2002) responses to female sex pheromone.

Octopamine increases the response of male *Grapholita molesta* (Lepidoptera: Tortricidae) (Linn and Roelofs 1984) and *Trichoplusia ni* (Lepidoptera: Noctuidae) (Linn and Roelofs 1986) to pheromone. Octopamine treatment increases the sensitivity of male *T. ni* to pheromone, while serotonin treatment causes males to broaden their pheromone response time window and respond to pheromone throughout the entire scotophase period (Linn and Roelofs 1986). Octopamine acts directly on the olfactory receptor neurons (ORNs) of male *Antheraea polyphemus* (Lepidoptera: Saturniidae) (Pophof 2000) to increase sensitivity of males to pheromone. *In situ* hybridization studies with cloned octopamine receptors show that octopamine receptors are located in the epithelium near the base of pheromone-specific sensilla in the moths *Bombyx mori* (Lepidoptera: Bombycidae) and *Heliothis virescens* (Lepidoptera: Noctuidae) (Nickisch-Rosenegk et al. 1996). It is likely that octopamine acts directly on these receptors to impact male antennal response to pheromone. In male *A. polyphemus*, octopamine treatment increases the nerve impulse frequency of male antennal response to pheromone, but only the spike generator located in the soma region of the receptor neurons was impacted by octopamine treatment suggesting that octopamine acts directly on receptors in this part of the ORN (Pophof 2000). The antennal tissues of *Mamestra brassicae* (Lepidoptera: Noctuidae) express an octopamine receptor protein (Grosmaitre et al. 2001). Octopamine treatment increases the ORN nerve impulse frequency to pheromone, and likely acts directly on octopamine receptors in the ORN (Grosmaitre et al. 2001). Serotonin also impacts the nerve impulse frequency of ORNs in male *M.*

brassicae, however serotonin lowers the spike frequency (Grosmaitre et al. 2001). The result suggests that both octopamine and serotonin work to modulate antennal sensitivity of male *M. brassicae* to pheromone directly at the level of the antenna (Grosmaitre et al. 2001). Octopamine and serotonin along with photoperiod cues impact male response to pheromone in *Lymantria dispar* (Lepidoptera: Lymantriidae) (Linn et al. 1992). Both octopamine and JH are necessary to elicit a behavioural response in reproductively active male *A. ipsilon* to female sex pheromone, and octopamine increases male behavioural response in reproductively active males that are 3-5 days old (Jarriault et al. 2009). However, octopamine treatment alone has no impact on the behavioural response of newly-eclosed, sexually immature *A. ipsilon* males (Jarriault et al. 2009). To date, the effect of BAs on pheromone response has not been tested on moths that experience a period of reproductive inactivity longer than several days.

Caloptilia fraxinella (Ely) is a pest of ash trees (*Fraxinus spp.*), on which early instar larvae are leafminers. *Caloptilia fraxinella* spends the majority of its univoltine lifecycle, approximately nine months, as an adult (Pohl et al. 2004). At adult eclosion in July, the majority of the population is in reproductive diapause (Evenden et al. 2007). After a summer aestivation, adults overwinter away from their ash hosts in an unmated state, and emerge in the spring in a reproductively active state. In the spring, adults mate and females oviposit on newly flushed ash leaflets (Pohl et al. 2004). Male response to the female-produced sex pheromone is plastic, and male response is highest in the spring when moths are reproductively active (Evenden and Gries 2008). Juvenile hormone enhances

male electroantennogram (EAG) and behavioural response to pheromone when the males are in reproductive diapause in the fall, but not in the summer (Lemmen and Evenden 2009).

This study examines the effects of two JHA and three BA treatments on male *C. fraxinella* response to pheromone when males are in reproductive diapause in the summer and fall, and the effects of BA treatments on males that are reproductively active in the spring. Although JHA treatment is already known to enhance male *C. fraxinella* EAG and behavioural response to pheromone in the fall, this response is not completely restored to the same level as in reproductively active males (Lemmen and Evenden 2009). In the current study, time post-JHA treatment is tested on male response to pheromone when males are in reproductive diapause. The impact of two different JHAs, methoprene and pyriproxyfen, is tested at two different application rates on male response to pheromone. The effect of JHAs on male pheromone response is also tested when males are held under two different environmental conditions prior to treatment to determine if an interaction between hormone and environment impacts diapause termination in this species. Finally, the impact of treatment with various BAs, octopamine, dopamine and serotonin, with and without JHA, is tested to determine if BAs alone are important in the regulation of pheromone response, and to determine if the BA and JHA treatments interact to affect male response to pheromone when males are in reproductive diapause.

Materials and Methods

Moth Collection

Pupae were collected in leaf rolls from green ash, *Fraxinus pennsylvanica* at various sites in Edmonton, Alberta (53^{o} 34'N 113^{o} 31'W) from 28 June-7 July 2010 and 30 June-5 July 2011. Individual leaf rolls were kept under a LD 16:8 h, 24^{o}C in 30 ml transparent plastic cups grouped in transparent plastic bags with a damp paper towel to maintain humidity. Moth eclosion occurred from 7 July-19 July 2010 and 6 July-20 July 2011. Cups were checked at least twice weekly for adult eclosion, and adults were removed and separated by sex and males were prepared for subsequent use in summer and fall bioassays.

Adult moths were also collected on the wing from various sites in Edmonton, Alberta in a reproductively active state in the spring, 20 April-12 May 2010, and in reproductive diapause in the fall, 2-10 September 2010. Adults were separated by sex, and males were placed in individual 30 ml transparent cups and provided access to 10% sugar solution through a dental wick and held under LD 16:8 h, 24^{o}C until use in a bioassay.

Experiments and Treatments

<u>Experiment 1 – Length of time post-JHA treatment</u>

Experiment 1 tested the hypothesis that time post-JHA treatment of males in reproductive diapause would influence male *C. fraxinella* pheromone responsiveness. This experiment was designed to determine if JHA treatment had an immediate effect on male moth pheromone response when moths were in diapause or if the response was delayed possibly due to upregulation of endogenous JH production triggered by the application of the JHA.

In the summer, male *C. fraxinella* were held from eclosion in July in individual 30 ml cups with access to 10% sugar solution, under a LD 16:8 h, 24^{o}C photoregime until treatment and the bioassay (Table 2-1). Males used in the fall were held outdoors in cages after eclosion in July to experience natural environmental conditions with access to 10% sugar solution until September when they were transferred indoors and held in the same manner as males in the summer experiment until they were treated and used in the bioassay (Table 2-1).

Based on previous experiments (Evenden et al. 2007; Lemmen and Evenden 2009), male *C. fraxinella* that were in reproductive diapause in the summer and fall of 2010 were treated with: (1) 1 μg of the JHA methoprene (94.3% pure, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 1 μl of high-performance liquid chromatography (HPLC) grade acetone (Fisher Scientific, Ottawa, Ontario, Canada) from a stock solution, (2) 1 μl acetone alone, or (3) were left untreated. Male moths were held using a gentle vacuum stream while treatments were applied exogenously to the ventral side of the abdomen. After treatment, males were transferred back to appropriate conditions for either one, three, seven or nine days post-JHA treatment or for seven days for the control acetone-treated or untreated males prior to use in a wind tunnel bioassay (Table 2-1).

Experiment 2 – Multiple JHA Treatments and Applications

Experiment 2 tested the hypothesis that treatment with two JHAs, methoprene or pyriproxyfen, at two different application rates would differentially impact pheromone response of male *C. fraxinella* in reproductive diapause.

Male *C. fraxinella* used in summer experiments were held indoors post-eclosion in individual 30 ml cups with access to 10% sugar solution, and were maintained under a LD 16:8 h, 24^{o}C photoregime until treatment and use in wind tunnel or EAG experiments (Table 2-1). Males used in fall experiments were divided into two groups after eclosion in July, one group was held in cages indoors next to windows to experience a natural photoregime but a constant ambient temperature, and the second group was held in cages outdoors to experience natural photoregime and temperature conditions until the fall. In the fall, males in both groups received all treatments and individuals were returned post-treatment to the original environmental conditions for the appropriate length of time (Table 2-1) before being used in wind tunnel or EAG experiments. Males that were held indoors and outdoors were tested in each experiment on the same day to enable a comparison of the pheromone response of males that had been treated with the JHAs after experiencing different environmental conditions.

Male *C. fraxinella* in reproductive diapause in the summer and fall 2010, were treated with: (1) 1 μg of methoprene diluted in 1 μl of acetone one week before the bioassay, (2) one application of 1 μg of methoprene diluted in 1 μl of acetone one week before the bioassay, and a second application one day before the bioassay, (3) 1 μg of pyriproxyfen (99% pure, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 1 μl of acetone one week before the bioassay, (4) one application of 1 μg of pyriproxyfen diluted in 1 μl of acetone one week before the bioassay, and a second application one day before the bioassay, (5) 1 μl of acetone alone one week before the bioassay, or (6) were left untreated. Male

moths were held using a gentle vacuum stream while treatments were applied exogenously to the ventral side of the abdomen. Males were briefly removed from housing conditions for treatment, and were then replaced and held until use in a wind tunnel or EAG bioassay (Table 2-1).

Experiment 3 – Biogenic Amines

Experiment 3 tested the hypothesis that BAs impact male pheromone response when males are reproductively active in the spring, and that both BAs and JH regulate male pheromone responsiveness when males are in reproductive diapause.

Reproductively active moths used in spring experiments were held indoors after collection under a LD 16:8 h, 24^{o}C photoregime until treatment and use in a wind tunnel or EAG bioassay (Table 2-1). In the summer, males were held indoors after eclosion in July under a LD 16:8, 24^{o}C photoregime and provided with a 10% sugar solution until treatment prior to use in a wind tunnel or EAG experiment (Table 2-1). Males used in the fall experiments were either held indoors in cages next to windows after eclosion in July 2010, or were collected in the fall as free-flying adults in reproductive diapause in September 2010 (Table 2-1). Adults from the two different collection-types in the fall were equally distributed among treatments, and males were all provided a 10% sugar solution.

Octopamine, serotonin and dopamine were all diluted so that the same number of moles/μl of each compound was applied. Following (Rafaeli 2004), males in the spring, summer and fall were treated with one of the following: (1) 0.019 μg of octopamine hydrochloride (≥95%, Sigma-Aldrich, MO, USA) diluted

in 1 μl methanol (≥99.8%, Sigma-Aldrich, MO, USA), (2) 0.021 μg serotonin hydrochloride (≥98%, Sigma-Aldrich, MO, USA) diluted in 1 μl methanol, (3) 0.019 μg dopamine hydrochloride (98.2%, Sigma-Aldrich, MO, USA) diluted in 1 μl methanol, (4) 1 μl methanol alone, or (5) were left untreated. Additional treatments were tested on males in reproductive diapause in the summer and fall including: (6) 0.019 μg of octopamine diluted in 1 μl methanol plus 1 μg of methoprene diluted in 1 μl of acetone, (7) 0.021 μg serotonin diluted in 1 μl methanol plus 1 μg of methoprene diluted in 1 μl of acetone, (8) 0.019 μg dopamine diluted in 1 μl methanol plus 1 μg of methoprene diluted in 1 μl of acetone, or (9) 1 μg of methoprene diluted in 1 μl of acetone. The solvent control (treatment 4) for the summer and fall experiments consisted of 1 μl methanol plus 1 μl acetone. Male moths were held using a gentle vacuum stream while treatments were applied exogenously to the ventral side of the abdomen. Males were treated five hours before the start of the experiment, based on a similar experiment (Linn and Roelofs 1984). Males were briefly removed from housing conditions for treatment, and were then replaced and held for five hours until the start of the wind tunnel or EAG bioassay. In the spring, the number of seconds it took male moths to contact the pheromone source was timed in order to test whether BA treatment had an impact on how quickly males could locate the pheromone source in the wind tunnel.

Wind Tunnel Bioassays

The wind tunnel used in behavioural assays had a flight section 1.7 m long and 0.85 m high. Six, 15-watt bulbs diffused through white paper dimly

illuminated the tunnel. Wind speed was 0.32-0.34 m/s and temperature was maintained at 24-26^{o}C. Males were acclimatized to experimental conditions 30 min prior to initiation of the behavioural assay. Flights were conducted during the last hour of the photophase and the first two hours of the scotophase to a pheromone source of 10 μg (*Z*)-11-hexadecenal and 1 μg (*Z*)-11-hexadecen-1-ol (Pherobank, Wageningen, The Netherlands) in HPLC grade hexane (Fisher Scientific, Ottawa, Ontario, Canada) released from a pre-extracted grey rubber septum (Contech Enterprises Inc., Delta, BC, Canada). Males were introduced individually into the wind tunnel in cylindrical wire cages (5 cm diameter x 6 cm height) on a platform 20 cm from the downwind end. Once the moth was positioned in the pheromone plume, the lid of the cage was removed and males were allowed three minutes to respond to the pheromone source. Males from the different treatments were flown alternately and each moth was flown only once. Behavioural responses to pheromone were recorded as: wing fanning, lock-on to the pheromone plume, upwind oriented flight and contact with the pheromone source.

Electroantennogram Recordings

Electroantennogram recordings were made using an IDAC-02 data acquisition controller system, and EAG 2000 software (SYNTECH, Hilversum, The Netherlands). In preparation for EAG recordings, male moths were chilled at 4^{o}C for at least 20 min before one antenna was excised and positioned onto an antenna holder using a small quantity of Spectra 360 conductive gel (Parker Laboratories Inc., Orange, NJ), that was attached to a SYNTECH EAG probe

(Type PRG-2, internal gain 10x). Pheromone loadings consisted of a 10:1 ratio of (*Z*)-11-hexadecenal and (*Z*)-11-hexadecen-1-ol (Pherobank, Wageningen, The Netherlands) serially diluted in HPLC-grade hexane to obtain decadal solutions between 0.0001 μg and 1 μg (*Z*)-11-hexadecenal μl^{-1} hexane. Fifty μl of each solution, and 50 μl of a hexane control, were pipetted individually onto 7 × 0.2 cm strips of folded Whatman no. 1 filter paper and allowed to evaporate in a fume hood. As a standard, 50 μl of the plant volatile (*E*)-2-hexenal (>95%, Aldrich Chemical Co., WI, USA) (1 μg μl^{-1} hexane) was pipetted onto filter paper and allowed to evaporate. Treated strips were inserted into disposable Pasteur pipettes. Stimulus puffs were generated with a SYNTECH CS-55 stimulus controller with a pulse duration of 0.2 sec and flow of 10 ml/sec. Electroantennogram responses were measured as the maximum amplitude of depolarization elicited by the stimulus applied. Each antenna received a series of puffs delivered once every minute in the following order: hexane; 50 μg plant volatile; 0.005 μg pheromone; 50 μg plant volatile; 0.05 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.

Statistical Analysis

The raw EAG responses (mV) recorded in EAG experiments were log transformed after conducting a Box Cox analysis to determine the most appropriate transformation of the data. The log-transformed data showed greatly improved analysis based on AIC values. Electroantennogram responses of males that were exposed to the different treatments in all experiments and tested to the

increasing doses of pheromone were compared using a repeated measures ANOVA (R Development Core Team, 2012). When necessary, a post-hoc multiple comparison Honestly Significant Differences (HSD) test was performed among treatments (R-package: agricolae). In the figures depicting EAG responses presented here, means and standard errors were back-transformed to reflect the original untransformed EAG responses that are pooled by pheromone dose and include response to the solvent control (see Appendix 2-1 for an example of EAG traces, and see Appendices 2-2 - 2-4 for raw EAG dose response curves).

In the wind tunnel experiments, behavioural responses of males in the wind tunnel were recorded as "yes" or "no", so the resulting data were binomially distributed. Behavioural response was compared using a generalized linear model (GLM) with a logit link function (R Development Core Team, 2012). Analysis of deviance was used to test whether male response was dependent on any of the experimental variables. When the GLM was significant, multiple comparisons (R-package: Multcomp) were made using Tukey-contrasts. In Experiment 3, the length of time if took males that had been treated with different biogenic amines in the wind tunnel to contact the pheromone source was recorded in seconds. A two-way ANOVA (R Development Core Team, 2012) including BA treatment and replicate as independent variables was performed to determine whether treatment impacted the number of seconds it took males to contact the pheromone source.

Results

Experiment 1 – Length of time post-JHA treatment

Overall, JHA treatment enhanced male pheromone response when males were in reproductive diapause in the fall, but not in the summer. When the overall impact of JHA treatment on male behavioural response in the summer was compared to acetone-treated and untreated controls, JHA treatment had a marginally significant impact on lock-on behaviour (GLM: $X_{2,0.05}$=6.1, P=0.05), but did not impact wing fanning (GLM: $X_{2,0.05}$=0.5, P=0.8), upwind flight (GLM: $X_{2,0.05}$=5.4, P=0.07), or source contact (GLM: $X_{2,0.05}$=3.0, P=0.2) (Fig. 2-1A). The length of time male *C. fraxinella* were held after JHA treatment before use in a wind tunnel bioassay did not impact the number of males that conducted any of the pheromone-mediated behaviours in the wind tunnel when males were tested in the summer early in reproductive diapause (Fig. 2-1A). Treatment with a JHA in the fall did enhance wing fanning (GLM: $X_{2,0.05}$=9.1, P=0.01), lock-on (GLM: $X_{2,0.05}$=11.3, P=0.004) and upwind flight (GLM: $X_{2,0.05}$=8.8, P=0.01) behaviours, but did not enhance source contact behaviour (GLM: $X_{2,0.05}$=4.7, P=0.1) compared with acetone-treated and untreated controls (Fig. 2-1B). The length of time post-JHA treatment did not significantly impact wing fanning, lock-on, upwind flight or source contact behaviours when males were tested late in reproductive diapause in the fall (Fig. 2-1B). Although it was not significant, there is a trend for males tested in the wind tunnel 7 days post-JHA treatment to have the highest response to pheromone in the fall, compared with males held for 1, 3 or 9 days post-JHA treatment (Fig. 2-1B).

Experiment 2 – Multiple JHA Treatments and Applications

Juvenile hormone analogue treatment did not impact male *C. fraxinella* behavioural response in the wind tunnel early in reproductive diapause in the summer when males were treated with either one or two applications of either of the JHAs tested (Fig. 2A). Juvenile hormone analogue treatment did impact behavioural response late in reproductive diapause in the fall when male *C. fraxinella* were held either indoors or outdoors post-eclosion. Juvenile hormone analogue treatment did not impact wing fanning, but did impact lock-on, upwind flight and source contact when males were held indoors post-eclosion (Fig. 2-2B). A higher percentage of males treated with one application of pyriproxyfen locked-on to the pheromone source compared to acetone-treated and not treated males (Fig. 2-2B). A post-hoc Tukey-contrast test could not separate significant treatments for upwind flight and source contact behaviours, however, for all pheromone-mediated behaviours, more males treated once with either methoprene or pyriproxyfen responded compared to males exposed to other treatments (Fig. 2-2B). Juvenile hormone analogue treatment did not impact wing fanning or source contact behaviours, but did impact lock-on and upwind flight behaviours when male *C. fraxinella* were held outdoors post-eclosion and tested in the fall (Fig. 2-2C). A post-hoc Tukey-contrasts test was unable to separate the effects of JHA treatments for lock-on and upwind flight behaviours, however for all of the pheromone-mediated behaviours, more males treated with one application of methoprene responded to the pheromone compared to males in the other treatment groups (Fig. 2-2C).

The EAG response of male *C. fraxinella* early in reproductive diapause in the summer was impacted by JHA treatment (Fig. 2-3A) and pheromone dose (repeated measures ANOVA: $F_{5,295}$=740.95, P<0.0001) (Appendix 2-2). Males treated with acetone had a lower EAG response to pheromone compared with males treated with two applications of pyriproxyfen, one application of methoprene and untreated males in the summer, but none of the other treatments were significantly different from each other, and JHA treatment did not enhance EAG response above the response of untreated males (Fig 3A). Juvenile hormone analogue treatment (Fig. 2-3B) and pheromone dose (repeated measures ANOVA: $F_{5,290}$=415.86, P<0.0001) impacted male *C. fraxinella* EAG response when males were held indoors post-eclosion and tested in the fall (Appendix 2-2). Males treated with one application of either methoprene or pyriproxyfen had a higher EAG response compared to males exposed to other treatments (Fig. 2-3B). Similar to males held indoors, both JHA treatment (Fig. 2-3C) and pheromone dose (repeated measures ANOVA: $F_{5,280}$=506.11, P<0.0001) impacted the EAG response of males held outdoors post-eclosion prior to the EAG experiment in the fall (Appendix 2-2). Males held outdoors and treated with one application of pyriproxyfen had the highest EAG response compared to males exposed to other treatments (Fig. 2-3C). The EAG results correspond to the wind tunnel results in the fall, as males treated with one application of either methoprene or pyriproxyfen typically had higher behavioural responses and higher EAG responses compared to males in the other treatment groups.

The environmental conditions that males were held under after eclosion in July significantly impacted male *C. fraxinella* wind tunnel and EAG responses. More males held indoors post-eclosion conducted wing fanning, lock-on, upwind flight and source contact compared to males held outdoors under natural conditions (Fig. 2-4A). In contrast, males held outdoors before the fall EAG experiment had slightly higher EAG responses compared to males held indoors (Fig. 2-4B) (Appendix 2-3).

Experiment 3 – Biogenic Amines

When male *C. fraxinella* were reproductively active in the spring, none of the BA treatments impacted male behavioural or EAG response to pheromone. Biogenic amine treatment did not impact wing fanning, lock-on, upwind flight, or source contact behaviours (Table 2-2). Electroantennogram response in the spring was impacted by pheromone dose (repeated measures ANOVA: $F_{5,230}$=328.03, P<0.0001), but not by BA treatment (Fig. 2-5A) (Appendix 2-4). Biogenic amine treatment also did not impact the length of time it took males to contact the pheromone source in the spring (Table 2-3).

When males were in reproductive diapause in the summer and fall, male *C. fraxinella* were treated with octopamine, dopamine or serotonin alone, or treated with a BA plus the JHA methoprene to test for a synergistic effect of both treatments on male pheromone response. Early in reproductive diapause in the summer, treatment did not impact wing fanning or upwind flight, but did impact lock-on and source contact (Table 2-2). Significant differences between treatments could not be separated for lock-on and source contact behaviours with

a Tukey-contrasts test, however more males treated with JHA alone conducted lock-on and source contact behaviours compared to males in the other treatment groups (Table 2-2). Electroantennogram response by males in the summer was impacted by both pheromone dose (repeated measures ANOVA: $F_{5,445}$=712.34, P<0.0001) and BA treatment alone or combined with a JHA treatment (Fig. 2-5B) (Appendix 2-4). Males that were either not treated or treated with the JHA methoprene had higher EAG responses compared to males given the other treatments (Fig. 2-5B). Treatment with octopamine or serotonin alone, or JHA plus octopamine, dopamine or serotonin lowered EAG response in the summer compared with untreated males (Fig. 2-5B). In the fall, treatment did not impact wing fanning, but did impact lock-on, upwind flight and source contact (Table 2-2). More males treated with the JHA methoprene or octopamine plus JHA locked-on to the pheromone compared to males that were either not treated or treated with a BA alone (Table 2-2). For upwind flight and source contact behaviours, treatment effects could not be separated with a Tukey-contrasts test, however in all cases, more JHA-treated males conducted each behaviour compared to the control or BA alone treatments, indicating that the increase in behavioural response with BA+JHA is likely due to the effect of JHA (Table 2-2). In the fall, male EAG response was impacted by both pheromone dose (repeated measures ANOVA: $F_{5,445}$=687.85, P<0.0001) and treatment (Fig. 2-5C) (Appendix 2-4). Males treated with either JHA alone or BA+JHA had higher EAG responses compared to males that were not treated with a JHA, and males

treated with a BA alone had the lowest EAG responses compared to males in the other treatment groups (Fig. 2-5C).

Discussion

Regulation of male pheromone response in insects that delay mating can be complex (Anton et al. 2007). The aim of this study was to further examine the role of JH in induction of pheromone response during reproductive inactivity in male *C. fraxinella*, and to test whether BAs are involved in male pheromone response plasticity in this species. One time treatment of male *C. fraxinella* in reproductive diapause with either methoprene or pyriproxyfen seven days before use in a bioassay, results in the highest subsequent pheromone response of males in reproductive diapause in the fall, but not earlier in the summer. Treatment with the JHA methoprene alone or JHA+BA also increases male pheromone response in the fall, however this increase can be attributed to treatment with the JHA alone, as treatment with the individual BAs does not enhance male response to pheromone at any time of year. Biogenic amine treatment does not affect pheromone processing of reproductively active males in the spring, but lowers response during reproductive diapause, indicating that there is a modulatory role for BAs in male odour processing.

Methoprene treatment of male *C. fraxinella* in reproductive diapause enhances behavioural and EAG response to pheromone in the fall but not summer (Lemmen and Evenden 2009). In the current study, pheromone response of males is enhanced by treatment with either methoprene or pyriproxyfen late in reproductive diapause. Male *C. fraxinella* tested seven days post-JHA treatment

have the highest behavioural and EAG response to pheromone in the fall. In insects, JH homologues are secreted by the *corpora allata*, and transported *via* JH binding proteins in the hemolymph to receptors in the cell membranes of specific tissues (Gilbert et al. 2000). Juvenile hormones are highly lipophilic, and JH binding proteins are necessary to transport JH through the aqueous hemolymph to target tissues (Gilbert et al. 2000). In adult Lepidoptera, JHIII is thought to be the most likely gonadotropic hormone, although other homologues are also produced by adults (Cusson et al. 1993). Three categories of JH binding proteins are known from the hemolymph of different insects (Gilbert et al. 2000). A potential JH receptor that binds JHIII, methoprene and pyriproxyfen has recently been identified (Charles et al. 2011).

Juvenile hormone analogues including methoprene and pyriproxyfen act either by preventing JH degradation or as JH-agonists (Ramaseshadri et al. 2012). There are two pathways to metabolize or degrade JH, the main pathway is *via* JH esterase, and the secondary pathway is *via* JH epoxide hydrolase (Gilbert et al. 2000, Minakuchi and Riddiford 2006). Although the mechanisms are not well understood, the JH esterase pathway plays an important role in the regulation of JH titre in the hemolymph (Gilbert et al. 2000). In general, JH is metabolized by JH esterase to JH acid, and then JH acid is metabolized by JH epoxide hydrolase to JH acid diol (Gilbert et al 2000). However, this metabolic sequence does not occur in all insects, and there are many water-soluble JH metabolites that differ among species of insects and others that have not yet been identified (Gilbert et al. 2000). After exogenous application, JHAs bind to JH-binding proteins and

prevent the degradation of JH by forming a JHA-protein complex that protects the JHA from natural degrading enzymes present in the hemolymph (Gilbert et al. 2000; Ramaseshadri et al. 2012). In the absence of endogenous JH, JHAs can act as a hormone replacement, which can lead to long-term effects after only one application (Ramaseshadri et al. 2012). Prevention of endogenous JH degradation or hormone replacement by JHA treatment can both lead to increased titres of JH in insects, which has been linked to higher pheromone response in some male moths (Cusson et al. 1993; Gadenne et al. 1993; Anton et al. 2007). In *C. fraxinella*, it is more likely that JHAs are acting as a JH hormone replacement than in the prevention of JH degradation, since it is expected that males would have low titres of or lack endogenous JH during reproductive diapause, as occurs in other male moths during a period of reproductive inactivity (Cusson et al. 1993; Duportets et al. 1998). The JHAs may act as a hormone replacement by binding to JH-binding proteins and receptors and initiating the upregulation of JH-regulated physiology, including pheromone response, for approximately 7 days, after which time they degrade and are not replaced, since the *corpora allata* of male *C. fraxinella* may not be able to produce endogenous JH during reproductive diapause. Reproductive diapause is not completely terminated in JHA-treated males in the fall, since male pheromone response declines 9 days after JHA treatment. If reproductive diapause was terminated and the *corpora allata* were stimulated to produce endogenous JH, then similar levels or an increase in pheromone response would be expected between 7 and 9 days. A single application of methoprene or pyriproxyfen stimulates vitellogenin synthesis in

female *Locusta migratoria* (Orthoptera: Acrididae) 1-2 days post-JHA treatment (Edwards et al. 1993). Hemolymph vitellogenin level slowly declines until 15 days post-JHA treatment (Edwards et al. 1993). In male *C. fraxinella*, there is an increase in pheromone response over 7 days post-JHA treatment, after which it declines. The lower pheromone response of male *C. fraxinella* when bioassayed >7 days post treatment suggests that JHAs may only act as a hormone replacement for a limited time.

Treatment with one application of either methoprene or pyriproxyfen enhances male *C. fraxinella* behavioural and EAG response to pheromone in the fall. Males treated with two applications of either JHA in the fall have lower behavioural and EAG responses than males treated with only one application. If JHAs act as a hormone replacement in male *C. fraxinella*, additional application of JHAs would be predicted to increase the length of time that male response to pheromone is high. In the current study, there is a decrease in pheromone response after moths receive multiple JHA applications. It is possible that the second application of JHA in the current study initiates high JH esterase activity that leads to rapid metabolism and breakdown of the JHAs and a reduced subsequent pheromone response (Gilbert et al. 2000). Methoprene is more structurally similar to endogenous JH than pyriproxyfen (Minakuchi and Riddiford 2006), but both have high activity in insects, and are commonly used in endocrine experiments (Ramaseshadri et al. 2012) (see Appendix 2-5 for chemical structures). The results here, suggest that both methoprene and pyriproxyfen are

equally able to enhance the responsiveness of male *C. fraxinella* to pheromone late in reproductive diapause.

The effect of methoprene and pyriproxyfen treatment on male *C. fraxinella* response to pheromone in the fall did differ depending on the environmental conditions under which males were held prior to the bioassay. In the fall, more treated males conduct pheromone-mediated behaviours than control males in the wind tunnel after one application of either JHA if they are held indoors under summer conditions prior to the bioassay. Only treatment with one application of methoprene enhances behavioural response to pheromone when males were held outdoors under a naturally declining photoregime in the fall. A similar difference in the effect of the two JHAs is evident from EAG bioassays. Males held indoors post-eclosion until the time of bioassay in the fall had higher EAG responses when treated with one application of either JHA. Similarly treated males held outdoors prior to the bioassay exhibit increased response to pheromone as compared to control males only after treatment with one application of pyriproxyfen. The two JHAs may have slightly different affinities for the JH binding proteins and/or JH receptors in this species, and the binding of the JHAs may be impacted by environmental conditions. It is clear that there are different JH binding proteins in the hemolymph of different insects (Gilbert et al. 2000, Minakuchi and Riddiford 2006), but it is not clear what regulates the concentration of the various proteins in the hemolymph, and whether they are static or plastic (Gilbert et al. 2000).

Male *C. fraxinella* behavioural and EAG pheromone response was impacted by the environmental conditions that males were held under after eclosion in July until use in a bioassay in the fall. More males held indoors expressed pheromone-mediated behaviours in the wind tunnel compared with males held outdoors, while males held outdoors had higher EAG responses compared with males held indoors. The results of behavioural response in the current study is supported by another study on *C. fraxinella*, in which males held indoors post-eclosion had higher behavioural response to pheromone in the fall compared to males held outdoors (Chapter 3). The environmental cues that males receive when held outdoors prepare them for overwintering. Male *C. fraxinella* should have a low pheromone response in the fall as all moths that overwinter are in reproductive diapause (Evenden et al. 2007). Males held indoors next to windows experience constant ambient warm temperature conditions, which could break reproductive diapause, as occurs in other species (Tauber and Tauber 1976; Herman and Dallmann 1981). Male EAG response is impacted by environmental conditions in the current study but those did not correlate with the behavioural response. Males held indoors have lower EAG responses but higher behavioural response to pheromone compared to males held outdoors. It is likely that some modulation occurs in the central nervous system of male *C. fraxinella*, so that the central nervous system is more sensitive to pheromone after moths are housed under indoor conditions that results in a higher behavioural output. Modulation of pheromone processing that is dependent on the physiological state of the moth is known to occur in other moth species in the central nervous system (Anton et al.

2007). Although EAG response of male *A. ipsilon* moths is similar in sexually immature and mature adults, a large portion of antennal lobe pheromone-responding neurons are much more sensitive to pheromone in sexually mature males compared to sexually immature males (Anton et al. 2007; Anton and Gadenne 1999). In male *C. fraxinella*, there may be similar modulation of pheromone perception in the antennal lobe that would correlate pheromone perception with behavioural output.

Treatment with the BAs octopamine, dopamine and serotonin did not increase behavioural or EAG response to pheromone in male *C. fraxinella* during any reproductive state. The BAs tested in the current study do not appear to be important neuromodulators of pheromone responsiveness following the termination of reproductive diapause. Exogenous application of octopamine increases the sensitivity of male *G. molesta* to pheromone (Linn and Roelofs 1984), and increases oviposition in virgin female *Plodia interpunctella* (Lepidoptera: Pyralidae) (Rafaeli 2004). Biogenic amine treatments typically impact male moth response to pheromone when the moths are reproductively active (Linn and Roelofs 1984; 1986; Linn et al. 1992; 1996; Pophof 2000; 2002; Jarriault et al. 2009). In male *A. ipsilon*, octopamine treatment increases male pheromone response in reproductively active males, but not in newly-eclosed sexually immature males (Jarriault et al. 2009). In male *C. fraxinella*, treatment with any of the BAs does not impact male response to pheromone when males are reproductively active in the spring, but only when males are in reproductive diapause in the summer and fall.

Biogenic amine treatment lowers male *C. fraxinella* response to pheromone when males are in reproductive diapause in the summer and fall. In the summer, treatment with octopamine or serotonin lowers EAG response compared to untreated males, whether the BA is applied alone or in combination with JHA. Treatment with JHA alone slightly enhances the number of males that conduct pheromone-mediated behaviours in the summer compared to untreated males. Whereas, BA treatment slightly reduces the number of males that exhibit these behaviours compared to untreated males. In the fall, JHA treatment enhances male EAG and behavioural response compared to untreated males, while treatment with octopamine, dopamine or serotonin lowers male EAG response. Biogenic amine treatment did not lower male behavioural response below that of untreated males in the fall, likely because so few untreated individuals respond at this time of year. When the BA treatment was combined with JHA treatment in the fall, male EAG and behavioural response was enhanced to the same level as the JHA treatment alone.

Induction of pheromone response in male *C. fraxinella* during reproductive diapause is achieved with JHA treatment alone, while treatment with a BA reduces pheromone responsiveness. There is no interaction between the JHA and BA treatments. The JHA treatment overrides the negative effect of the BAs in the fall, as EAG and behavioural response is enhanced after treatment with both BA+JHA. Binding of chemical messengers, such as BAs and JH to receptors causes a channel pore to open, which can excite or inhibit the specific target cell (Blenau and Bauman 2001). In the fall, JHA treatment causes excitation of ORNs

in male *C. fraxinella*, as evidenced by the increase in EAG response compared to similarly treated males in the summer in this and previous (Lemmen and Evenden 2009) studies. Whereas, treatment with BAs seems to inhibit ORNs, as EAG response after treatment is consistently lower, regardless of when males are treated throughout the period of reproductive diapause. This suggests that BAs play a role in the modulation of the peripheral nervous system in male *C. fraxinella* to ensure that male pheromone response is low when males are in reproductive diapause. Pheromone responsiveness is typically enhanced with octopamine treatment in insects (Grosmaitre et al. 2001; Linn et al. 1992; Linn and Roelofs 1986; Pophof 2000; Pophof 2002; Zhukovskaya 2008), however response to a general odorant is suppressed with octopamine treatment in male *Periplaneta americana* (Blattodea: Blattidae) (Zhukovskaya 2008). Serotonin treatment increases pheromone response in some male moths (Linn et al. 1992), and decreases response in other moths (Grosmaitre et al. 2001). Although the role of octopamine has been investigated in *A. ipsilon* (Barrozo et al. 2010; Jarriault et al. 2009), a moth with delayed reproduction that requires the presence of both octopamine and JH to respond to pheromone, this delay does not involve a period of reproductive diapause as occurs in *C. fraxinella*. It is essential that males do not respond to pheromone in a reproductively inactive state. Biogenic amines may be important modulators that prevent mating encounters of *C. fraxinella* during reproductive diapause, as all of the BA treatments lower male pheromone response in the current study.

The effect of JHA and BA treatments on male *C. fraxinella* EAG response is similar to the effect the treatments have on male behavioural response. Male behavioural response may be modulated separately from EAG response with JHA and BA treatments that impact the peripheral and central nervous systems independently, or the behavioural response of males could be a direct result of modulation at the level of the antenna. The impact of JHA and BA on the central nervous system could be tested in male *C. fraxinella* directly with intracellular recordings of antennal lobe interneurons as has been done to test the role of JH in the modulation of pheromone processing in male *A. ipsilon* (Anton and Gadenne 1999; Gadenne and Anton 2000). The levels of endogenous BAs in *C. fraxinella* could also be measured at different times of year and at different periods during the photophase and scotophase to determine whether endogenous levels of the BAs change rhythmically or seasonally, which would provide a better indication of their function in odour modulation in *C. fraxinella*.

In this study, the role and mechanism of JHAs in enhancing pheromone response of male *C. fraxinella* in reproductive diapause is examined, and a role for BAs in odour modulation is determined. Juvenile hormone analogue treatment enhances male EAG and behavioural response to pheromone when males are in reproductive diapause in the fall and that effect is greatest one week after a single JHA application. In contrast, BA treatment lowers male EAG and behavioural response to pheromone throughout the period of reproductive diapause in both the summer and fall. Juvenile hormone is important in the termination of reproductive diapause, as measured by enhanced pheromone

response in this species. Whereas, BAs inhibit response to pheromone by males in reproductive diapause, which ensures males do not mate at an inappropriate time. Exposure to different environmental conditions impacts pheromone response of JHA-treated males in the fall, which indicates an important role for temperature and photoperiod in diapause termination in *C. fraxinella* (Chapter 3). During the period of reproductive diapause, JH and BAs both function to modulate male *C. fraxinella* pheromone response to appropriately time male response to pheromone with female receptivity.

Table 2-1. The number of *Caloptilia fraxinella* males tested in juvenile hormone analogue and biogenic amine experiments at various times of year.

Season	Dates	Bioassay	Treatments	N
Experiment 1				
Summer	27-29 July 2011	Wind Tunnel	1-day post-JHA[a]	31
			3-day post-JHA	25
			7-day post-JHA	29
			9-day post-JHA	22
			Acetone	29
			Not Treated	29
Fall	1-2 October 2011	Wind Tunnel	1-day post-JHA	46
			3-day post-JHA	44
			7-day post-JHA	37
			9-day post-JHA	32
			Acetone	48
			Not Treated	49
Experiment 2				
Summer	22-27 July 2010	Wind Tunnel	Methoprene x1[b]	37
			Methoprene x2	35
			Pyriproxyfen x1	40
			Pyriproxyfen x2	34
			Acetone	47
			Not Treated	47
	26-27 July 2010	EAG[c]	Methoprene x1	10
			Methoprene x2	10
			Pyriproxyfen x1	10
			Pyriproxyfen x2	10
			Acetone	10
			Not Treated	10
Fall Indoors	20 September-4 October 2010	Wind Tunnel	Methoprene x1	32
			Methoprene x2	33
			Pyriproxyfen x1	33
			Pyriproxyfen x2	29
			Acetone	32
			Not Treated	33
	14-23 September 2010	EAG	Methoprene x1	10
			Methoprene x2	10
			Pyriproxyfen x1	10
			Pyriproxyfen x2	9
			Acetone	10
			Not Treated	10
Fall Outdoors	20 September-4 October 2010	Wind Tunnel	Methoprene x1	33
			Methoprene x2	32
			Pyriproxyfen x1	32
			Pyriproxyfen x2	29

			Acetone	32
			Not Treated	32
	14-23 September 2010	EAG	Methoprene x1	9
			Methoprene x2	10
			Pyriproxyfen x1	8
			Pyriproxyfen x2	10
			Acetone	10
			Not Treated	10
Experiment 3				
Spring	19-21 May 2010	Wind Tunnel	Octopamine	46
			Dopamine	47
			Serotonin	44
			Methanol	50
			Not Treated	50
	5-8 May 2010	EAG	Octopamine	10
			Dopamine	9
			Serotonin	9
			Methanol	10
			Not Treated	9
Summer	28 July-5 August 2010	Wind Tunnel	Octopamine	45
			Dopamine	43
			Serotonin	41
			Octopamine+JHA	39
			Dopamine+JHA	39
			Serotonin+JHA	37
			JHA	35
			Methanol+Acetone	45
			Not Treated	38
	27-28 July 2010	EAG	Octopamine	10
			Dopamine	10
			Serotonin	10
			Octopamine+JHA	10
			Dopamine+JHA	10
			Serotonin+JHA	10
			JHA	10
			Methanol+Acetone	10
			Not Treated	10
Fall	27 September-13 October 2010	Wind Tunnel	Octopamine	39
			Dopamine	37
			Serotonin	37
			Octopamine+JHA	38
			Dopamine+JHA	37
			Serotonin+JHA	37
			JHA	42
			Methanol+Acetone	39
			Not Treated	35
	26 September-2 October 2010	EAG	Octopamine	10
			Dopamine	10

	Serotonin	10
	Octopamine+JHA	10
	Dopamine+JHA	10
	Serotonin+JHA	10
	JHA	10
	Methanol+Acetone	10
	Not Treated	10

[a] JHA=Juvenile hormone analogue, methoprene. [b] x1 indicates one application of the treatment, x2 indicates two applications of the treatment.

[c] EAG=Electroantennogram.

Table 2-2. Percentage behavioural response of male *C. fraxinella* to a 10 μg pheromone lure in wind tunnel bioassays after exposure to various biogenic amine treatments with and without JHA. Males were tested while reproductively active in the spring, and while in reproductive diapause in the summer and fall.

Season	Behaviour[a]	Deviance$_{df,\ \alpha}$	P	Controls (% Response)			Biogenic Amine[d] (% Response)			Biogenic Amine+JHA (% Response)		
				Not Treated	Acetone+ Methanol[b]	JHA[c]	Oct	Dop	Ser	Oct+ JHA	Dop+ JHA	Ser+ JHA
Spring	WF	$X_{4,0.05}$=8.2	0.08	94	74	n/a	87	85	86	ND[e]	ND	ND
	LO	$X_{4,0.05}$=8.2	0.08	94	76	n/a	83	77	82	ND	ND	ND
	UWF	$X_{4,0.05}$=8.8	0.07	90	72	n/a	80	68	75	ND	ND	ND
	SC	$X_{4,0.05}$=3.4	0.5	72	66	n/a	76	60	68	ND	ND	ND
Summer	WF	$X_{8,0.05}$=3.9	0.9	21	16	26	29	19	20	23	23	16
	LO	$X_{8,0.05}$=23.1	0.003	16	2	29	9	9	5	18	5	3
	UWF	$X_{8,0.05}$=15.3	0.6	16	2	20	9	9	5	15	5	3
	SC	$X_{8,0.05}$=16.6	0.03	11	2	17	9	9	0	13	5	3
Fall	WF	$X_{8,0.05}$=9.7	0.3	26	31	24	15	16	24	32	38	35
	LO	$X_{8,0.05}$=43.9	<0.0001	6 (b)[f]	26 (ab)	50 (a)	10 (b)	8 (b)	16 (ab)	45 (a)	32 (ab)	30 (ab)
	UWF	$X_{8,0.05}$=41.3	<0.0001	3	23	41	10	3	16	40	19	30
	SC	$X_{8,0.05}$=34.3	<0.0001	3	23	33	3	3	16	29	19	19

[a]Behaviour abbreviations: WF=Wing Fanning; LO=Lock-on to pheromone plume; UWF=upwind flight towards pheromone source; SC=Source contact with the pheromone. [b]In the spring, this control treatment was methanol alone, in the summer and fall, this control treatment was acetone+methanol. [c]JHA=Juvenile hormone analogue, methoprene. [d]Biogenic amine abbreviations: Oct=Octopamine; Dop=Dopamine; Ser=Serotonin. [e]ND=Experiment not done. [f]Different letters indicate significant differences among treatments within the same behaviour based on a Tukey-contrasts test ($P<0.05$).

Table 2-3. Length of time it took reproductively active male *C. fraxinella* to contact the pheromone source when treated with various biogenic amines in the spring. Biogenic amine treatment did not impact the length of time it took males to contact the pheromone source (ANOVA: $F_{4,155}$=1.86, P=0.1).

Treatment	Mean (+SE) (Seconds)
Not Treated	46 (5.95)
Methanol	71 (9.05)
Octopamine	51 (5.43)
Dopamine	64 (10.20)
Serotonin	51 (8.06)

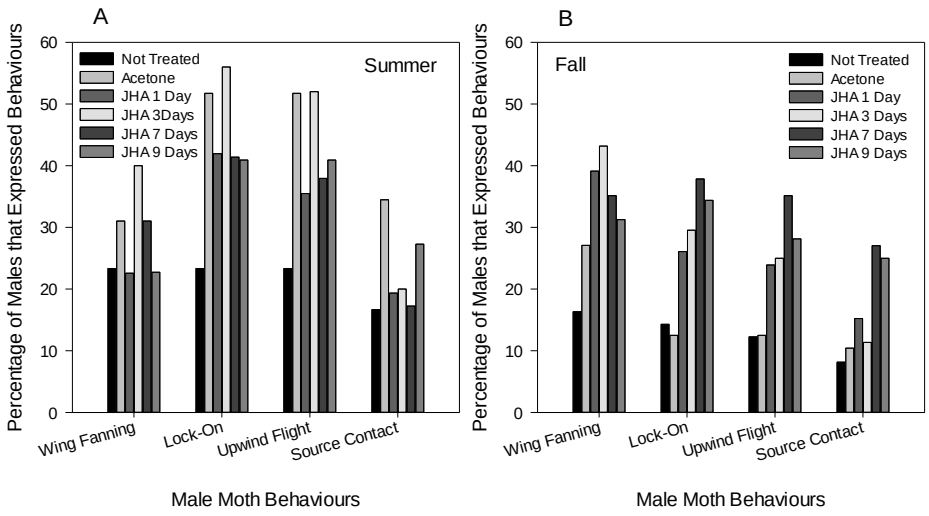

Fig. 2-1. Percentage of male *C. fraxinella* in reproductive diapause in the (A) summer and (B) fall that expressed behaviours in the wind tunnel 1, 3, 7, or 9 days post-treatment with the JHA methoprene. (A) In the summer the number of days post-JHA treatment did not impact any pheromone-mediated behaviour: wing fanning (GLM: $X_{3,0.05}$=2.5, P=0.5), lock-on (GLM: $X_{3,0.05}$=1.6, P=0.7), upwind flight (GLM: $X_{3,0.05}$=1.7, P=0.6) and source contact (GLM: $X_{3,0.05}$=0.8, P=0.9). (B) In the fall, the number of days post-JHA treatment did not impact wing fanning (GLM: $X_{3,0.05}$=1.3, P=0.7), lock-on (GLM: $X_{3,0.05}$=1.5, P=0.7), upwind flight (GLM: $X_{3,0.05}$=1.5, P=0.7) or source contact (GLM: $X_{3,0.05}$=4.5, P=0.2).

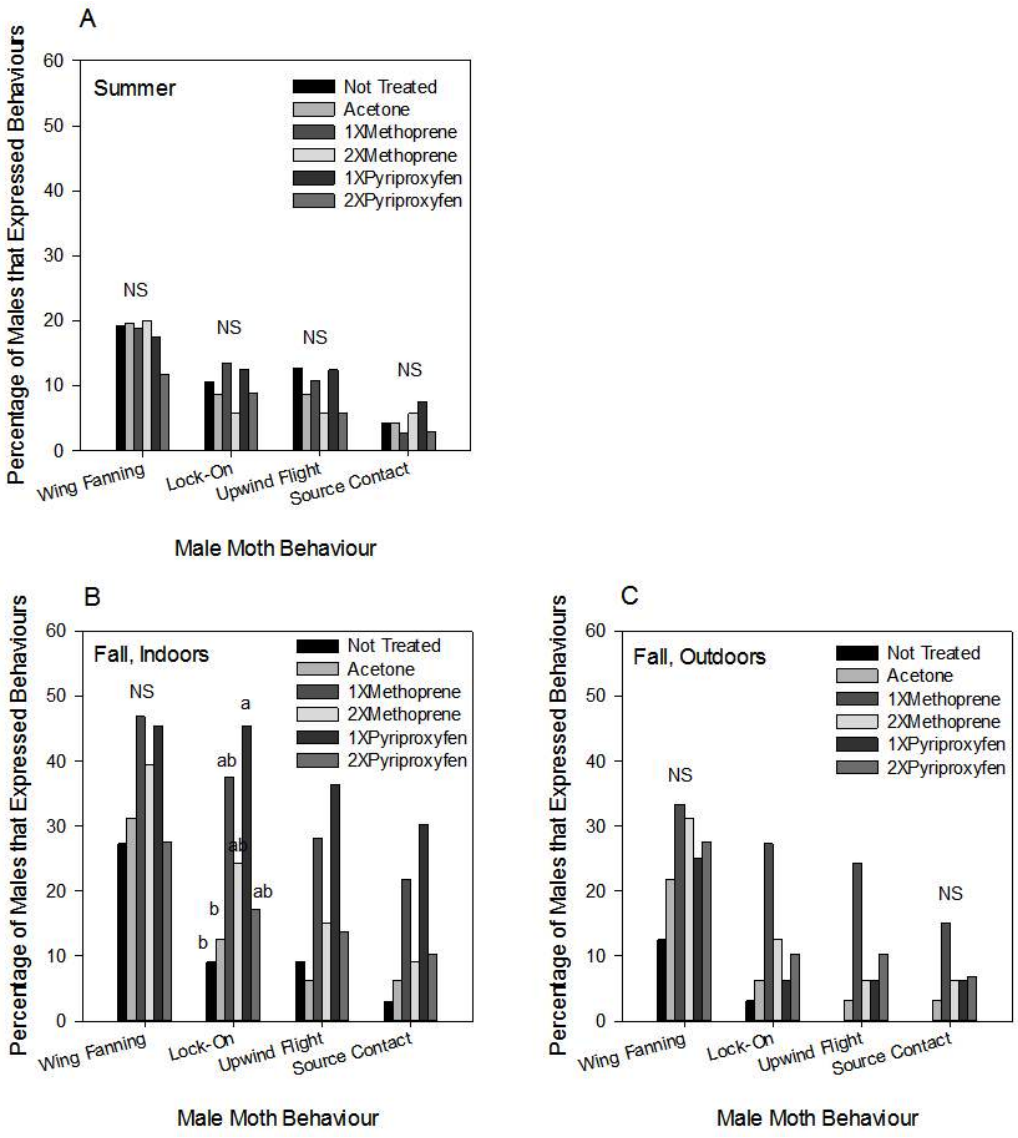

Fig. 2-2. Percentage of male *C. fraxinella* in reproductive diapause in the (A) summer, (B) fall (indoors) and (C) fall (outdoors) that expressed behaviours in the wind tunnel after treatment with one or two applications of the JHA methoprene or pyriproxyfen one week before the bioassay. (A) In the summer, JHA treatment did not impact any pheromone-mediated behaviours: wing fanning (GLM: $X_{5,0.05}$=1.23, P=0.9), lock-on (GLM: $X_{5,0.05}$=1.72, P=0.9), upwind flight (GLM: $X_{5,0.05}$=2.28, P=0.8) or source contact (GLM: $X_{5,0.05}$=1.36, P=0.9). (B) When males were held indoors post-eclosion and tested in the fall, JHA treatment did

not impact wing fanning (GLM: $X_{5,0.05}$=5.36, P=0.4), but did impact lock-on (GLM: $X_{5,0.05}$=18.46, P=0.002), upwind flight (GLM: $X_{5,0.05}$=14.65, P=0.01) and source contact (GLM: $X_{5,0.05}$=14.85, P=0.01). (C) When males were held outdoors post-eclosion and tested in the fall, JHA treatment did not impact wing fanning (GLM: $X_{5,0.05}$=5.13, P=0.4) or source contact (GLM: $X_{5,0.05}$=8.06, P=0.2), but did impact lock-on (GLM: $X_{5,0.05}$=11.19, P=0.05) and upwind flight (GLM: $X_{5,0.05}$=15.12, P=0.01). Different letters indicate significant differences between JHA treatments for lock-on behaviour (HSD≤0.06). NS=treatment were not significant for that behaviour. Behaviours with no letters or NS indicate a significant overall model, but significance between JHA treatments could not be determined with post-hoc HSD test.

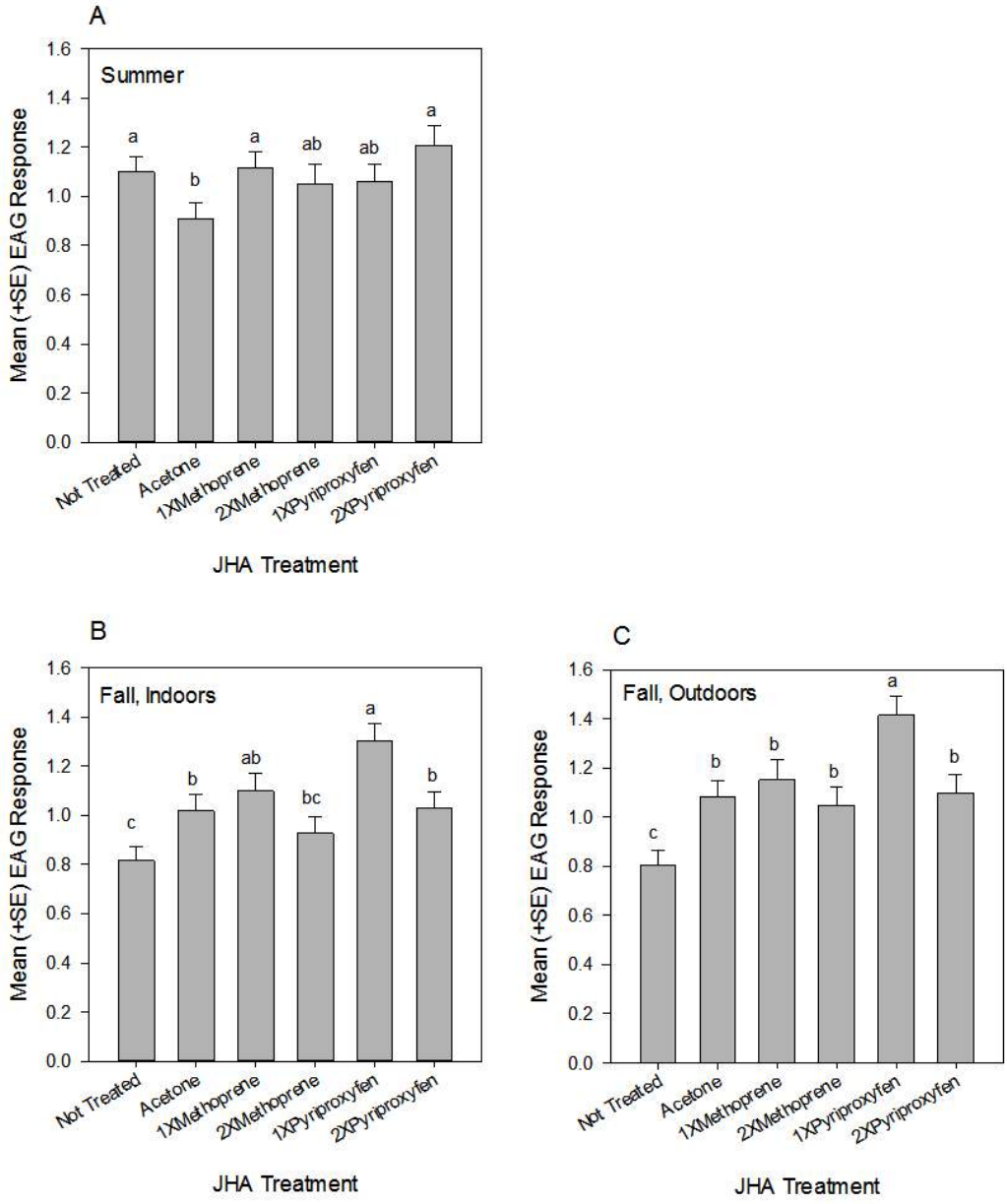

Fig. 2-3. Mean (+SE) EAG response of antennae from male *C. fraxinella* in reproductive diapause in the (A) summer, (B) fall (indoors) and (C) fall (outdoors) after treatment with one or two applications of the JHA methoprene or pyriproxyfen one week before the bioassay. (A) In the summer, JHA treatment impacted male EAG response (repeated measures ANOVA: $F_{5,295}$=6.02, P<0.0001). (B) When males were held indoors prior to fall experiments, JHA treatment impacted EAG response (repeated measures ANOVA: $F_{5,290}$=11.16,

P<0.0001). (C) When males were held outdoors prior to fall experiments, JHA treatment impacted male EAG response (repeated measures ANOVA: $F_{5,280}$=17.84, P<0.0001). Response to the different doses (including the control) is pooled and y-axes are back-transformed to reflect raw mean EAG responses. Different letters indicate significant differences between JHA treatments (HSD=0.05).

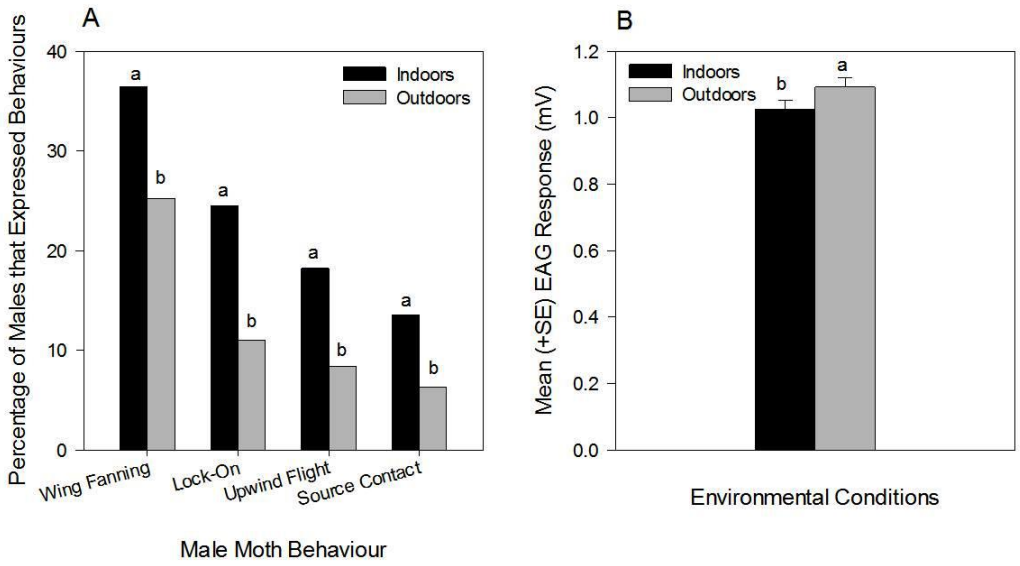

Fig. 2-4. Male *C. fraxinella* (A) behavioural and (B) EAG response to pheromone when males were held either indoors or outdoors post-eclosion and tested in the fall. (A) More males held indoors conducted wing fanning (GLM: $X_{1,0.05}$=5.63, P=0.02), lock-on (GLM: $X_{1,0.05}$=12.02, P=0.0005), upwind flight (GLM: $X_{1,0.05}$=8.12, P=0.004) and source contact (GLM: $X_{1,0.05}$=5.69, P=0.02). (B) Males held outdoors had higher EAG responses to pheromone compared to males held indoors (repeated measures ANOVA: $F_{1,575}$=5.67, P=0.02). Response to the different pheromone doses (including the control) is pooled and the y-axis is back-transformed to reflect raw mean EAG responses. Different letters indicate a significant difference between the two treatments.

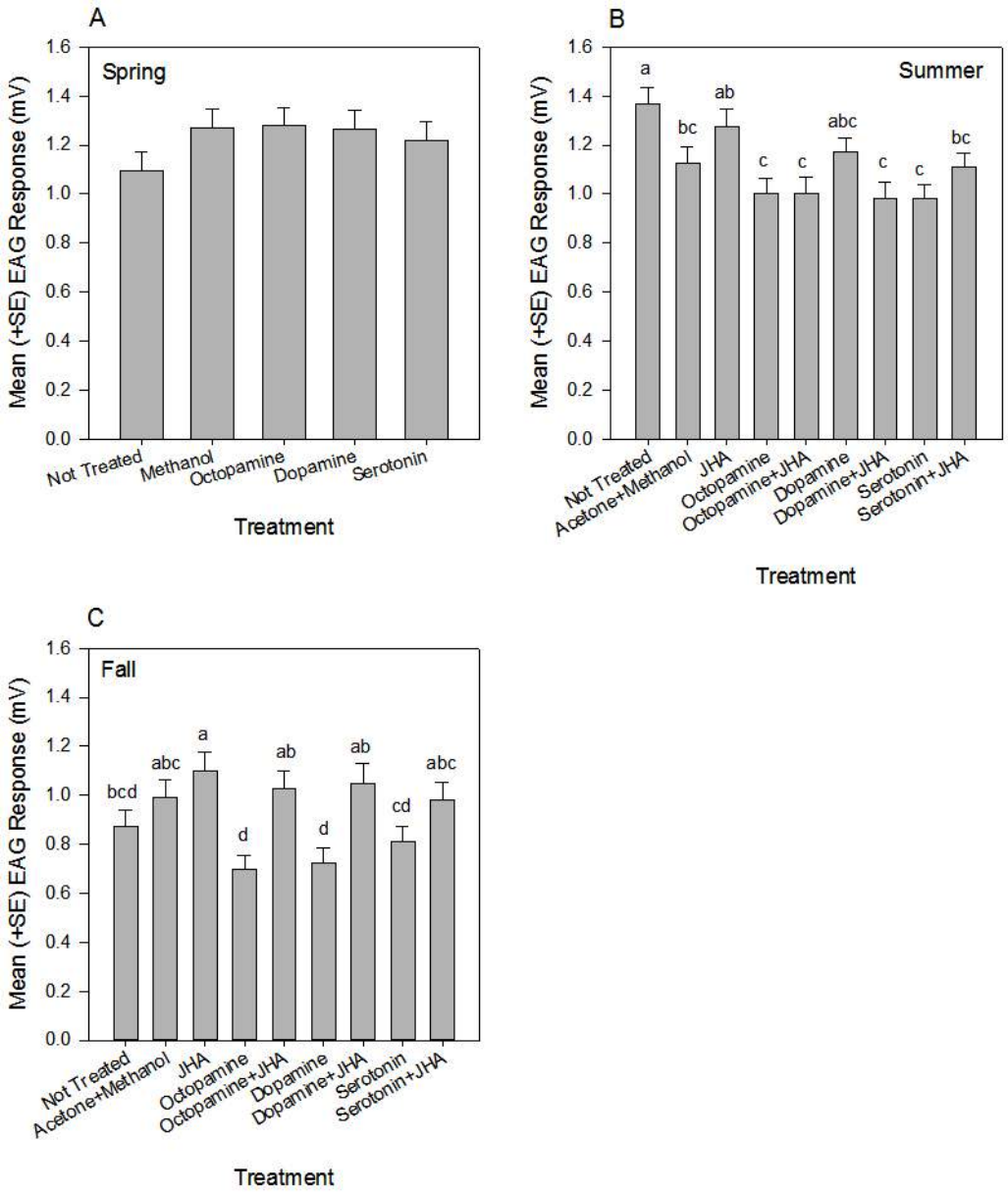

Fig. 2-5. Mean (+SE) EAG response of antennae from male *C. fraxinella* treated with a variety of BA and BA+JHA treatments and tested in the (A) spring, (B) summer and (C) fall. (A) In the spring, biogenic amine treatment did not impact EAG response (repeated measures ANOVA: $F_{4,230}$=1.71, P=0.2). (B) In the summer, treatment impacted EAG response (repeated measures ANOVA: $F_{8,445}$=9.64, P<0.0001). (C) In the fall, treatment impacted EAG response

(repeated measures ANOVA: $F_{8,445}=10.84$, $P<0.0001$). For all graphs, EAG responses to the different doses (including the control) are pooled and y-axes are back-transformed to reflect a pooled raw mean EAG response. Different letters indicate significant differences between JHA treatments (HSD=0.05).

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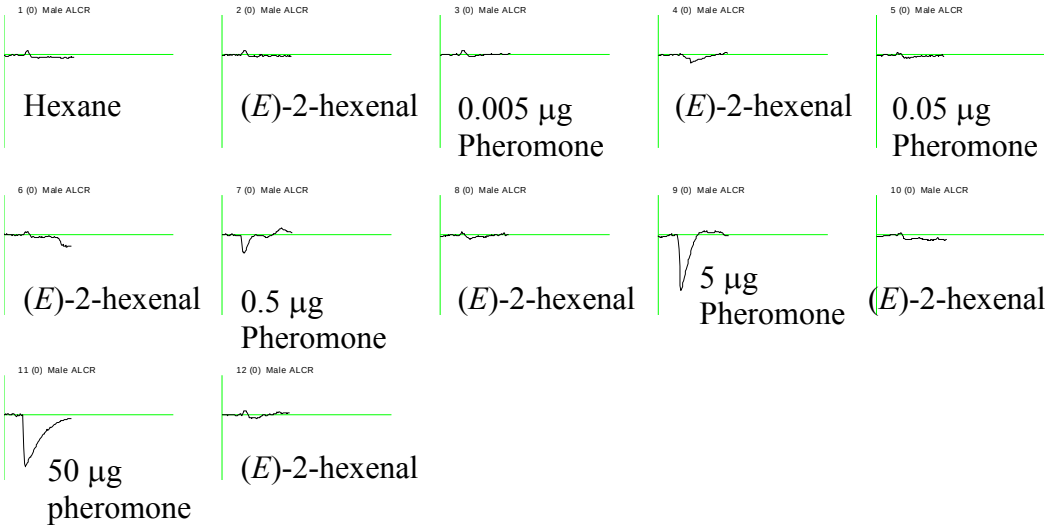
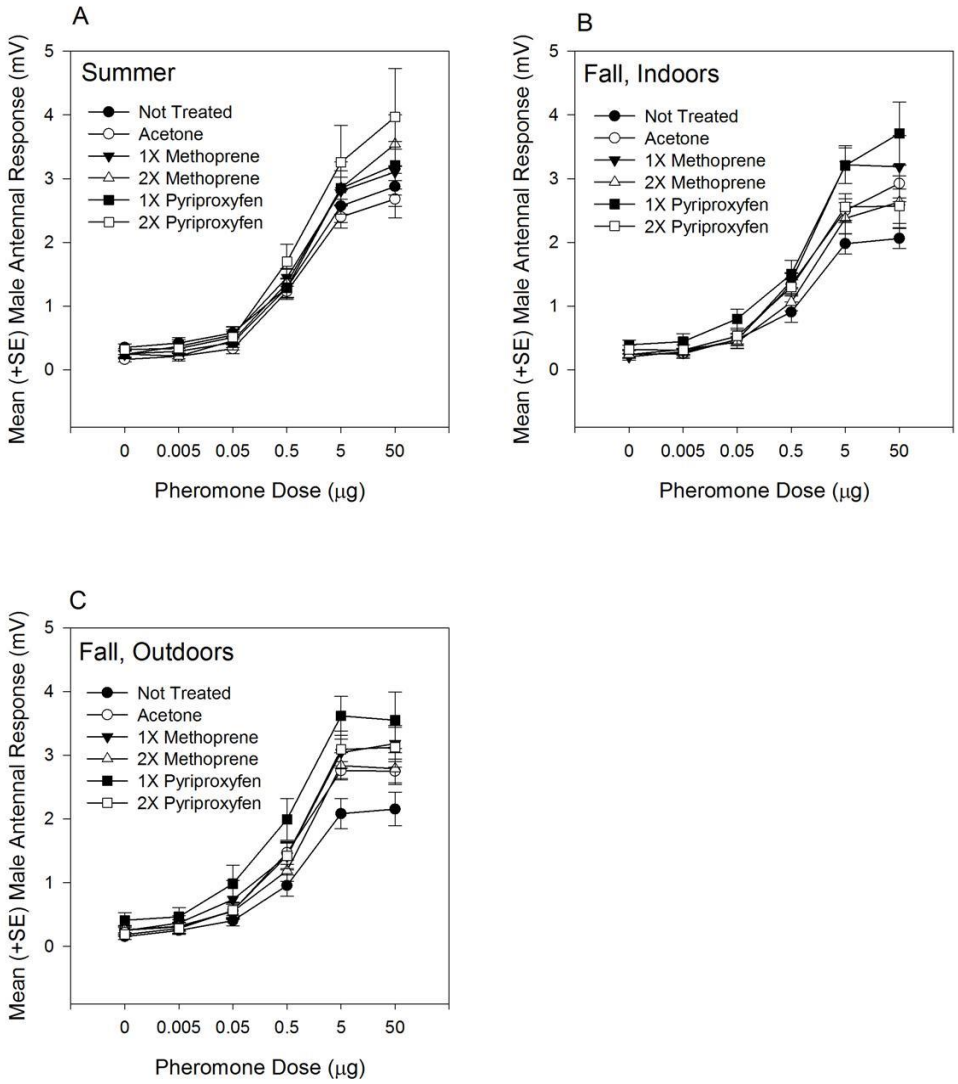
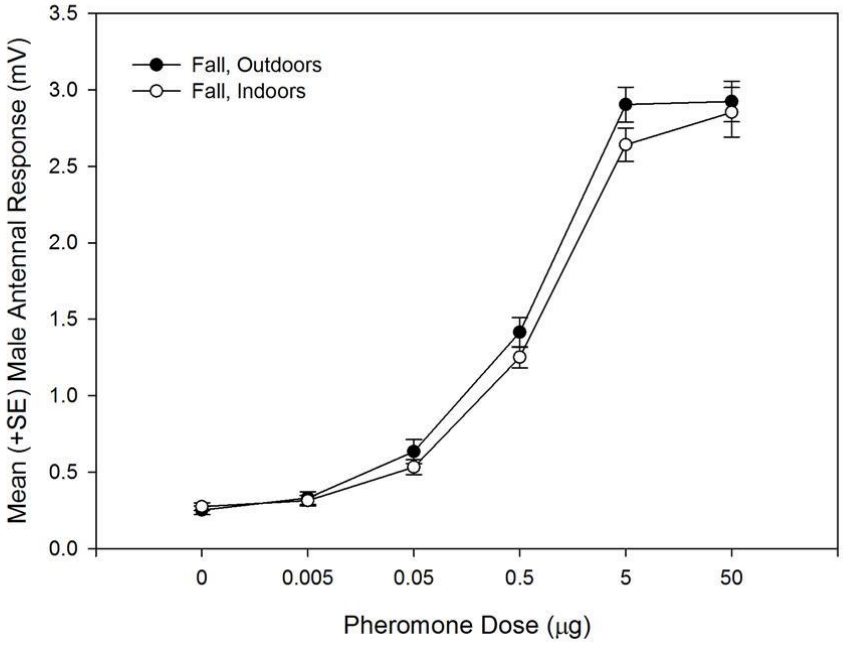


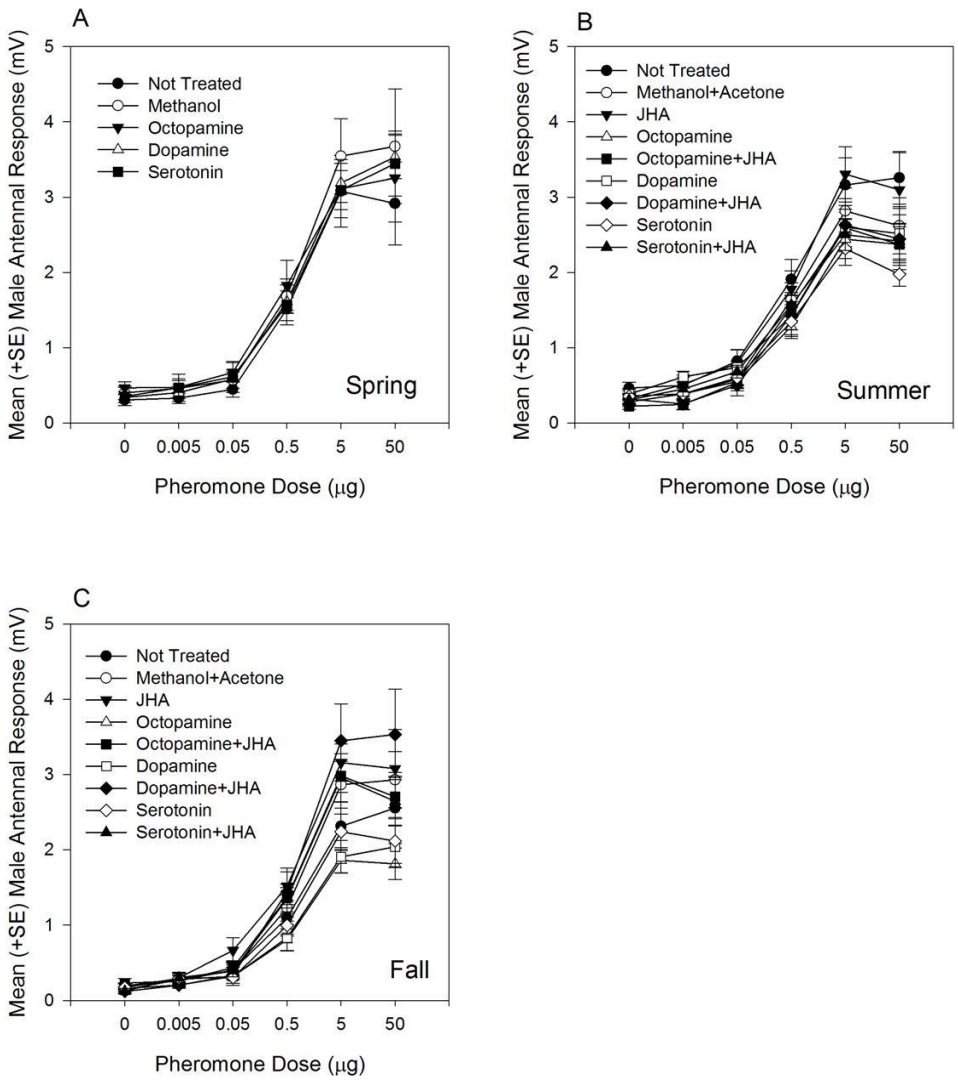
Appendix 2-1. Representative EAG traces for the series of stimuli presented to male *C. fraxinella* single antennae. Male antenna tested here was from a spring, untreated individual tested on 5 May 2010.



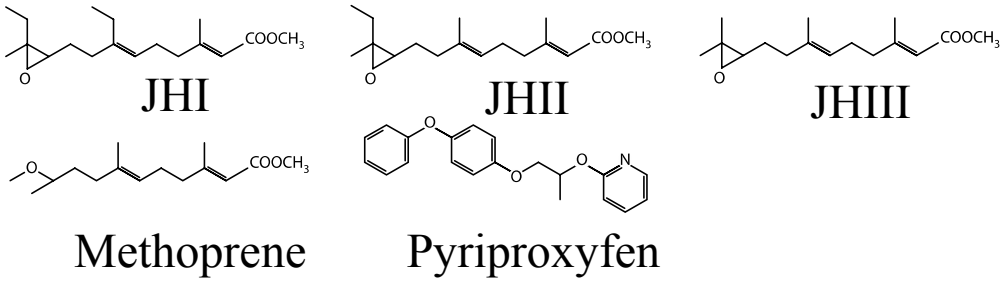
Appendix 2-2. Dose response curves for male EAG response to pheromone when males were in reproductive diapause in the (A) summer and (B, C) fall. Males in the fall were held either (A) indoors in the laboratory or (B) outdoors in cages under natural environmental conditions from eclosion in the summer until time of testing. Legend indicates treatments applied to male moths one week before testing. 1X indicates one application of the treatment, 2X indicates two applications of the treatment. Graph depicts raw EAG data.



Appendix 2-3. Dose response curve of male EAG response to pheromone from males in reproductive diapause in the fall. Males were held either indoors in the laboratory or outdoors in cages to experience natural environmental conditions from eclosion in the summer until time of testing. Juvenile hormone analogue treatment and controls are pooled, and the graph depicts raw EAG data.



Appendix 2-4. Dose response curves of male *C. fraxinella* EAG responses to pheromone when males were reproductively active in the (A) spring, and while in reproductive diapause in the (B) summer and (C) fall. Legend indicates biogenic amine, control and JHA treatments. JHA=Juvenile hormone analogue, methoprene. Graphs depict raw EAG data.



Appendix 2-5. Structures of juvenile hormones and the juvenile hormone analogues methoprene and pyriproxyfen. (Adapted from: Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annual Review of Entomology **58**, 181-204.)

Chapter 3

Environmental conditions terminate reproductive diapause and influence pheromone perception in the long-lived moth, *Caloptilia fraxinella* (Lepidoptera: Gracillariidae)

Introduction

Insects exhibit seasonal adaptations throughout their life cycles to environmental conditions such as temperature and photoperiod (Danks 1991, 2006). In response to environmental cues, insects can advance or delay development in order to conserve energy and protect themselves under adverse conditions. Plasticity in development time also allows for optimization of resource and mate location under conditions that maximize success (Danks 2002, 2006). Diapause is a state of arrested development in insects that is controlled centrally or hormonally, and is a seasonal adaptation that occurs at a specific stage of development in response to an environmental stimulus, such as photoperiod or temperature (Denlinger et al., 2005; Hahn and Denlinger, 2011; Kostal et al., 2008; Kostal, 2011, Pener 1992). Many insects experience a facultative adult reproductive diapause that depends on temperature and/or photoperiod conditions to control the timing of reproduction so that it occurs when environmental conditions are suitable for mating and offspring development (Danks, 2006; Denlinger et al., 2005; Kostal et al., 2008; Macrae, 2005; Macrae, 2010). Reproductive diapause is diagnosed mainly by a cessation in reproduction, an inability of males to locate and mate with receptive females, reduced accessory

gland development in males and females and arrestment of oocyte development in females (Denlinger, 2005, Pener 1992).

Juvenile hormone (JH) released from the *corpora allata* is the main hormone that controls the onset or termination of reproductive diapause in insects (Kopper et al., 2001; Shiga et al., 2003). In blow flies, *Protophormia terraenovae* (Diptera: Calliphoridae) (Matsuo et al., 1997; Shiga et al., 2003) and bean bugs, *Riportus clavatus* (Heteroptera: Alydidae) (Morita and Numata, 1997; Numata, 1992), adults eclose in reproductive diapause under short-day conditions proximately caused by suppression of JH synthesis (Matsuo et al., 1997; Morita and Numata, 1997). The solitary bee, *Osmia rufa* (Hymenoptera: Megachilidae) undergoes an overwintering reproductive diapause, and application of the juvenile hormone analogue (JHA), methoprene, and increased temperature either separately or together terminate diapause (Wasielewski et al., 2011). Photoperiod and temperature conditions maintain reproductive diapause by suppression of JH secretion by the *corpora allata* in autumn morphs of the nymphalid butterfly, *Polygonia c-aureum* (Lepidoptera: Nymphalidae) (Endo, 1972; Fujita et al., 2009). Monarch butterflies, *Danaus plexippus* (Lepidoptera: Nymphalidae), experience a reproductive diapause that is initiated and maintained by cold temperatures and short day lengths and can be terminated with JH treatment (Barker and Herman, 1976; Goehring and Oberhauser, 2002; Herman, 1981). Butterflies exposed to multiple diapause-inducing cues are more likely to enter reproductive diapause compared to butterflies exposed to only one cue (Goehring and Oberhauser, 2002).

Access to nutrition can also trigger the endogenous changes to regulate or terminate reproductive diapause (Hahn and Denlinger, 2011; Kostal, 2006). The nut weevil, *Curculio nucum* (L.) (Coleoptera: Curculionidae), and many other weevils that overwinter as adults, must feed before mating and reproductive development (Bel-Venner et al. 2009). In the rice bug, *Leptocorisa chinensis* (Dallas) (Hemiptera: Alydidae), while food is not required to terminate diapause, it is required for females to resume and complete gonad development after reproductive diapause (Tachibana and Watanabe, 2008). *Bruchus rufimanus* (Boh.) (Coleoptera: Bruchidae) overwinter as adults in reproductive diapause, and a combination of food and increasing photophase terminates diapause (Tran and Huignard, 1992).

The induction of sexual communication with pheromones after the termination of reproductive diapause in insects is also under the regulation of exogenous factors such as temperature and photoperiod (Kostal, 2011) that trigger endogenous factors including hormones (Anton et al., 2007; Goehring and Oberhauser, 2002; Simonet et al., 2004). Environmental conditions that trigger the production and release of JH can also regulate male response to sex pheromone (Anton et al., 2007; Cusson et al., 1993; Duportets et al., 1996; Lemmen and Evenden, 2009) and accessory gland development (Duportets et al., 1998) in moths with delayed reproduction. Newly-eclosed male *Agrotis ipsilon* (Lepidoptera: Noctuidae) do not respond to female sex pheromone, however treatment with a JHA induces response in wind tunnel assays (Gadenne et al., 1993). *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) also have a pre-

reproductive period after adult eclosion, and JH acid production coincides with male responsiveness to pheromone (Cusson et al., 1994).

Caloptilia fraxinella (Ely) (Lepidoptera: Gracillariidae) adults eclose in July when the majority of the population is reproductively inactive (Evenden et al., 2007). Adults overwinter in reproductive diapause that is maintained until the following spring when adults emerge from overwintering sites to mate and lay eggs on newly-flushed ash leaflets (Pohl et al., 2004). Female *C. fraxinella* require a carbohydrate nutrition source to develop eggs after overwintering (Evenden et al., 2007), but it is not known whether males also require any nutrition before mating in the spring. Male *C. fraxinella* exhibit plasticity in response to sex pheromone that is dependent on physiological state and their response is most acute in the spring when females are reproductively active (Evenden and Gries, 2008). During reproductive diapause from July-October, males have lower electroantennogram (EAG) and behavioural responses to sex pheromone compared to reproductively active males in the spring (Lemmen and Evenden, 2009). It is also known that treatment of males in reproductive diapause with a JHA, methoprene, enhances male EAG and behavioural response to pheromone in the fall, but not earlier in the summer (Lemmen and Evenden, 2009).

The current study examines the effect of environmental conditions on male *C. fraxinella* pheromone responsiveness during reproductive diapause, and the impact of nutritional status on pheromone response of reproductively active males. The temperature and photoperiod conditions are manipulated to determine

how environmental conditions impact pheromone response during reproductive diapause in both the summer and fall. The hypotheses tested include: 1) the environmental conditions that male *C. fraxinella* experience during reproductive diapause impact male pheromone responsiveness during this time; 2) males held under natural outdoors conditions during reproductive diapause will respond to pheromone differently than males held indoors under long day/warm conditions; 3) reproductively active males require a carbohydrate nutrition source to respond to pheromone. In addition, males held under these different environmental conditions are subjected to treatment with the JHA methoprene to determine the conditions necessary for males to break diapause.

Materials and Methods

Moth Collection

Moths presumed to be in reproductive diapause (Evenden et al., 2007) were reared from pupae collected in leaf rolls from green ash, *Fraxinus pennsylvanica* at various sites in Edmonton, Alberta (53^{o} 34'N 113^{o} 31'W) in June 2008 and 2011. Individual leaf rolls were kept under long day/warm conditions (LD 16:8 h, 24^{o}C) in 30 ml transparent plastic cups grouped in transparent plastic bags with a damp paper towel to maintain humidity. Moth eclosion occurred from 9-23 July 2008 and 6-20 July 2011. Cups were checked at least twice weekly for adult eclosion, and adults were removed and separated by sex and males were prepared for subsequent use in bioassays. Adults were acclimatized to experimental conditions for at least one week after eclosion prior to JHA treatment and use in any bioassay.

JHA Treatment

Based on previous experiments (Evenden at al., 2007; Lemmen and Evenden, 2009), male moths in reproductive diapause were treated with one of the following: (1) 1 μg of methoprene (94.3% pure, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 1 μl of high-performance liquid chromatography (HPLC) grade acetone (Fisher Scientific, Ottawa, Ontario, Canada) from a stock solution, (2) 1 μl acetone alone, or (3) were left untreated. Male moths were held using a gentle vacuum stream while treatments were applied exogenously to the ventral side of the abdomen. After treatment, males were transferred to experimental conditions and provided with a 10% sugar solution or water, depending on the experiment, for one week prior to use in EAG or wind tunnel bioassays.

Experiments

Controlled Conditions

The hypothesis that environmental conditions impact pheromone response of male *C. fraxinella* in reproductive diapause was tested. In July 2008, newly eclosed males in reproductive diapause were held in individual cups in growth chambers set at one of three temperature and photoperiod regimes: LD 16:8 h 24^{o}C (long day/warm), 16:8 h 10^{o}C (long day/cool), 12:12 h 10^{o}C (short day/cool). Males were held for different lengths of time throughout the summer and fall under these conditions (Table 3-1) before use in EAG or wind tunnel bioassays. Males were temporarily removed from environmental conditions for

treatment with methoprene, acetone or to remain untreated and were then re-distributed to the appropriate conditions for one week before use in the bioassay.

An additional experiment tested the hypothesis that a change in temperature and photoperiod cues predisposes male *C. fraxinella* to break reproductive diapause and respond to sex pheromone when treated with a JHA. Newly eclosed males in reproductive diapause were initially held under long day/cool or short day/cool conditions for six weeks (18 July-5 September, 2008) and were then treated with either methoprene, acetone or were left untreated, and subsequently transferred to long day/warm conditions for one week (Table 3-1) before use in an EAG or wind tunnel bioassay.

Natural vs. Controlled Conditions

The hypothesis that males held under natural conditions outside during the period of reproductive diapause would respond to pheromone differently compared to males held under long day/warm conditions indoors was tested using a wind tunnel bioassay in 2011. In July 2011, newly eclosed males in reproductive diapause were placed either in cages (H 80 cm x W 40 cm x D 40 cm) with a green ash seedling (*Fraxinus pennsylvanica*, Jeffries Nurseries, Portage La Prairie Manitoba) outdoors to experience the natural changing photoperiod and temperature conditions, or were held in individual cups in a growth chamber set at LD 16:8 h 18^{o}C in 2011 (long day/warm). Males were held for different lengths of time throughout the summer and fall under these conditions (Table 3-1) before use in a wind tunnel bioassay. Males were temporarily removed from environmental conditions for treatment with

methoprene, acetone or to remain untreated and were then re-distributed to the appropriate conditions for one week before use in the wind tunnel bioassay.

Reproductively Active Males

The hypothesis that reproductively active male *C. fraxinella* require a carbohydrate nutrition source in the spring to respond to pheromone was tested. Free-flying moths in reproductive diapause were collected from 9-11 September 2010 at various sites in Edmonton and were transferred to individual 30 ml transparent cups, and ~ 100 cups were grouped in transparent bags with damp paper toweling to overwinter. Bags were held under winter conditions at LD 0:24 h 2^{o}C for 12 weeks until December 2010 when males were provided individually with either water or a 10% sugar solution through a dental wick, and transferred to long day/warm conditions for at least one week before use in EAG and wind tunnel bioassays. In addition, moths that experienced natural overwintering conditions were collected as free-flying adults in April 2010 and provided with water or 10% sugar solution and were transferred to long day/warm conditions for at least one week before use in EAG or wind tunnel bioassays.

Electroantennogram Recordings

Electroantennogram recordings were made using an IDAC-02 data acquisition controller system, and EAG 2000 software (SYNTECH, Hilversum, The Netherlands). In preparation for EAG recordings, male moths were chilled at 4^{o}C for at least 20 min before one antenna was excised and positioned onto an antenna holder using a small quantity of Spectra 360 conductive gel (Parker Laboratories Inc., Orange, NJ), that was attached to a SYNTECH EAG probe

(Type PRG-2, internal gain 10x). Pheromone loadings consisted of a 10:1 ratio of (*Z*)-11-hexadecenal and (*Z*)-11-hexadecen-1-ol (>99%, Pherobank, Wageningen, The Netherlands) serially diluted in HPLC-grade hexane to obtain decadal solutions between 0.0001 μg and 1 μg (*Z*)-11-hexadecenal μl^{-1} hexane. Fifty μl of each solution, and 50 μl of a hexane control, were pipetted individually onto 7 × 0.2 cm strips of folded Whatman no. 1 filter paper and allowed to evaporate in a fume hood. As a standard, 50 μl of the plant volatile (*E*)-2-hexenal (>95%, Aldrich Chemical Co., WI, USA) (1 μg μl^{-1} hexane) was also pipetted onto filter paper and allowed to evaporate. Treated strips were inserted into disposable Pasteur pipettes. Stimulus puffs were generated with a SYNTECH CS-55 stimulus controller with a pulse duration of 0.2 sec and flow of 10 ml/sec. Electroantennogram responses were measured as the maximum amplitude of depolarization elicited by the stimulus applied. Each antenna received a series of puffs delivered once every minute in the following order: hexane; 50 μg plant volatile; 0.005 μg pheromone; 50 μg plant volatile; 0.05 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.

Wind Tunnel Bioassays

The wind tunnel used in behavioural assays had a flight section 1.7 m long and 0.85 m high. Six, 15-watt bulbs diffused through white paper dimly illuminated the tunnel. Wind speed was 0.32-0.34 m/s and temperature was maintained at 24-26^{o}C. Males were acclimatized to experimental conditions 30 min prior to initiation of the behavioural assay. Flights were conducted during the

last hour of the photophase and the first two hours of the scotophase to a pheromone source of 10 μg (*Z*)-11-hexadecenal and 1 μg (*Z*)-11-hexadecen-1-ol (>99%, Pherobank, Wageningen, The Netherlands) in HPLC grade hexane (Fisher Scientific, Ottawa, Ontario, Canada) released from a pre-extracted grey rubber septum (Contech Enterprises Inc., Delta, BC, Canada). Males were introduced individually into the wind tunnel in cylindrical wire cages (5 cm diameter x 6 cm height) on a platform 20 cm from the downwind end. Once the moth was positioned in the pheromone plume, the lid of the cage was removed and males were allowed three minutes to respond to the pheromone source. Methoprene-treated, acetone-treated and untreated males were flown alternately and each moth was flown only once. Behavioural responses to pheromone were recorded as: wing fanning, lock-on to the pheromone plume, upwind oriented flight and contact with the pheromone source.

Statistical Analyses

Raw EAG responses (mV) were log transformed after conducting a Box Cox analysis to determine the most appropriate transformation of the data. The log-transformed data showed greatly improved analysis based on AIC values. Electroantennogram responses of males that were exposed to the different treatments and tested to five doses of pheromone were compared using a repeated measures ANOVA (R Development Core Team, 2012). All two-way interactions were included in the models, and non-significant interaction terms were removed to achieve the most parsimonious model. When a variable was significant in the model, a post-hoc multiple comparison Honestly Significant Differences (HSD)

test was performed among treatments (R-package: agricolae). In the figures depicting EAG responses presented here, means and standard errors were back-transformed to reflect original EAG responses (see Appendix 3-1 for representative EAG traces and Appendix 3-2 for EAG dose response curves not already depicted in figures).

In the wind tunnel experiments, behavioural responses of males were recorded as "yes" or "no", so the resulting data were binomially distributed. Behavioural response was compared using a generalized linear model (GLM) with a logit link function (R Development Core Team, 2012). Analysis of deviance was used to test whether male response was dependent on any of the experimental variables. All two-way interactions were included in the models (Table 3-2) and non-significant interactions terms were removed to achieve the most parsimonious model. When a variable in the GLM was significant, multiple comparisons (R-package:Multcomp) were made using a Tukey-contrasts test.

Results

Controlled Conditions

Electroantennogram response of males in reproductive diapause was impacted by a significant interaction between the temperature and photoregime males were held under and the pheromone dose to which antennae were exposed. Photoregime did not influence EAG responses to the lower pheromone doses (0 μg-5 μg) but did to the two highest doses tested (5 μg-50 μg) (Fig. 3-1A). Males in reproductive diapause held under short day/cool conditions had the highest EAG responses to the high pheromone doses, followed by males held under long

day/warm conditions, and males held under long day/cool conditions had the lowest EAG responses (Fig. 3-1A). A significant interaction between hormone treatment and pheromone dose on EAG response was recorded (Fig. 3-1B). Hormone treatment did not affect male EAG response to the lower pheromone doses tested (0 μg-5 μg), but did affect EAG response to the highest pheromone dose tested (50 μg) (Fig. 3-1B). Males in reproductive diapause that were treated with methoprene had a higher EAG response to the 50 μg dose of pheromone than acetone-treated and untreated males (Fig. 3-1B). An interaction between the length of time males were held under the various environmental conditions and the pheromone dose to which antennae were exposed significantly influenced EAG response (Fig. 3-1C). Time held under each temperature and photoregime did not affect EAG response to the lower pheromone doses tested (0 μg-0.5 μg) but did affect response to the two highest doses of pheromone (5 μg-50 μg) (Fig. 3-1C). Males in reproductive diapause tested after one week under the various temperature and photoregimes had the highest EAG responses, followed closely by males tested after two and three weeks (Fig. 3-1C). Males tested after four weeks under the various temperature and photoperiod regimes had much lower EAG responses (Fig. 3-1C).

Male EAG response was also impacted by the significant main effects of the environmental conditions that males were held under while in reproductive diapause (repeated measures ANOVA: F=3.9, 2 df, P=0.02), hormone treatment (repeated measures ANOVA: F=3.8, 2 df, P=0.02), length of time that males were

held prior to the bioassay (repeated measures ANOVA: F=11.7, 3 df, P<0.0001) and pheromone dose (repeated measures ANOVA: F=3401.0, 5 df, P<0.0001).

Male behavioural response to pheromone was significantly impacted by the temperature and photoregime under which males were held while in reproductive diapause (Fig. 3-2). A higher percentage of males held under long day/warm conditions expressed all pheromone-mediated behaviours (wing fanning, lock-on to the pheromone plume, upwind flight and source contact) compared to males held under long day/cool or short day/cool conditions (Fig. 3-2). A small percentage of males held under long day/cool conditions expressed wing fanning behaviour, but none displayed any other pheromone-mediated behaviour, and no males held under short day/cool conditions expressed any of the pheromone-mediated behaviours in the wind tunnel. The length of time males were held under the temperature and photoregime conditions prior to the wind tunnel bioassay did impact wing fanning behaviour (GLM: $X_{3,0.05}$=9.1, P=0.03). The percentage of males that conducted wing fanning behaviour increased each week (24 July = 0%; 29-31 July = 3.4%; 5-7 August = 5.9%; 12-14 August = 9.1%), however length of time held under controlled conditions did not impact lock-on (GLM: $X_{3,0.05}$=5.5, P=0.1), upwind flight (GLM: $X_{3,0.05}$=4.9, P=0.2) or source contact (GLM: $X_{3,0.05}$=6.1, P=0.1) behaviours. Male behavioural response was not impacted by hormone treatment: wing fanning (GLM: $X_{2,0.05}$=2.1, P=0.3), lock-on (GLM: $X_{2,0.05}$=3.7, P=0.2), upwind flight (GLM: $X_{2,0.05}$=3.5, P=0.2), source contact (GLM: $X_{2,0.05}$=2.4, P=0.3).

Pheromone response after male *C. fraxinella* in reproductive diapause were transferred from short day/cool or long day/cool conditions to long day/warm conditions was tested with both EAG and wind tunnel bioassays. Male EAG response was impacted by a significant interaction between the initial photoregime males were held under and the pheromone dose to which their antennae were exposed (Fig. 3-3). Electroantennogram response of males to low doses of pheromone (0 μg-0.5 μg) did not differ with the initial conditions under which males were held (Fig. 3-3). Males held initially under long day/cool conditions had higher EAG responses compared to males held under short day/cool conditions to the two highest pheromone doses tested (5 μg-50 μg) (Fig. 3-3). As a main effect, initial environmental conditions (repeated measures ANOVA: F=2.2, 1 df, P=0.1) and hormone treatment (repeated measures ANOVA: F=0.06, 2 df, P=0.9) did not impact male EAG response to pheromone, but there was a significant main effect of pheromone dose to which antennae were exposed (repeated measures ANOVA: F=801.2, 5 df, P<0.0001).

Male behavioural response to pheromone was impacted by the initial environmental conditions under which males were held prior to the transfer to long day/warm conditions. Initial conditions did not impact the percentage of males that wing fanned or locked-on to the pheromone plume (Fig. 3-4). A higher percentage of males held initially under long day/cool conditions expressed upwind flight and source contact behaviours compared with males held initially under short day/cool conditions (Fig. 3-4). Hormone treatment did not impact any of the pheromone-mediated behaviours of the transferred males: wing fanning

(GLM: $X_{2,0.05}$=5.7, P=0.06), lock-on (GLM: $X_{2,0.05}$=0.4, P=0.8), upwind flight (GLM: $X_{2,0.05}$=1.4, P=0.5), and source contact (GLM: $X_{2,0.05}$=0.4, P=0.8).

Natural vs. Controlled Conditions

A comparison of males in reproductive diapause held outside under natural conditions to those held indoors over varying periods of time in the summer and fall showed that all pheromone-mediated behaviours in the wind tunnel were affected by a significant interaction between hormone treatment and the environmental conditions under which males were held (Fig. 3-5). Methoprene treatment resulted in the highest percentage of males that expressed pheromone-mediated behaviours in the wind tunnel followed by acetone and no treatment (Fig. 3-5). A higher percentage of males held indoors expressed all behaviours compared to males held outdoors (Fig. 3-5).

Pheromone-mediated behaviours in the wind tunnel were also significantly impacted by an interaction between the length of time males were held under the conditions prior to the wind tunnel bioassay and the environmental conditions under which males were held (Fig. 3-6). The percentage of males that responded to the pheromone in the wind tunnel decreased between July and August, regardless of the conditions under which they were held (Fig. 3-6). The percentage of males that responded behaviourally to pheromone later in the fall between August and September increased for males held indoors, and decreased for males held outdoors (Fig. 3-6). As a main effect, the environmental conditions under which males were held significantly impacted all of the pheromone-mediated behaviours, with males that were held indoors expressing all

behaviours more frequently compared with males held outdoors (Fig. 3-7A). Hormone treatment also significantly impacted male behavioural response, and a higher percentage of methoprene-treated males expressed all pheromone-mediated behaviours compared with acetone-treated and untreated males (Fig. 3-7B). The length of time males were held under the different environmental conditions prior to the wind tunnel bioassay impacted lock-on behaviour (GLM: $X_{2,0.05}$=7.2, P=0.03), which decreased over time (July = 29%; August = 23%; September = 19%). The length of time held did not impact wing fanning (GLM: $X_{2,0.05}$=3.6, P=0.2), upwind flight (GLM: $X_{2,0.05}$=5.8, P=0.06) or source contact (GLM: $X_{2,0.05}$=3.8, P=0.2) behaviours.

Reproductively Active Males

Nutrition provided to reproductively active males after overwintering under natural or laboratory conditions significantly influenced male EAG response to pheromone (Fig. 3-8) (Appendix 3-2). Males tested in the spring after overwintering under natural conditions had a significantly higher EAG response to pheromone if they were fed sugar water compared to similarly collected males provided only with water (Fig. 3-8A). In contrast, after overwintering under laboratory conditions, males provided with sugar had a lower EAG response to pheromone compared with males provided water alone (Fig. 3-8B). Male behavioural response to pheromone was not impacted by nutrition treatment in the spring after overwintering under natural conditions (Fig. 3-9A) or immediately after overwintering in the laboratory (Fig. 3-9B).

Discussion

The timing of reproduction in insects that experience reproductive diapause is controlled by exogenous and endogenous factors (Kostal, 2011). The present study tests the impact of temperature, photoperiod, JHA treatment and nutrition on diapause termination by measuring pheromone response of male *C. fraxinella*. As is known for other insects, temperature and photoperiod are important exogenous cues for regulating reproductive diapause in male *C. fraxinella* (Kostal, 2006; Kostal, 2011; Macrae, 2005). Pheromone response of male *C. fraxinella* is plastic throughout reproductive diapause (Evenden and Gries, 2008) suggesting that male physiological state changes over the course of the diapause period that lasts from eclosion in July until moths are ready to mate the following spring (Pohl et al., 2004). Treatment of male moths with a JHA in the current study does enhance male pheromone response in a manner similar to previous experiments (Lemmen and Evenden, 2009). Although nutritional status did impact male pheromone response, access to a carbohydrate nutrition source was not required for males to conduct mate location behaviours.

Caloptilia fraxinella males collected from leaf rolls in July in reproductive diapause require long day/warm conditions to break reproductive diapause and respond behaviourally to pheromone. Males held under short day/cool or long day/cool conditions maintain reproductive diapause and do not express mate-location behaviours in the wind tunnel. Temperature and photoperiod separately impact male pheromone responsiveness. Males held indoors under cool conditions do not subsequently respond in the wind tunnel, while some males in reproductive diapause held under warm conditions do express all pheromone-

mediated behaviours in the wind tunnel, confirming the impact of temperature on subsequent male pheromone response. Males held initially under long day/cool conditions and then transferred to long day/warm conditions have higher EAG responses to the two highest pheromone doses tested and a greater capacity to express mate-location behaviours than males held initially under short day/cool conditions. This indicates that long day photoperiod predisposes males in reproductive diapause to respond to pheromone under warm conditions. In other insects with facultative reproductive diapause, short day conditions induce adult diapause and long day conditions either terminate diapause or prevent individuals from entering diapause. This phenomenon is widespread among species in several orders of insects that overwinter as adults, including the Coleoptera (Berkvens et al., 2008), Hymenoptera (Kipyatkov and Lopatina, 2009), Orthoptera (Zhu et al., 2013), Hemiptera (Kostal et al., 2008; Morita and Numata, 1997; Musolin and Ito, 2008; Nakamura and Numata, 2000; Numata, 1992) and Lepidoptera (Barker and Herman, 1976; Fujita et al., 2009; Pieloor and Seymour, 2001; Pullin, 1986). Most *C. fraxinella* males eclose in reproductive diapause which is maintained when males are held under sub-optimal short day or cool conditions. A portion of these males are either reproductively active when they eclose or can become responsive to pheromonal cues when held under long day/warm conditions.

Males held indoors in the summer and fall after eclosion respond differently to pheromone in the wind tunnel than males held outside under natural conditions during the same period. This confirms the importance of

environmental conditions on regulating male reproductive diapause and subsequent pheromone responsiveness under natural conditions. A much higher percentage of males held under long day/warm conditions express pheromone-mediated behaviours in the wind tunnel compared to males held outdoors under natural conditions. Males perceive an important environmental cue sometime between August and September that determines whether they will break diapause and search for a mate or remain in diapause and prepare to overwinter. Environmental conditions cue the pathway that an insect takes during its lifecycle, such as whether to enter diapause or to continue development (Danks 1991; Danks 2002). Bean bugs, *R. clavatus*, exhibit an adult reproductive diapause that is maintained under short day conditions, and is terminated under long day conditions (Nakamura and Numata 2000). If the photophase is shortened, then diapause is prolonged in *R. clavatus*, presumably to prevent males from terminating diapause too early, as adults overwinter in reproductive diapause (Nakamura and Numata 2000). This may also be the case in male *C. fraxinella*, as the percentage of males held indoors under long day/warm conditions that expressed pheromone-mediated behaviours in the wind tunnel greatly increased between August and September, while males that were held outdoors and experienced a naturally decreasing photoperiod displayed reduced response between August and September. The natural decrease in day lengths and cooler temperatures must cue male *C. fraxinella* to prepare for overwintering, which includes a reduction in pheromone responsiveness. Fluctuating temperatures as

well as decreasing day lengths are also important for monarch butterflies to enter reproductive diapause (Goehring and Oberhauser, 2002).

Treatment with JHA is known to restore pheromone responsiveness in some other moths that delay mating (Anton et al., 2007; Cusson et al., 1994; Gadenne et al., 1993), and in previous experiments JHA treatment enhanced both antennal and behavioural response of male *C. fraxinella* to pheromone late in reproductive diapause in the fall, but not early in diapause in the summer (Lemmen and Evenden, 2009). In the current study, JHA treatment does increase EAG response to pheromone in male *C. fraxinella* that were held indoors in 2008, and also impacts male behavioural response when males were held either outdoors under natural conditions or indoors under long day/warm conditions in 2011. Male EAG response to the highest pheromone dose is highest in JHA-treated males compared to the controls, indicating that JH plays a role in modulating the peripheral nervous system in male *C. fraxinella*. The impact of JHA on male EAG response was not observed until later in reproductive diapause in previous experiments (Lemmen and Evenden, 2009), and the current results suggest that JHA treatment may impact EAG response earlier in the diapause period than was previously reported, and may also depend on the environmental conditions males experience. The increase observed in male EAG response with JHA treatment in 2008 was not translated into an increase in behavioural response in 2008 as treatment with JHA did not increase pheromone-mediated behaviours of males held under the various temperature and photoregimes indoors in 2008. Treatment of males in reproductive diapause with JHA did increase pheromone-mediated

behaviours in the wind tunnel in 2011 when males were held either outdoors or indoors. This difference may be attributed to the different timing of the experiments between the two years. In 2008, males were tested once a week over four weeks between the end of July to mid-August, while in 2011, males were tested once a month from the end of July to September (Table 1). The behavioural results of 2008 are consistent with previous work that showed JHA treatment only impacted male behavioural response late in reproductive diapause (Lemmen and Evenden, 2009). However, since there was no significant interaction between the length of time males were held prior to the wind tunnel bioassay and JHA treatment, we can assume that in the 2011 experiment, JHA treatment impacted male response to pheromone throughout the period of reproductive diapause in both summer and fall. It appears that the newly-established population of *C. fraxinella* in Edmonton may be experiencing a shift in which males in early reproductive diapause are now predisposed to break diapause earlier in the season than was previously reported (Lemmen and Evenden, 2009), or a larger proportion of the population eclose in a reproductively active state. This may be the first step to the development of multivoltinism of this species in its expanded range. Mating and egg-laying by *C. fraxinella* on newly flushed secondary growth at the base of ash trees has been observed during the summer months (Tyler Wist, unpublished data). The willow leaf beetle, *Phratora vulgatissima* (Linnaeus 1758) (Coleoptera: Chrysomelidae) has a similar life history in which it is usually univoltine, and adults overwinter in reproductive diapause. Beetles mate in the spring and the resulting offspring

enters reproductive diapause before overwintering (Dalin, 2011). A small portion of the willow leaf beetle population becomes reproductively active in the same season and produces a small second generation (Dalin, 2011). It is likely that that a summer generation of *C. fraxinella* will also be small, and will be constrained by access to freshly flushed ash leaflets (Evenden, 2009) on secondary growth.

The nutritional status of adult male *C. fraxinella* impacts male EAG but not behavioural response to pheromone. Adult nutrition can greatly increase the reproductive and flight capacity of many lepidopterans (Boggs, 2009). Access to a carbohydrate source increased male EAG response after overwintering under natural conditions and collection and testing in the spring but decreased response after overwintering in the laboratory. In nature, male and female *C. fraxinella* mate immediately as soon as temperatures warm up in the spring, usually before ash leaves begin to flush (Pohl et al., 2004). It is possible that the males collected in the spring after overwintering under natural conditions had already exerted energy into mate location and mating. Additional food resources may have increased their EAG responsiveness simply by providing more fuel to moths. Males that overwintered under laboratory conditions did not exert any energy prior to testing and sugar feeding significantly reduced EAG response. Feeding is required for egg development in female *C. fraxinella* (Evenden et al., 2007), but the current study shows feeding is not necessary for male mate location behaviours as both sugar-fed and water-fed reproductively active males held under both overwintering conditions were highly responsive to pheromone in wind tunnel assays. Since moths mate early in the spring, it may benefit males to

be ready to mate as soon as females begin calling in the spring. It remains to be tested if females require a carbohydrate source in the spring before they emit pheromone.

Diapause termination as measured by pheromone response in male *C. fraxinella* is controlled primarily by temperature and photoperiod, and is also impacted by JH. Long day/warm conditions enable males to break diapause, while short day, cool, or natural outdoors conditions maintain reproductive diapause into the fall overwintering period. This is crucial for the timing of mating with females in the spring. Environmental conditions strongly impact the level of pheromone responsiveness exhibited by male *C. fraxinella*. This study provides additional evidence that modulation of EAG and behavioural response to pheromone is hormone regulated. Sensitivity of males to JHA treatment occurs earlier in reproductive diapause than has previously been reported, and may indicate a shift in the predisposition of males to the termination of reproductive diapause. This research highlights the importance of testing insects under natural conditions as maintenance in the laboratory can significantly impact physiological and behavioural response. This study provides a solid foundation for future research into the mechanisms of pheromone response plasticity and the developmental biology of *C. fraxinella*.

Table 3-1. Sample size of male *C. fraxinella* used in each bioassay type of each experiment conducted under varying conditions and times post eclosion. Different males were tested at each time point in each experiment.

Experiment	Conditions	Bioassay	Dates of Experiment	N
Controlled Conditions 2008	LD 16:8 h 24°C	EAG	Week 1 – 25-26 July	30
			Week 2 – 1-2 Aug	29
			Week 3 – 8-9 Aug	30
			Week 4 – 15-16 Aug	15
		Wind Tunnel	Week 1 – 24 July	15
			Week2 – 29-31 July	28
			Week 3 – 5-7 Aug	27
			Week 4 – 12-14 Aug	21
	LD 16:8 h 10°C	EAG	Week 2 – 29-30 July	30
			Week 3 – 5-6 Aug	29
			Week 4 – 12-13 Aug	30
		Wind Tunnel	Week 1 – 24 July	15
			Week2 – 29-31 July	30
			Week 3 – 5-7 Aug	28
			Week 4 – 12-14 Aug	28
	LD 12:12 h 10°C	EAG	Week 1 – 24-25 July	30
			Week 2 – 31 July-1 Aug	30
			Week 3 – 7-8 Aug	30
			Week 4 – 14-15 Aug	30
		Wind Tunnel	Week 1 – 24 July	15
			Week2 – 29-31 July	30
			Week 3 – 5-7 Aug	30
			Week 4 – 12-14 Aug	28
Controlled Conditions 2008	Transfer: LD 16:8 h 10°C to LD 16:8 h 24°C	EAG	8-9 September	30
		Wind Tunnel	12 September	48
	Transfer: LD 12:12 h 10°C to LD 16:8 h 24°C	EAG	8-9 September	30
		Wind Tunnel	10 September	29
Natural Conditions 2011	Outdoors	Wind Tunnel	25-26 July	138
			29-30 August	138
			28-29 September	116
Controlled Conditions 2011	LD 16:8 h 18°C	Wind Tunnel	25-26 July	141
			29-30 August	132
			29 September	57
Reproductive Males	Overwintered Sugar vs. Water	EAG	6-7 December	20
		Wind Tunnel	7-8 December	71

Spring	EAG	28 April – 1 May	17
Sugar vs. Water	Wind Tunnel	30 April – 18 May	84

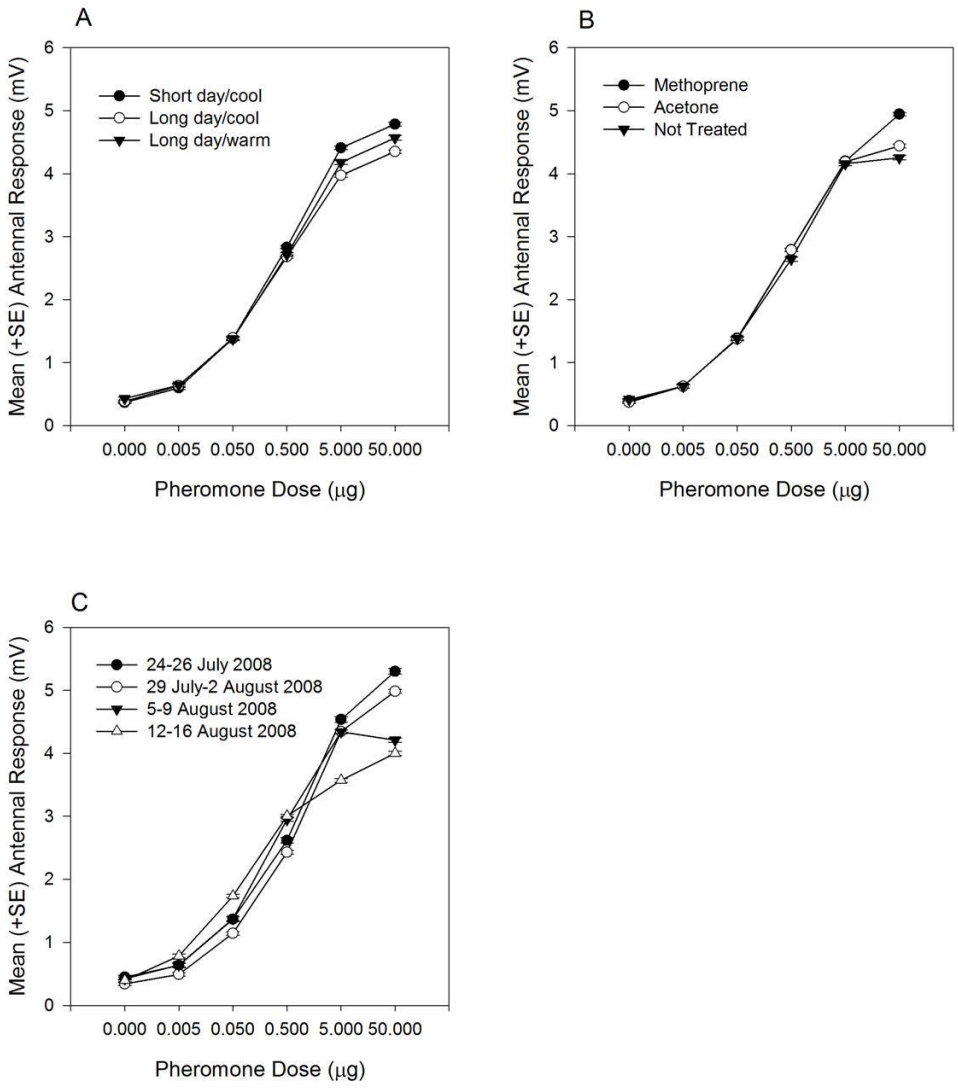


Fig. 3-1. Antennal response of male *C. fraxinella* held under one of three controlled environmental conditions, treated with a JHA and tested once a week for four weeks. Antennal response was impacted by significant interactions between (A) pheromone dose and the temperature and photoperiod regime that moths were held under (repeated measures ANOVA: F=2.6, 10 df, P=0.004); (B) pheromone dose and JHA treatment (repeated measures ANOVA: F=2.2, 10 df, P=0.02); and (C) pheromone dose and the length of time males were held under their environmental conditions prior to the experimental date (repeated measures ANOVA: F=18.2, 15 df, P<0.0001). Mean antennal response is pooled for (A) all JHA treatments and time points, (B)

environmental conditions and time points, and (C) environmental conditions and JHA treatments.

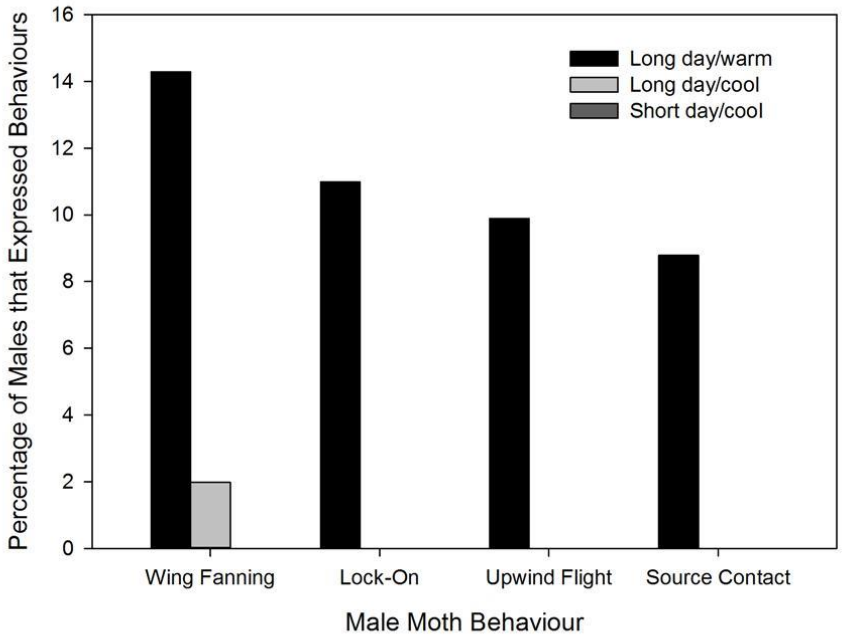


Fig. 3-2. Percentage of male *C. fraxinella* in reproductive diapause held under controlled environmental conditions that expressed pheromone-mediated behaviours in the wind tunnel. Males were held indoors in 2008 under one of three temperature and photoperiod regimes and were tested once a week from July-August. The percentage includes all males tested from July-August and all JHA treatments. Males held under long day/warm conditions had a significantly higher percentage of moths that expressed wing fanning (GLM: $X_{2,0.05}$=24.3, P<0.0001), lock-on (GLM $X_{2,0.05}$=24.3, P<0.0001), upwind flight (GLM: $X_{2,0.05}$=21.8, P<0.0001) and source contact (GLM: $X_{2,0.05}$=19.3, P<0.0001) behaviours compared with males held under either of the other two photoregimes.

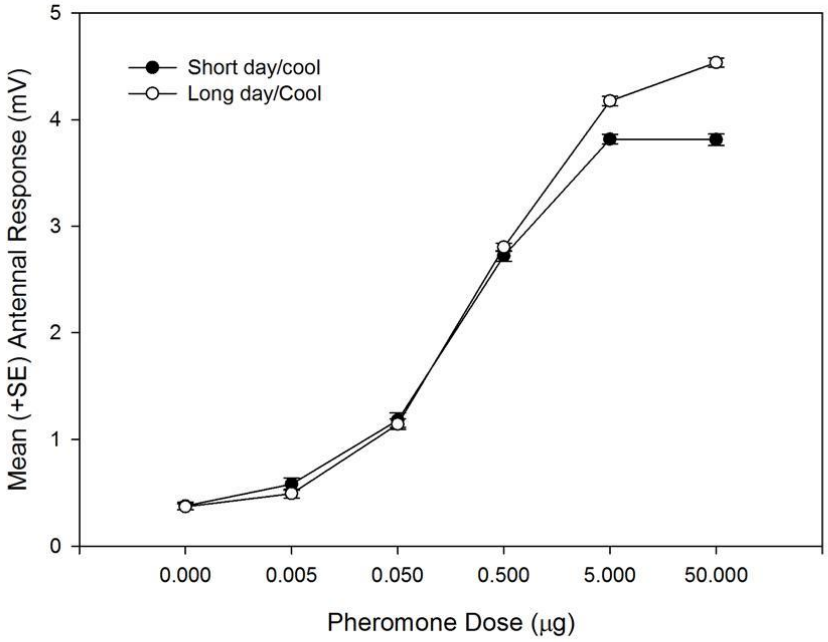


Fig. 3-3. Antennal response of male *C. fraxinella* that were held initially under short day/cool or long day/cool conditions and then transferred to long day/warm conditions prior to testing. Mean antennal response is pooled for males with different JHA treatments. Antennal response was impacted by a significant interaction between pheromone dose and the initial conditions under which males were held (repeated measures ANOVA: F=2.9, 5 df, P=0.02).

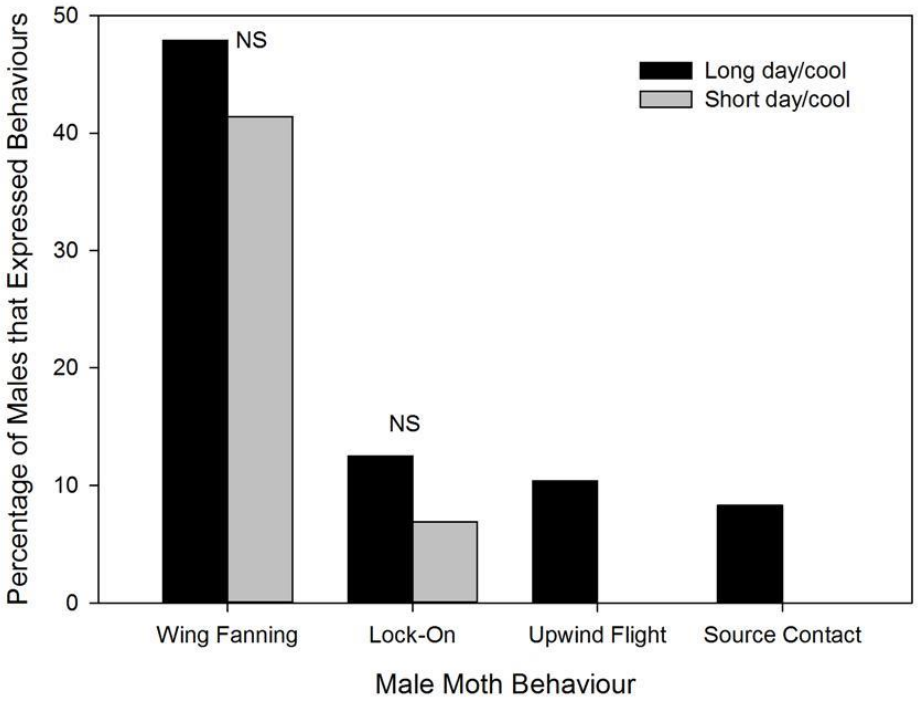


Fig. 3-4. Percentage of male *C. fraxinella* in reproductive diapause that expressed behaviours in the wind tunnel when held initially under short day/cool or long day/cool conditions and then transferred to long day/warm conditions. Response of males treated with the various JHA treatments is pooled. Initial photoregime did not impact wing fanning (GLM: $X_{1,0.05}$=0.3, P=0.6) or lock-on (GLM: $X_{1,0.05}$=0.7, P=0.4) behaviours, but did impact upwind flight (GLM: $X_{1,0.05}$=5.2, P=0.02) and source contact (GLM: $X_{1,0.05}$=4.0, P=0.045) behaviours.

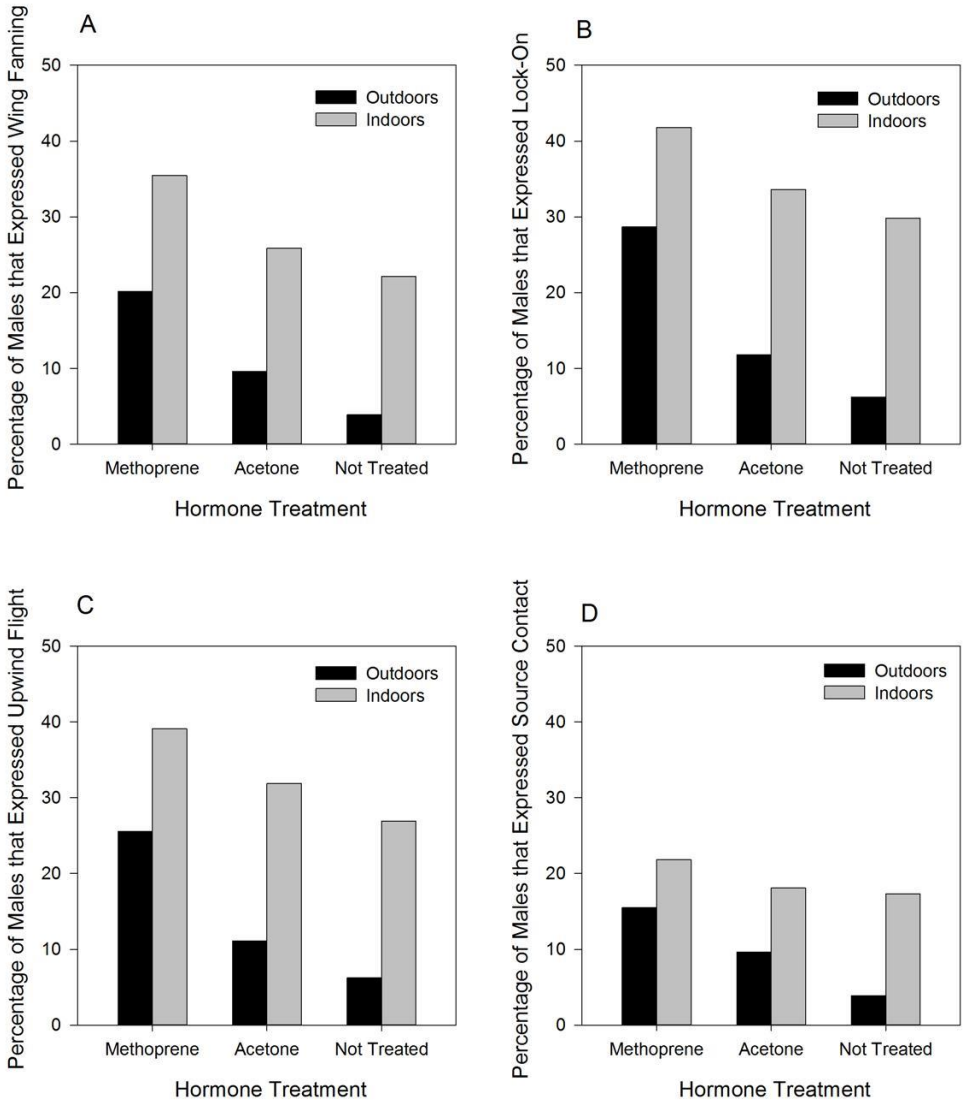


Fig. 3-5. Percentage of male *C. fraxinella* in reproductive diapause held under natural outdoors or controlled indoors conditions that expressed pheromone-mediated behaviours after exogenous treatment with JHA. All males that were tested from July-September were pooled. Behavioural response of male *C. fraxinella* was impacted by a significant interaction between JHA treatment and the environmental conditions under which moths were held. This interaction impacted (A) wing fanning (GLM: $X_{2,0.05}$=6.3, P=0.04), (B) lock-on (GLM: $X_{2,0.05}$=9.8, P=0.008), (C) upwind flight (GLM: $X_{2,0.05}$=7.5, P=0.02) and (D) source contact (GLM: $X_{2,0.05}$=6.1, P=0.05) behaviours.

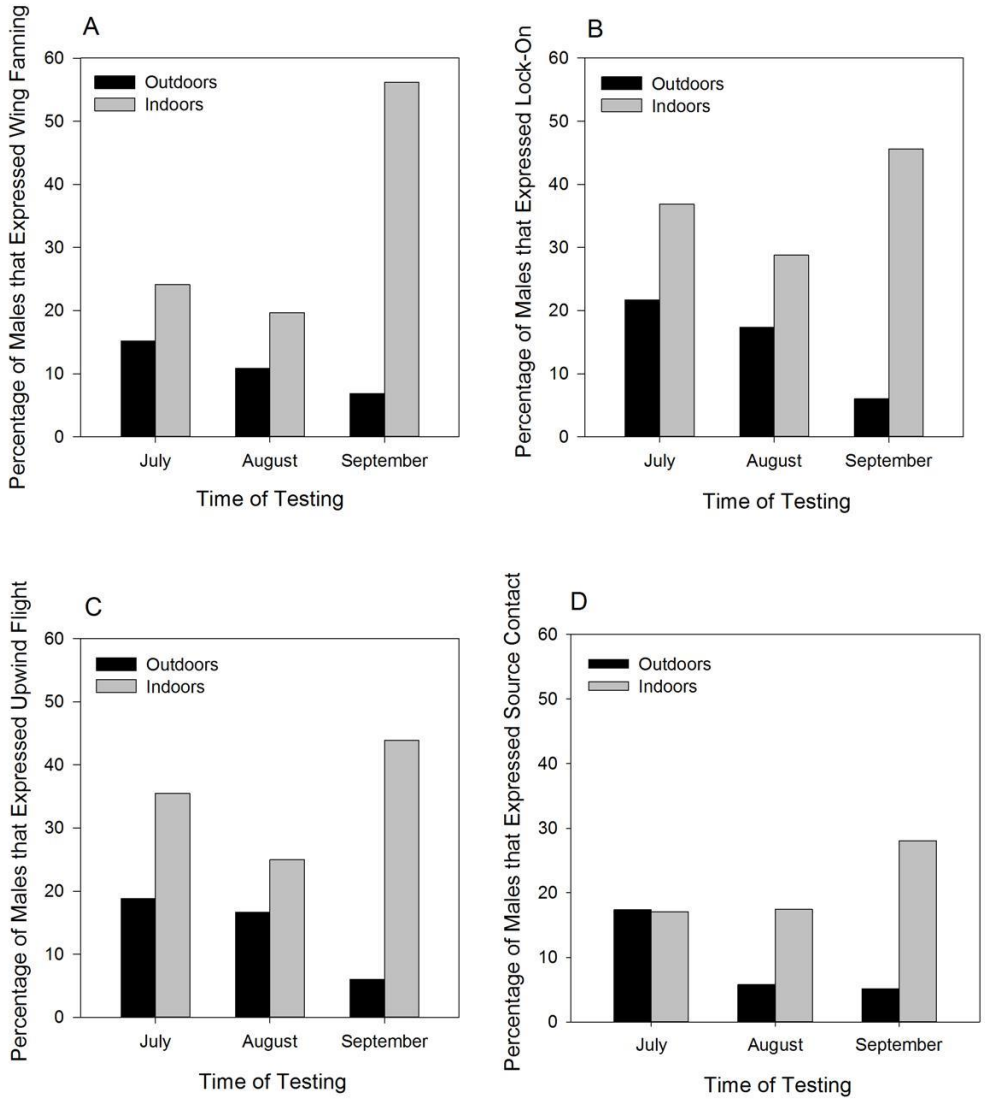


Fig. 3-6. Percentage of male *C. fraxinella* in reproductive diapause held under natural outdoors or controlled indoors conditions that expressed pheromone-mediated behaviours over time from July-September. Response of males treated with the various JHA treatments is pooled. Behavioural response of male *C. fraxinella* was impacted by a significant interaction between the length of time moths were held prior to the experimental date and the environmental conditions under which moths were held. This interaction impacted (A) wing fanning (GLM: $X_{2,0.05}$=19.0, P<0.0001), (B) lock-on

(GLM: $X_{2,0.05}$=13.6, P=0.001), (C) upwind flight (GLM: $X_{2,0.05}$=13.3, P=0.001) and (D) source contact (GLM: $X_{2,0.05}$=10.7, P=0.005) behaviours.

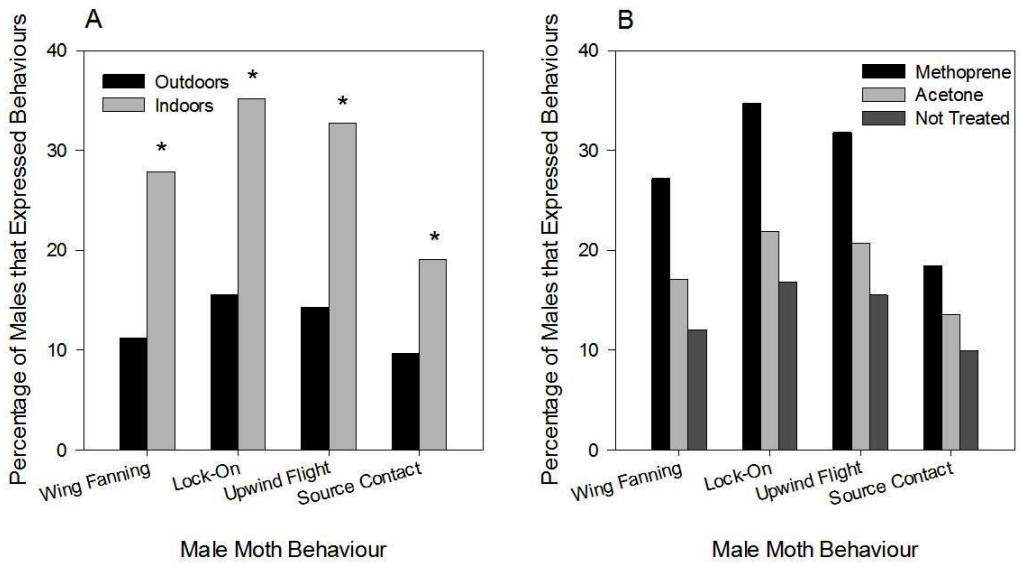


Fig. 3-7. Percentage of male *C. fraxinella* in reproductive diapause that expressed pheromone-mediated behaviours in the wind tunnel after being held under natural outdoors or controlled indoors conditions and treated with JHA. (A) Environmental conditions impacted the percentage of males that expressed wing fanning (GLM: $X_{1,0.05}$=36.7, P<0.0001), lock-on (GLM: $X_{1,0.05}$=35.2, P<0.0001), upwind flight (GLM: $X_{1,0.05}$=33.3, P<0.0001) and source contact (GLM: $X_{1,0.05}$=12.6, P=0.0004). * Indicates significantly different treatment. (B) JHA treatment impacted the percentage of males that expressed wing fanning (GLM: $X_{2,0.05}$=18.2, P=0.0001), lock-on (GLM: $X_{2,0.05}$=21.5, P<0.0001), upwind flight (GLM: $X_{2,0.05}$=18.4, P<0.0001) and source contact (GLM: $X_{2,0.05}$=7.1, P=0.03) behaviours.

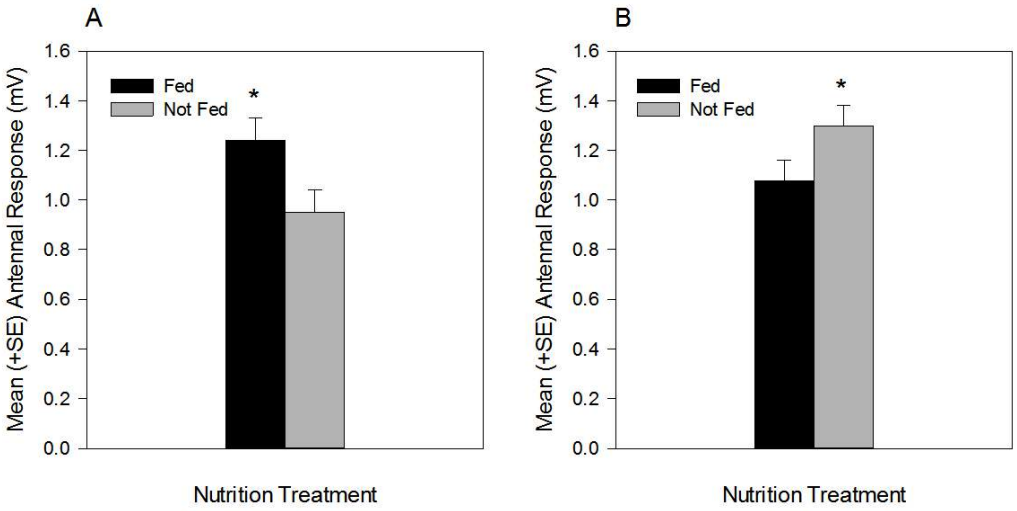


Fig. 3-8. EAG response of reproductively active male *C. fraxinella* that were provided either 10% sugar water (fed) or water only (not fed). Nutrition status significantly impacted male *C. fraxinella* antennal response to pheromone in (A) the spring (repeated measures ANOVA: F=27.7, 1 df, P<0.0001) after overwintering under natural conditions and (B) immediately after overwintering under simulated winter conditions in the laboratory (repeated measures ANOVA: F=13.1, 1 df, P=0.0005). Mean EAG response is the mean antennal response of all males that were tested to all doses of pheromone tested (0 μg-50 μg). * Indicates significantly different treatment.

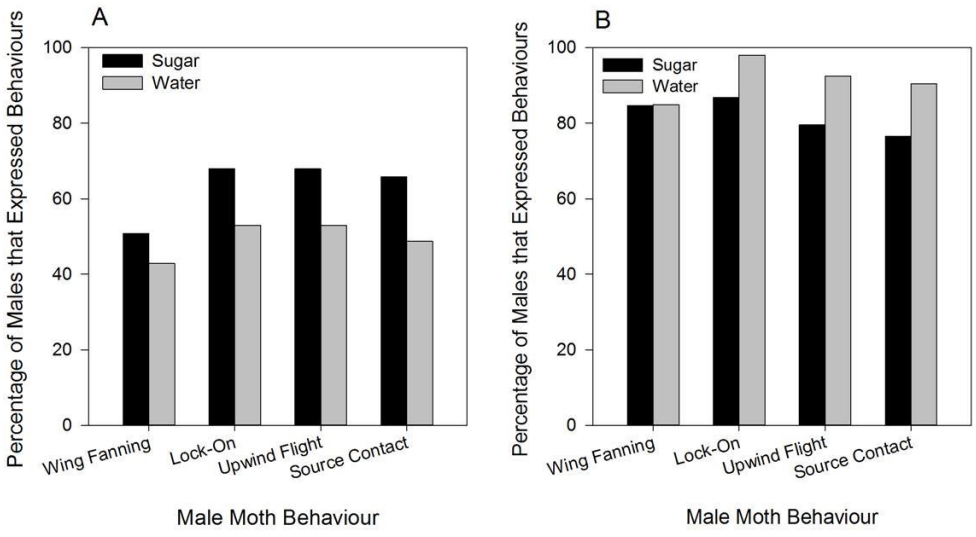


Fig. 3-9. Percentage of reproductively active male *C. fraxinella* that were fed 10% sugar water or remained unfed with access to water only that expressed pheromone-mediated behaviours in the wind tunnel. (A) In the spring after overwintering under natural conditions, none of the pheromone-mediated behaviours were impacted by nutrition treatment: wing fanning (GLM: $X_{1,0.05}$=1.8, P=0.2), lock-on (GLM: $X_{1,0.05}$=0.6, P=0.4), upwind flight (GLM: $X_{1,0.05}$=0.6, P=0.4), and source contact (GLM: $X_{1,0.05}$=0.6, P=0.5). (B) Immediately after overwintering under simulated winter conditions in the laboratory, none of the pheromone-mediated behaviours were impacted by nutrition treatment: wing fanning (GLM: $X_{1,0.05}$=0.002, P=0.96), lock-on (GLM: $X_{1,0.05}$=3.0, P=0.08), upwind flight (GLM: $X_{1,0.05}$=2.6, P=0.1), and source contact (GLM: $X_{1,0.05}$=2.2, P=0.1).

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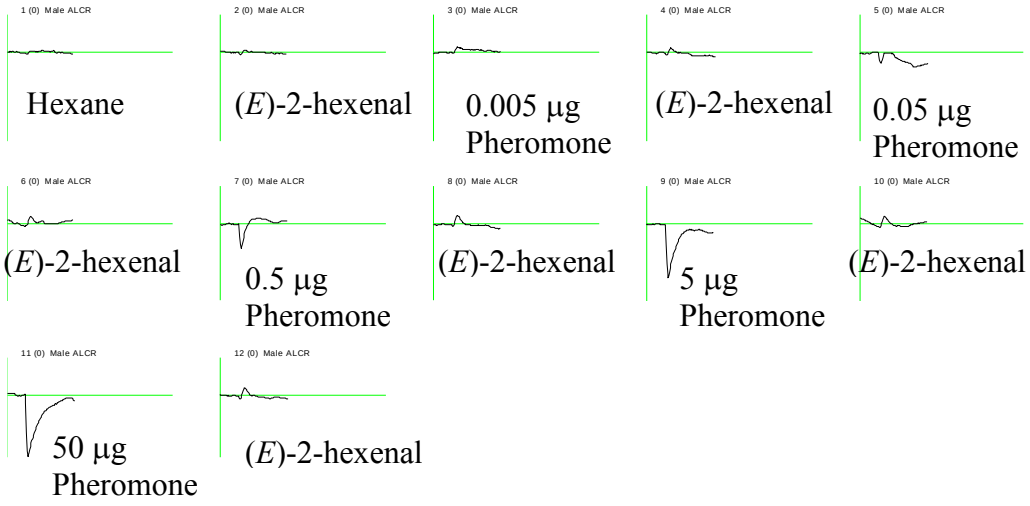
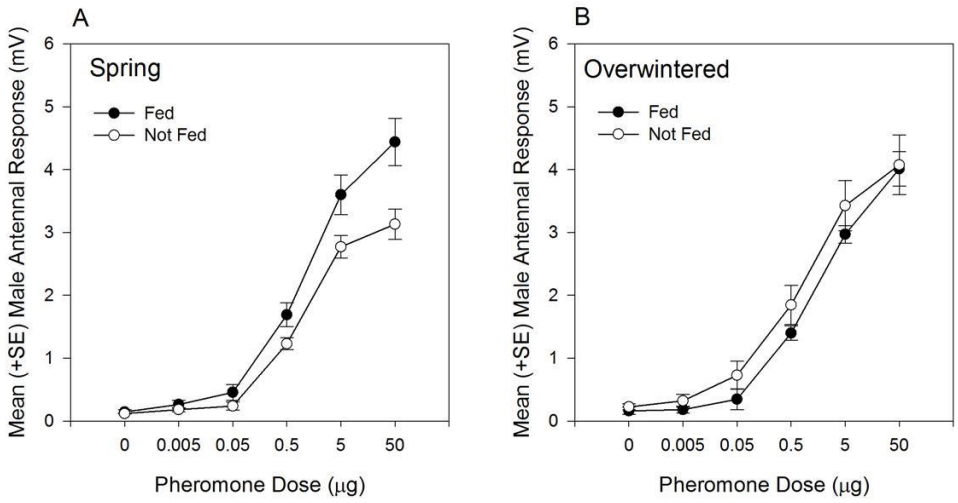


Appendix 3-1. Representative EAG traces for the series of stimuli presented to male *C. fraxinella* single antennae. Male antenna tested here was from a summer, untreated individual held under indoors 16:8 24^{o}C conditions and tested on 1 August 2008.



Appendix 3-2. Dose response curves of male *C. fraxinella* EAG response to female sex pheromone in reproductively active males tested after (A) overwintering in nature in the spring, and after (B) overwintering under laboratory conditions. Legend indicates treatment of males that were provided either 10% sugar solution or only water for one week prior to testing. Graphs depict raw EAG responses.

Chapter 4

Seasonal plasticity of response to host plant volatiles in a leaf-mining moth, *Caloptilia fraxinella* (Lepidoptera: Gracillariidae)

Introduction

Host plants are used by all herbivorous animals for food resources, and by many as mating sites and shelter. An individual's ability to locate these resources is essential for survival. Herbivorous insects rely to a large extent on olfactory cues to locate host plants for food, rendezvous locations, and oviposition substrates (Hansson 1995; Anton et al. 2007). Herbivorous insects can be generalists that feed on a wide variety of plant species from different plant families, or they can be specialists, and feed on a limited number or single plant species (Bruce et al. 2005). It is expected that generalists and specialists will respond to host plant odours differently, and specialists in particular, need to locate specific host plants against a background of non-host odours (Szendrei & Rodriguez-Saona 2010). Location of host plants for food or oviposition is widely studied in herbivorous insects, and the hypothesis with the most support is that insects use host cues made up of blends of common and ubiquitous host plant compounds (Bruce et al. 2005). What is not as well known is how or if herbivorous insects modulate response to host plant volatiles, so that plant location occurs at the appropriate time for food location or oviposition. Olfactory modulation or plasticity would be expected to occur more often in specialist

herbivores than generalists, as the timing of host plant location is constrained and important for offspring development.

Insects have specifically tuned olfactory receptor neurons (ORNs) on their antennae to detect relevant host plant odour cues in the environment (Bruce et al. 2005; Bruce & Pickett 2011), and this odour information is processed in the antennal lobe by ordinary glomeruli before transmission to higher brain centers that control behaviour (Hansson 1995). Response to host plant volatile cues has been studied in several species of moths (Lepidoptera) because of their almost ubiquitous herbivorous feeding habits and their important pest status. Both male and female adult moths can detect host plant volatiles (Coracini et al. 2004; Von Arx et al. 2011) and male moths have additional ORNs to detect female-produced pheromone cues (Hansson 1995). Since female moths often release pheromone on or near host plants, the presence of both host plant volatiles and sex pheromone simultaneously in the environment can impact female moth foraging for oviposition sites or the search for mates by male moths (Reddy & Guerrero 2004; Deisig et al. 2012).

Plasticity in response to host plant volatiles can help to time reproductive events such as mating and oviposition. A plastic response would be adaptive if mating occurs on or near host plants or if the oviposition substrate is ephemeral. Plasticity in response to odour cues can occur in the peripheral nervous system on the antennae (Lemmen and Evenden 2009), which can lead to a subsequent change in behavioural response (Browne 1993; Anton et al. 2007). Such plasticity is well documented in mosquitoes, as female antennal response to host

cues decreases after a blood meal, which leads to a behavioural shift in preference from food source to oviposition substrate (Takken et al. 2001; Biessmann et al. 2005). Modulation of antennal response to host plant volatiles is known to occur in some moth species. Female *Dioryctria abietivorella* (Lepidoptera: Pyralidae) antennal response to host plant volatiles increases with age and after mating (Shu et al. 1997). Female cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) show a similar plasticity, as antennal and behavioural response to host plant volatiles increases with moth age and after mating (Martel et al. 2009; Saveer et al. 2012). Unmated female *S. littoralis* are attracted to nectar sources while mated females are attracted to host plants used as oviposition sites (Saveer et al. 2012).

Plasticity in response to odour cues is ultimately realized in the behavioural response of the insect. The response of female tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), moths to volatile cues released by plants used for oviposition increases after mating and also with moth age (Mechaber et al. 2002). Mated female codling moths, *Cydia pomonella* (Lepidoptera: Tortricidae), have a stronger attraction to apple volatiles compared to unmated females (Yan et al. 1999). In moth species that mate on or near host plants, host plant volatiles can refine mate finding by male moths and increase their likelihood of encountering a receptive female (Landolt & Phillips 1997; Deisig et al. 2012). Simultaneous perception of host plant volatiles and female-produced sex pheromone increases mate finding behaviours of male *Lobesia botrana* (Lepidoptera: Tortricidae) (Von Arx et al. 2012), *C. pomonella* (Light et

al. 1993; Yang et al. 2004), *Grapholita molesta* (Lepidoptera: Tortricidae) (Varela et al. 2011), *Spodoptera exigua* (Deng et al. 2004), *Helicoverpa zea* (Lepidoptera: Noctuidae) (Light et al. 1993), and *Eupoecilia ambiguella* (Lepidoptera: Tortricidae) (Schmidt-Busser et al. 2009). The synergism of response to host plant volatiles and sex pheromone by male *C. pomonella* occurs neurophysiologically, in the macroglomerular complex in the antennal lobe of males, which is typically the location of pheromone processing alone in other moths (Trona et al. 2013). Host plant volatiles and pheromone molecules synergize to produce a higher firing rate in pheromone-specific ORNs of male *H. zea* compared to that produced by exposure to pheromone alone (Ochieng et al. 2002). Such evidence indicates that male mate finding behaviour can be enhanced by plasticity in response to odour cues in the peripheral nervous system at the antenna.

The nutritional (den Otter et al. 1991; Browne 1993; Takken et al. 2001) and hormonal (Anton et al. 2007) status of insects contributes to the plasticity of antennal sensitivity and behavioural response to host plant volatiles in some species. In most cases, the effect of nutritional state on host plant location has been tested in conjunction with foraging for food rather than oviposition resources (Browne 1993; Martel et al. 2009). Starved individuals should be more motivated to locate food compared to satiated individuals in order to increase their likelihood of locating a food source (Browne 1993). This is the case in many insects, including tsetse flies (den Otter et al. 1991), locusts (Moorhouse 1971) and the Colorado potato beetle (Visser 1988). The impact of nutrition on female

moth response to host volatiles for the purposes of oviposition is not as well known. Nutrition is required by many female insects in order to initiate oogenesis and reproductive behaviours after a period of delayed mating or reproductive diapause (Tran & Huignard 1992; Wheeler 1996; Evenden et al. 2007). It is likely that nutrition would also impact female response to oviposition cues from host plants, to ensure that females are physiologically primed to exploit the oviposition substrate. Along with nutrition, juvenile hormone (JH) synthesis and release can regulate oogenesis and reproductive behaviours in adult females and sex pheromone response and sex accessory gland development in male moths and butterflies with delayed reproduction (Barker & Herman 1976; Cusson et al. 1993; Duportets et al. 1996; Evenden et al. 2007; Ramaswamy et al. 1997; Duportets et al. 1998; Denlinger 2002; Anton et al. 2007).

Female moths typically have higher antennal responses to host plant volatiles and are able to respond to lower concentrations of pertinent compounds than male moths, due to a higher number of ORNs tuned to host plant volatiles on female antennae compared to that on male antennae (Bruce & Pickett 2011). Such quantitative sexual dimorphism in the sensory physiology of the peripheral nervous system occurs in several moth species (Shu et al. 1997; Fraser et al. 2003; Das et al. 2007). Qualitative sexual dimorphism in response to host plant volatiles also occurs in moths such as the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). Antennae of male and female *O. nubilalis* are differentially responsive to host plant volatiles (Sole et al. 2010). A similar response to host plant volatiles by both males and females can indicate a

similarity in sensory ecology and habitat requirements (Von Arx et al. 2012). The antennae of male and female *C. pomonella* respond similarly to host plant volatile compounds (Anesbo et al. 2004). Male and female European grape berry moth, *E. ambiguella* also show similar antennal response to individual host plant volatiles and to biologically significant blends of the same compounds (Schmidt-Busser et al. 2011).

Caloptilia fraxinella (Lepidoptera: Gracillariidae) (Ely) is a leaf miner as larvae on leaflets of ash trees (*Fraxinus spp.*). Early instar larvae mine the leafets and later instars roll up a leaflet in preparation for pupation. Because of the leaf mining habit of the larvae, female oviposition behaviour is constrained to occur when ash trees are flushing in early spring. Upon eclosion in July, most adult *C. fraxinella* are in reproductive diapause (Evenden et al. 2007). After a summer aestivation, unmated adults overwinter away from their ash hosts. Adults emerge from overwintering in a reproductively active state and mate before orientation to ash trees, where females oviposit on newly flushed ash leaflets (Pohl et al. 2004). Male response to the female-produced sex pheromone is plastic, and is highest in the spring when moths are reproductively active (Evenden & Gries 2008). Exogenous treatment with a JH analogue (JHA) can terminate reproductive diapause and induce females to mate and produce vitellogenic oocytes in both the summer and in the fall (Evenden et al. 2007). Treatment with a JHA also enhances male antennal and behavioural response to pheromone when the males are in reproductive diapause in the fall, but not immediately following eclosion in the summer (Lemmen & Evenden 2009).

In this study, I test the hypothesis that male and female *C. fraxinella* response to host plant volatiles is plastic, and is expected to be highest in the spring when moths orient to host trees. I also test the response to ash volatiles of both male and female moths in various physiological states during their extended adult life. Electroantennographic (EAG) techniques are used to assess response of the peripheral nervous system in addition to behavioural bioassays in a wind tunnel. I also attempt to understand the mechanisms underlying plasticity in response to olfactory cues by testing moths under different nutritional and hormonal treatments.

Methods

Moths in different physiological states were tested for their response to ash host volatiles using EAG and behavioural bioassays (Tables 4-1, 4-2). Moths tested in the summer and fall were in reproductive diapause and those tested after overwintering in December and in the spring were reproductively active (Evenden et al. 2007).

Moth Collection and Treatment

Moths were reared from pupae collected in leaf rolls from green ash, *Fraxinus pennsylvanica* at various sites in Edmonton, Alberta (53^{o} 34'N 113^{o} 31'W) in June 2009 and 2010. Each leaf roll containing one pupa was held in a 30 ml transparent plastic cup and ~ 70 cups were placed in transparent plastic bags with a damp paper towel to maintain humidity under a 16:8 h light:dark

(L:D) photoperiod at 24°C. Moth eclosion was monitored 2-3 times per week, and newly-eclosed moths were separated by sex and prepared for use in subsequent bioassays.

Moths used in summer experiments were held individually in 30 ml cups supplied with a 10% sugar solution through a dental wick. To avoid exposure to conspecific cues, cups containing male and female moths were held in separate growth chambers both maintained at 16:8 h L:D photoperiod and 24°C for at least one week before use in a bioassay. Moths used in fall experiments were transferred outdoors after eclosion and were held in cages (H 80 cm x W 40 cm x D 40 cm) separated by sex under natural temperature and photoperiod conditions and supplied with a 10% sugar solution until one week before use in bioassays when they were transferred indoors and maintained as described for the summer experiments before experimentation. *Caloptilia fraxinella* moths are in reproductive diapause in the summer and the fall.

Moths tested after a period of overwintering in the laboratory (overwintered moths) were either reared from pupae and held outdoors under natural conditions post-eclosion until late September, as described for the fall experiments, or were collected free-flying in the fall at various sites in Edmonton, AB. Moths were transferred indoors in late September and held under winter conditions of 0:24 h L:D photoperiod at 2°C for two to three months. At least one week before bioassays, moths were transferred to summer conditions and maintained as described for moths tested in the summer and fall. To determine if adult nutrition influenced female response to ash volatiles, female moths tested

after overwintering were provided with either a 10% sugar solution or water alone through a dental wick. Additional moths were collected in flight during spring at various sites in Edmonton after overwintering under natural conditions outside (spring moths). Collected moths were set up individually in 30 ml transparent cups and were maintained as in the other experiments at 16:8 h L:D photoperiod at 24^{o}C for at least one week before use in a bioassay. Reproductively active overwintered and spring moths were both included in these experiments to test the importance of natural overwintering conditions of about six months compared with controlled overwintering conditions in the laboratory of about two months on subsequent moth response to host plant volatiles.

Hormone Treatment

To directly test if physiological state influenced moth response to ash volatiles, based on previous experiments (Evenden et al. 2007; Lemmen & Evenden 2009), moths in reproductive diapause in the summer and fall experiments were exposed to one of three treatments prior to use in bioassays. (1) 1 μg of the JHA methoprene (94.3% pure, Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 1 μl of high-performance liquid chromatography (HPLC) grade acetone (Fisher Scientific, Ottawa, Ontario, Canada) from a stock solution, (2) 1 μl acetone alone, or (3) were left untreated. Moths were held under a gentle vacuum stream while treatments were applied exogenously to the ventral side of the abdomen. After treatment, individuals were transferred to experimental conditions and provided with a 10% sugar solution or water, depending on the

experiment, for one week prior to the EAG or wind tunnel bioassays. In all experiments, moths were treated with methoprene seven days before the EAG or wind tunnel bioassay. Moths used in overwintering and spring experiments were already in a reproductively active state and were not subjected to hormone treatment.

Wind Tunnel Bioassays

The wind tunnel used in behavioural assays had a flight section 1.7 m long and 0.85 m high. Six, 15-watt bulbs diffused through white paper dimly illuminated the tunnel. Wind speed was 0.32-0.34 m/s and temperature was maintained at 24-26^{o}C. Moths were acclimatized to experimental conditions 30 min prior to initiation of the behavioural assay. Flights were conducted during the last hour of the photophase and the first two hours of the scotophase. Moths were introduced individually into the wind tunnel in cylindrical wire cages (5 cm diameter x 6 cm height) on a platform, 20 cm from the downwind end. Once the moth was positioned in the odour plume, the lid of the cage was removed and individuals were allowed three minutes to respond to the odour source. In experiments conducted in the summer and fall, methoprene-treated, acetone-treated and untreated moths were flown alternately and each moth was flown only once. Behavioural responses to odours were recorded as: wing fanning, lock-on to the odour plume, upwind oriented flight and contact with the odour source.

Wind Tunnel Experiments: Males

Males were flown to host plant odour sources with and without female sex pheromone in the spring, summer and fall to test whether male response to host plant volatiles is plastic throughout the course of its adult life. Males were flown to two, three or four odour stimuli depending on the experiment (Table 4-1): 1) a green ash seedling (*Fraxinus pennsylvanica* Marsh. var. *subintegerrima* (Vahl) Fern., Jeffries Nurseries, Portage La Prairie Manitoba) planted individually in pots containing Sunshine 4 potting mix (Sun Gro Horticulture, Agawam, MA, USA); 2) a synthetic plastic "tree" that resembled an ash seedling in height, colour and architecture and was potted in the same soil as the real seedling; 3) a pheromone source consisting of 10 μg (*Z*)-11-hexadecenal and 1 μg (*Z*)-11-hexadecen-1-ol (>99%, Pherobank, Wageningen, The Netherlands) in HPLC grade hexane (Fisher Scientific, Ottawa, Ontario, Canada) released from a pre-extracted grey rubber septum (Contech Enterprises Inc., Delta, BC, Canada) or 4) a pheromone source plus an ash seedling.

Experiment 1 tested for a synergistic effect of sex pheromone plus ash tree volatiles on reproductively active male behavioural response in the wind tunnel in the spring (Table 4-1). Experiment 2 tested reproductively active male response in the wind tunnel to an ash seedling with and without pheromone, and also to a synthetic seedling to test whether visual cues are sufficient to attract male *C. fraxinella* (Table 4-1). Experiments 3 and 4 were conducted when males were in reproductive diapause in the summer and fall, respectively, and moths were treated with methoprene, acetone, or were left untreated. Male *C. fraxinella*

behavioural response was tested to an ash seedling with and without pheromone in the summer (Experiment 3) and in the fall (Experiment 4) (Table 4-1).

Electroantennography Bioassays

Electroantennogram recordings were made using an IDAC-02 data acquisition controller system, and EAG 2000 software (SYNTECH, Hilversum, The Netherlands). In preparation for the antennal recordings, moths were chilled at 4^{o}C for at least 20 min before one antenna was carefully excised and attached to a stainless steel antenna holder using a small quantity of Spectra 360 conductive gel (Parker Laboratories Inc., Orange, NJ), and attached to a SYNTECH EAG probe (Type PRG-2, internal gain 10x). Five ash volatiles known to be detected by *C. fraxinella* (Wist et al. unpublished data) were tested individually: methyl salicylate (≥99%, Sigma-Aldrich, St. Louis, MO), β-ocimene (70/30% E/Z, Contech International Inc., Delta, B.C.), linalool (97%, Aldrich Chemical Co. Inc., Milwaukee, WI), (*E*)-2-hexenal (95%, Aldrich Chemical Co. Inc., Milwaukee, WI) and (*E*,*E*)-α-farnesene (67.2%, with 23.1% (*Z*,*E*)-α-farnasene, Contech International Inc., Delta, B.C.). Each tested compound was serially diluted in HPLC-grade hexane to obtain decadal solutions between 100 μg and 0.001 μg/μl hexane. Ten μl of each compound at the various doses, and 10 μl of a hexane control, were pipetted individually onto 7 × 0.2 cm strips of folded Whatman no. 1 filter paper and allowed to evaporate in a fume hood. Additionally, 10 μl of the sex pheromone (Evenden and Gries 2008) made up of 0.1 μg/μl (*Z*)-11-hexadecenal and 0.01 μg/μl (*Z*)-11-hexadecen-1-ol (>99%,

Pherobank, Wageningen, The Netherlands) diluted in hexane was pipetted onto filter paper and used as a standard. Treated strips were inserted into disposable Pasteur pipettes. Stimulus puffs were generated with a SYNTECH CS-55 stimulus controller with a pulse duration of 0.2 sec and flow of 10 ml/sec. Antennal responses were measured as the maximum amplitude of depolarization elicited by the stimulus odour applied. Each antenna received a series of puffs delivered once every minute in the following order: blank; hexane; 0.01 μg plant volatile; 1 μg pheromone; 0.1 μg plant volatile; 1 μg pheromone; 1 μg plant volatile; 1 μg pheromone; 10 μg plant volatile; 1 μg pheromone; 100 μg plant volatile; 1 μg pheromone; 1000 μg plant volatile; blank.

Electroantennography Experiments: Males and Females

Adult *C. fraxinella* were presented with the five individual green ash volatiles in the spring, summer, fall and after overwintering (Table 4-2) to test the hypothesis that male and female antennal response to host plant volatiles is plastic over the course of the adult life.

The first experiment tested the antennal response of female and male *C. fraxinella* to the five individual volatiles at different times of year (Table 4-2). Moths in reproductive diapause were tested in the summer and fall, and were either left untreated, or treated with methoprene or acetone. Reproductively active moths were tested in the spring and after overwintering and were not treated with the JHA (Table 4-2).

The second experiment tested the antennal response of overwintered female *C. fraxinella* that were presumed to be reproductively active, and were provided with either a 10% sugar solution as a nutrition source, or water alone for at least one week after removal from overwintering conditions prior to the EAG experiment (Table 4-3). Females were tested to the same five host volatiles (Table 4-3).

Statistical Analyses

In the wind tunnel experiments, behavioural responses were recorded as "yes" or "no" for each moth so the resulting data were binomially distributed. Each behaviour was analyzed separately with a generalized linear model (GLM) with a logit link function and binomial errors terms (R Development Core Team 2012). The resulting analysis of deviance table was used to determine if male response was dependent on the experimental variables. All two-way interactions were included in the models and non-significant interactions terms were removed to achieve the most parsimonious model. When the GLM was significant, multiple comparisons were made using a Tukey-contrasts test (R-package: Multcomp).

The raw mV antennal responses obtained in the EAG experiments were log transformed after conducting a Box Cox analysis to determine the most appropriate transformation of the data. The log-transformed data showed greatly improved analysis based on AIC values. The EAG responses of individuals were compared using a repeated measures ANOVA (R Development Core Team 2012),

to account for repeated measurement of different volatile doses on each antenna tested. Separate models were used to test: (1) the effect of time of year on EAG response of untreated male and female moths to all five host plant volatiles at the four different times of year; (2) the effect of moth sex on EAG response to host plant volatiles; (3) the effect of hormone treatment on male and female EAG response to host plant volatiles in the summer and the fall; and (4) the effect of adult nutrition on female EAG response to host plant volatiles after overwintering. All two-way interactions were included in the models and non-significant interaction terms were removed from the models to achieve the most parsimonious model. When necessary, a post-hoc multiple comparison Honestly Significant Differences (HSD) test was performed to determine differences by treatment (R-package: agricolae). In the figures depicting EAG responses presented here, means and standard errors have been back-transformed. (See Appendix 4-1 for representative EAG traces of males and females to host plant volatiles, see Appendices 4-2 and 4-3 for EAG dose response curves of females and males to all host volatiles tested in all season, and see Appendices 4-3 – 4-13 for EAG dose response curves for experiments when not presented in the Figures.)

Results

Wind Tunnel Experiments: Males

In the spring, reproductively active males responded similarly in all behavioural categories to pheromone alone or to pheromone plus an ash seedling positioned in the wind tunnel in Experiment 1 (Fig. 4-1A). The type of odour source did impact the behavioural response of reproductively active males in Experiment 2. As would be expected, a higher proportion of males expressed all behaviours to the pheromone plus ash seedling compared to the ash seedling alone or the synthetic seedling (Fig. 4-1B). A high percentage of reproductively active males (80-100%) expressed behaviours in the wind tunnel when tested in the spring (Fig. 4-1).

The type of odour source impacted behavioural response of males in reproductive diapause that were tested in the summer in the wind tunnel. For all behaviours, more males responded to the pheromone alone or to the pheromone plus ash seedling compared to those that responded to the ash seedling alone or the synthetic seedling (Fig. 4-2A). Hormone treatment did not impact wing fanning (X^2_2=4.13, P=1), lock-on (X^2_2=2.75, P=0.3), upwind flight (X^2_2=2.41, P=0.3) or source contact (X^2_2=2.44, P=0.3) in the summer. The majority of males were in reproductive diapause in the summer, and only 30-40% of males expressed behaviours to semiochemical sources in the wind tunnel (Fig. 4-2A).

The type of odour source also impacted male behavioural response when males in reproductive diapause were tested in the fall, but male response was different from that observed in the summer. In the fall, more males expressed lock-on, upwind flight and source contact to pheromone alone, compared to the proportion of males that responded to pheromone plus an ash seedling or to an ash

seedling alone (Fig. 4-2B). Wing fanning behaviour was not impacted by odour source in the fall (Fig. 4-2B). Hormone treatment did impact male lock-on, upwind flight and source contact behaviours in the fall (Fig. 4-3). More methoprene-treated males locked-on to the odour source compared to acetone-treated and untreated males (Fig. 4-3); however the significant effects of hormone treatment for upwind flight and source contact behaviours identified in the overall model could not be separated with a post-hoc analysis. Wing fanning was not impacted by hormone treatment in the fall (Fig. 4-3). Males tested in the fall were assumed to be in reproductive diapause, and less than 20% of males expressed behaviours in the wind tunnel (Figs. 4-2B, 4-3).

Electroantennography Experiments: Males and Females

The physiological state, or season in which moths were tested significantly impacted the antennal response of female (Appendix 4-2) and male (Appendix 4-3) *C. fraxinella* to all five host plant volatiles tested. In most cases, moth antennal response was lower in the summer and fall when moths were in reproductive diapause compared to antennal response when moths were reproductively active in the spring and immediately after overwintering. Females had the highest mean antennal responses to both (*E,E*)-α-farnesene and β-ocimene in the spring, and highest responses to (*E*)-2-hexenal, linalool and methyl salicylate when tested immediately after overwintering (Table 4-4). Male *C. fraxinella* had the highest antennal responses to (*E,E*)-α-farnesene, β-ocimene and methyl salicylate in the

spring, and the highest responses to (*E*)-2-hexenal and linalool immediately after overwintering (Table 4-4).

Significant interactions between the season moths were tested in and host plant volatile dose impacted male antennal response to (*E*)-2-hexenal (Fig. 4-4A), (*E,E*)-α-farnesene (Fig. 4-4B) and linalool (Fig. 4-4C), and female antennal response to (*E*)-2-hexenal (Fig. 4-4D) and (*E,E*)-α-farnesene (Fig. 4-4E). Antennal response to the lowest five doses remained mostly constant for male and females to all of the host plant volatiles tested. In most seasons, there was a sharp increase in antennal response to the 100 μg and 1000 μg doses of the host plant volatiles (Fig. 4-4). When moths were tested immediately after overwintering, males had the highest antennal responses to the two highest doses of (*E*)-2-hexenal (Fig. 4-4A) and linalool (Fig. 4-4C), and females had the highest antennal responses to the two highest doses of (*E*)-2-hexenal (Fig. 4-4D). In response to (*E,E*)-α-farnesene, males had the highest antennal response to the two highest doses in the spring, however males tested immediately after overwintering also had a high response to the highest dose test (Fig. 4-4B). Females showed similar responses to (*E,E*)-α-farnesene at the four different times of year (Fig. 4-4E).

Male and female moths responded differently to host plant volatiles when moths were tested in the spring, immediately after overwintering, in the summer and in the fall (Appendices 4-4 – 4-8). In the spring, reproductively active male *C. fraxinella* had higher antennal responses to (*E,E*)-α-farnesene, (*E*)-2-hexenal and methyl salicylate compared to reproductively active females, and females had higher antennal responses to linalool and β-ocimene compared to males (Fig. 4-

5A). After a period of overwintering, when moths were presumed to be reproductively active, males had higher antennal responses to (*E*,*E*)-α-farnesene, and females had higher antennal responses to linalool, methyl salicylate and β-ocimene compared to males (Fig. 4-5B). Moth sex did not impact antennal response of reproductively active moths to (*E*)-2-hexenal (Fig. 4-5B). In the summer, males had higher antennal responses to (*E*,*E*)-α-farnesene and β-ocimene compared to females (Fig. 4-5C). Moth sex as a main factor did not impact antennal response to (*E*)-2-hexenal, linalool or methyl salicylate (Fig. 4-5C). In the fall, females in reproductive diapause had higher antennal responses to all of the host plant volatiles tested, (*E*,*E*)-α-farnesene, (*E*)-2-hexenal, linalool, methyl salicylate and β-ocimene compared to males in reproductive diapause (Fig. 4-5D).

Moth antennal response was impacted by significant interactions between sex and host plant volatile dose in the spring, summer and fall (Fig. 4-6). In the spring, antennal response was impacted by interactions between moth sex and (*E*)-2-hexenal dose (Fig. 6A), linalool dose (Fig. 4-6B) and methyl salicylate dose (Fig. 4-6C). When reproductively active moths were tested to (*E*)-2-hexenal and to methyl salicylate in the spring, male and female antennal responses were similar to each other at all of the doses except for the highest 1000 μg dose for which male antennal response was higher than female response (Figs. 4-6A,C). In the spring, male and female antennal responses were similar to each other at the lower doses of linalool, but at the higher doses, beginning at 1 μg, female antennal

response was higher compared to male response, especially at the highest 1000 μg dose (Fig. 4-6B).

In the summer, an interaction between moth sex and host plant volatile dose impacted moth response to (*E,E*)-α-farnesene (Fig. 4-6D). Males in reproductive diapause responded more to the lower doses (0 μg-10 μg) compared to females in reproductive diapause. Males and females had a similar response to the 100 μg dose, and female response was higher to the 1000 μg dose of (*E,E*)-α-farnesene compared to males when tested in the summer (Fig. 4-6D). When moths were tested in the fall, significant interactions between moth sex and host plant volatile dose impacted moth antennal response to (*E,E*)-α-farnesene (Fig. 4-6E) and linalool (Fig. 4-6F). In both cases in the fall, male and female antennal responses were similar to each other at the lower doses tested (0 μg-10 μg), but at the two highest doses, female antennal response was higher compared to that of males (Figs. 4-6E, F).

The impact of hormone treatment on male and female antennal response to all five host plant volatiles was tested in the summer and the fall, when the moths were in reproductive diapause (Appendices 4-9 – 4-13). Hormone treatment impacted the antennal responses of both male and female *C. fraxinella* to host plant volatiles in the summer and the fall, but the effect of the hormone treatment differed by sex and the times of year in which they were tested. Methoprene-treated females in reproductive diapause tested in the summer had higher antennal responses to (*E,E*)-α-farnesene compared to untreated females. Methoprene-treated females responded more to methyl salicylate and β-ocimene compared to

both acetone-treated and untreated females in the summer (Fig. 4-7A). Treatment with methoprene in the summer enhanced male antennal response to (*E*,*E*)-α-farnesene and (*E*)-2-hexenal compared with acetone-treated and untreated males, and to methyl salicylate compared with untreated males (Fig. 4-7B). Acetone-treated males had the highest antennal response to linalool and untreated males had the highest antennal response to β-ocimene (Fig. 4-7B). Hormone treatment did not impact female response to (*E*)-2-hexenal in the summer (Fig. 4-7A). Acetone-treated females had higher responses to linalool compared with methoprene-treated and untreated females in the summer (Fig. 4-7A).

Hormone treatment had no impact on female antennal response to linalool or methyl salicylate in the fall (Fig. 4-7C). Acetone-treated females had higher responses to (*E*,*E*)-α-farnesene compared with methoprene-treated and untreated females in the fall (Fig. 4-7C). Methoprene-treated and acetone-treated females had similar antennal responses to both (*E*)-2-hexenal and β-ocimene, and the responses were higher to both host plant volatiles compared with untreated females (Fig. 4-7C). In the fall, methoprene treatment enhanced male response to (*E*,*E*)-α-farnesene and methyl salicylate compared with acetone-treated and untreated males (Fig. 4-7D). Methoprene treatment enhanced response to (*E*)-2-hexenal compared with the response of untreated males, and to linalool compared with the response of acetone-treated males (Fig. 4-7D). Acetone-treated males had a higher antennal response to β-ocimene compared to untreated males, and the response of methoprene-treated males was intermediate (Fig. 4-7D).

Significant interactions between hormone treatment and host plant volatile dose impacted female response to (*E,E*)-α-farnesene in the summer (Fig. 4-8A) and male response to (*E,E*)-α-farnesene in the fall (Fig. 4-8B). In the summer, untreated females had the lowest antennal responses to (*E,E*)-α-farnasene at all doses tested (Fig. 4-8A). Methoprene-treated and acetone-treated females had similarly low responses to the lower volatile doses of (*E,E*)-α-farnasene (0 μg-0.1 μg), and to the higher doses tested (1 μg-1000 μg). Methoprene-treated females had the highest antennal responses, and acetone-treated females had intermediate antennal responses (Fig. 4-8A). In the fall, untreated and acetone-treated males had similar antennal responses to (*E,E*)-α-farnesene, and at doses of 1 μg and higher, methoprene-treated males had higher antennal responses compared to the other two treatments.

Adult moth access to sugar water impacted the antennal response of reproductively active females to four of the five host plant volatiles tested (Appendix 4-14). Females provided with sugar water had higher antennal responses to (*E,E*)-α-farnesene, (*E*)-2-hexenal and methyl salicylate compared to females provided only water (Fig. 4-9). Reproductively active females provided with water alone had higher antennal responses to linalool compared to females that were provided sugar water (Fig. 4-9). Nutrition status did not impact female antennal response to β-ocimene (Fig. 4-9).

Discussion

The current study tested the hypothesis that response to host plant volatiles by adult *C. fraxinella* is plastic and depends on the physiological state of adult moths. Variation in hormone titre with physiological state is a potential mechanism driving such plasticity and that possibility was tested in the current study. Male and female *C. fraxinella* antennal response and male behavioural response to host volatiles is plastic. Responsiveness varies with moth physiological state, moth sex and hormone treatment in males and females, and access to nutrition in females. Moth response to host volatiles is modulated at the level of the antenna, and this modulation corresponds to subsequent behaviour in the wind tunnel experiments and also in nature (Evenden and Gries, 2008; Pohl et al. 2004).

Male *C. fraxinella* behavioural response to ash seedlings changes depending on the physiological state of the male, and whether a pheromone source is present. Males orient to the pheromone plus ash seedling odour source in the spring and the summer, but not in the fall. In the spring, males do not respond to the ash seedling alone in the wind tunnel, suggesting that their presence in ash trees in the spring (Pohl et al. 2004; Evenden & Gries 2008) is the result of response to female-produced sex pheromone. The hypothesis is supported by the high number of males that conduct all pheromone-mediated behaviours in the spring to a pheromone source alone in the wind tunnel. The presence of an ash seedling in addition to a source of pheromone did not enhance male response to the pheromone source in the spring. Host volatiles often enhance response of male moths to pheromone if a quantitatively or qualitatively

suboptimal signal is provided but provide no synergism to an optimal pheromone signal (Schmidt-Busser et al. 2009; Von Arx et al. 2012). The high level of male response to the pheromone signal used in this study (Evenden & Gries 2008) was not improved with addition of cues from an ash seedling when the males were reproductively active. Ash volatiles may enhance male *C. fraxinella* response to a suboptimal pheromone dose or blend, but this hypothesis remains to be tested.

Male response to odour sources is reduced when males are in reproductive diapause in the summer and fall compared to that of reproductively active males in the spring. Further, male response differs with time in the season that they are tested in reproductive diapause. In the summer, males in reproductive diapause are equally attracted to a pheromone source alone or a pheromone source with an ash seedling in wind tunnel bioassays. In the fall, male *C. fraxinella* do not orient to pheromone when an ash seedling is present. Ash volatiles appear to negatively impact male pheromone processing, as some males do respond to the pheromone source alone in the fall. Males tested in the fall have a lower antennal response to the ash volatiles compared to males tested at the other times of year and to females tested in the fall. Reduced responsiveness to host cues at this time of year may indicate reduced reliance on host cues as moths prepare to overwinter away from their ash hosts (Pohl et al. 2004).

There may be some interaction between pheromone processing and host volatile processing in male *C. fraxinella*. Inhibitory or repellent effects of host volatiles on orientation to pheromone are known to occur, but it is not as well-studied as the synergistic effects of host volatiles and pheromone signals (Reddy

& Guerrero 2004). Pheromone response of southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Curculionidae) to its aggregation pheromone is reduced when the pheromone is released with the pine host volatile, 4-allyl anisole, which could play a role is host selection by the beetle (Hayes et al. 1994; Reddy & Guerrero 2004). Male *Agrotis ipsilon* (Lepidoptera: Noctuidae) show highly reduced behavioural and central nervous system response to pheromone immediately after mating, which lasts until the following night when pheromone response is restored (Barrozo et al. 2010). Mating status of this species does not impact male behavioural response to linden flower extracts in the absence of pheromone. Presentation of flower extracts with above-threshold doses of pheromone enhances orientation to the odour source in virgin but not mated males (Barrozo et al. 2010). In mated *A. ipsilon* males, behavioural response to floral volatiles is inhibited by simultaneous perception of female sex pheromone (Barrozo et al. 2010). Electrophysiological response of male *A. ipsilon* antennae to components of the floral extract show that exposure of antennae to host volatiles reduces pheromone perception regardless of mating status perhaps as a result of non-competitive inhibition of the olfactory receptors (Deisig et al. 2012). In the current study, the presence of the ash seedling inhibits male *C. fraxinella* response to the pheromone in the fall. The inhibitory effects of host volatiles on male *C. fraxinella* response to pheromone in the fall may ensure that males do not orient to trees at an inappropriate time of year, even in the presence of a sex pheromone cue. Further experiments should be conducted to test male antennal response to host volatiles with and without the presence of pheromone to confirm

whether the behavioural response observed in the fall is caused by modulation at the level of the antenna.

Antennal response of both males and females to individual ash volatiles is variable and plastic, and changes depending on physiological state. In all cases for all of the volatiles tested, season significantly impacts male and female antennal response. For both sexes, reproductively active moths have the highest antennal responses when tested either in spring or directly after a period of overwintering in the laboratory. *Caloptilia fraxinella* should respond more acutely to host cues in the spring when females are locating oviposition sites and males are searching for mates (Pohl et al. 2004). Antennally, males and females respond similarly to host volatiles when they are reproductively active which should enable host location by both sexes when ash leaves flush in the spring, despite the observation that male behavioural response is elicited by female sex pheromone rather than host volatiles in the wind tunnel bioassays. Similarity in antennal response to host volatiles by male and female moths also occurs in *E. ambiguella* (Schmidt-Busser et al. 2011) and *C. pomonella* (Anesbo et al. 2004).

Male and female *C. fraxinella* have comparable responses to host volatiles early in reproductive diapause when they are tested in the summer. Later in reproductive diapause when moths are preparing for overwintering in the fall, male antennal response is lower than that of females. Male *C. fraxinella* antennal response to pheromone also differs throughout the period of reproductive diapause (Lemmen & Evenden 2009). In male moths, host volatiles and pheromone are typically detected by separate ORNs, but it is possible that an

overall modulation of antennal response occurs across all ORNs. In mosquitoes that have a reduced antennal response to hosts after feeding, this modulation is due to a factor found only in the hemolymph of fed females (Klowden & Leah 1979; Davis 1984; Anton et al. 2007). The biogenic amines octopamine and serotonin can modulate the sensitivity of males to pheromone at the level of the antenna, but this has not been the case for moth response to host volatiles tested to date (Pophof 2000; Pophof 2002; Grosmaitre et al. 2011; Anton et al. 2007). Further experiments are required to determine which factors are involved in modulating antennal response in *C. fraxinella*. Multi-component blends of the host volatiles may result in higher antennal responses from male and female *C. fraxinella* as occurs in other moth species (Bruce & Pickett 2011; Von Arx et al. 2012). It remains to be determined if *C. fraxinella* respond to plant cues in order to orient to overwintering sites.

Hormone treatment of males in reproductive diapause enhances behavioural response to odour cues tested in the wind tunnel in the fall. Hormone treatment also enhances antennal response of males in reproductive diapause to some of the host volatiles in the summer and most of the tested volatiles in the fall. Since males did not conduct odour-mediated behaviours in the wind tunnel to the ash seedling alone, the increase in response of methoprene-treated males in the fall could be attributed to response to the pheromone alone. Male *C. fraxinella* antennal response to sex pheromone is enhanced after methoprene treatment in the fall but not summer (Lemmen & Evenden 2009). Reproductive diapause is a dynamic state, and insect physiological state (Kostal 2006) and

diapause intensity (Tauber & Tauber 1976) change throughout the period of diapause. Diapause intensity typically decreases over the course of the diapause season (Tauber & Tauber 1976), and hormone treatment more readily breaks reproductive diapause in male and female *C. fraxinella* in the fall than in the summer (Evenden et al. 2007; Lemmen & Evenden 2009). In the current study, hormone treatment enhances male *C. fraxinella* antennal response to individual host volatiles more readily in the fall than in the summer. Central nervous system processing of host volatile signals is always high and is not increased with JH treatment in another long-lived moth, *A. ipsilon* (Greiner et al. 2002). Although JH treatment does affect processing of pheromone signals in reproductively inactive *A. ipsilon* (Anton & Gadenne 1999, Gadenne et al. 1993), these moths need to feed throughout their adult life, and so less plasticity in response to the host would be adaptive in this species. It appears that response to host volatiles by male *C. fraxinella* is highest to most compounds in the spring when males are searching for mates and females are seeking oviposition locations. Although most mating occurs before females locate ash trees in the spring (Evenden et al. 2007), males are captured in trees throughout the adult flight where they presumably are seeking mates (Evenden & Gries 2008).

In contrast to the results with male *C. fraxinella* in reproductive diapause, hormone treatment enhanced antennal response of females in reproductive diapause to some of the volatiles when females were tested in the summer, but not in the fall. Since female and male antennal response is impacted by hormone treatment at different stages of reproductive diapause, this is evidence that

developmental physiology of the moths differ between the sexes. In some insects, there can be variation in diapause regulation between males and females (Denlinger et al. 2005). Similar to what was observed in male *C. fraxinella*, the impact of methoprene treatment on reproductive diapause termination in females varies between the summer and fall. Some female *C. fraxinella* that are treated with methoprene and provided with sugar as a nutrition source break diapause and produce vitellogenic oocytes in the summer, but more females produce vitellogenic oocytes and successfully mate when treated with methoprene in the fall (Evenden et al. 2007). Gene expression varies throughout the period of reproductive diapause (Denlinger 2002; Denlinger et al. 2005). As female antennal response to host volatiles increases with methoprene treatment in the summer but not in the fall, upregulation of genes as a result of JHA treatment that are involved in antennal sensitivity to host volatiles may occur only in the summer and not in the fall.

The nutritional status of female *C. fraxinella* impacted antennal response to four out of the five host plant volatiles tested. Nutrition may also modulate female antennal response to the whole host plant volatile mixture. Starved moths have higher responses to plant food cues compared to satiated individuals in species that must feed as adults (Den Otter et al. 1991; Browne 1993; Takken et al. 2001). The ash volatiles tested in the current study are likely exploited by female moths for location of their ephemeral oviposition site and not directly as food cues. As adult female *C. fraxinella* require a carbohydrate food source to mature eggs (Evenden et al. 2007), a heightened response to oviposition site cues

when females are physiologically capable of egg-laying should be adaptive as females are constrained by a narrow oviposition window. In several species of flies, females only become responsive to oviposition sites after oocyte maturation (Browne 1993). Female mosquitoes, *A. aegypti* (Diptera: Culicidae) become attracted to oviposition sites after ingesting a blood meal and subsequent egg development (Klowden 1990; Klowden et al. 1987). Female diamondback moths, *Plutella xylostella* (Lepidoptera: Plutellidae) provided with a carbohydrate nutrition source greatly increase their oviposition period and fecundity compared to females provided only water (Marchioro & Foerster 2013), and females also have a much higher rate of calling, mating and oviposition when they are in the presence of host plants (Pivnick et al. 1990).

Moth response to host volatiles in *C. fraxinella* is plastic, and response is modulated at the level of the peripheral nervous system. It could be that further modulation of response to host volatiles occurs in the central nervous system as in other moth species (Anton et al. 2007), and further experiments could test this hypothesis in *C. fraxinella*. Similar to male antennal response to pheromone (Lemmen & Evenden 2009), hormone treatment impacts male and female antennal responses to host volatiles, which to my knowledge is the first time this has been shown in a moth species. There is also evidence of pheromone and host volatile odour interaction that impacts male behavioural response to pheromone when males are in reproductive diapause in the fall. Further experiments could elucidate this interaction, and further the understanding of odour modulation in long-lived insects.

Table 4-1. Experimental dates, odour sources tested, hormone treatments and number of male *C. fraxinella* individuals flown to each odour source for all behavioural bioassays in the wind tunnel.

Experiment	Season	Dates of Experiment	Odour Source	Hormone Treatment[a]		
				NT 'N'	JHA 'N'	Acetone 'N'
1	Spring	17 May 2011	Pheromone	19	ND[b]	ND
			Pheromone + Ash Seedling	18	ND	ND
2	Spring	9-16 May 2011	Pheromone + Ash Seedling	50	ND	ND
			Ash Seedling	53	ND	ND
			Synthetic Seedling	50	ND	ND
3	Summer	15 July-3 August 2011	Pheromone	44	43	48
			Pheromone + Ash Seedling	47	43	50
			Ash Seedling	44	41	47
			Synthetic Seedling	44	41	47
4	Fall	28 September-1 October 2011	Pheromone	25	22	26
			Pheromone + Ash Seedling	27	21	25
			Ash Seedling	28	21	25

[a]Hormone treatment abbreviations: NT=Not Treated; JHA=Juvenile hormone analogue, methoprene. [b]ND=Treatment not done.

Table 4-2. Experimental dates, host plant volatiles tested, hormone treatments and number of female and male *C. fraxinella* antennae tested for seasonal EAG experiments.

				Hormone Treatment[a]		
Antennal Source	Host Volatile	Season	Dates of Experiment	NT 'N'	JHA 'N'	Acetone 'N'
Female EAG	(*E*,*E*)-α-farnesene	Spring	25 May 2010	10	ND[b]	ND
		Summer	22 July 2010	10	8	10
		Fall	5-7 Oct 2010	10	10	10
		Overwintered	14 Dec 2010	10	ND	ND
	(*E*)-2-hexenal	Spring	6 May 2009	10	ND	ND
		Summer	21 July 2010	10	9	10
		Fall	18 Sept 2009	11	8	8
		Overwintered	22 Jan 2010	8	ND	ND
	Linalool	Spring	7 May 2009	10	ND	ND
		Summer	20 July 2010	10	10	10
		Fall	19 Sept 2009	10	9	10
		Overwintered	21-22 Jan 2010	9	ND	ND
	Methyl Salicylate	Spring	2 May 2010	10	ND	ND
		Summer	19 July 2010	10	10	10
		Fall	20 Sept 2009	10	9	10
		Overwintered	20-21 Jan 2010	9	ND	ND
	β-ocimene	Spring	25 May 2010	10	ND	ND
		Summer	23 July 2010	10	10	10
		Fall	16 Sept-5 Oct 2010	10	10	10
		Overwintered	15 Dec 2010	10	ND	ND
Male EAG	(*E*,*E*)-α-farnesene	Spring	26 May 2010	10	ND	ND
		Summer	25 July 2010	10	11	10
		Fall	11 Sept 2010	10	10	10
		Overwintered	7 Dec 2010	10	ND	ND
	(*E*)-2-hexenal	Spring	8 May 2009	10	ND	ND
		Summer	24 July 2010	10	10	10
		Fall	7-9 Sept 2009	11	11	11
		Overwintered	14 Jan 2010	8	ND	ND
	Linalool	Spring	9 May 2009	10	ND	ND
		Summer	22-24 July 2010	10	10	10
		Fall	10 Sept 2009	11	9	11
		Overwintered	14 Jan 2010	9	ND	ND
	Methyl Salicylate	Spring	2 May 2010	10	ND	ND
		Summer	18-20 July 2010	10	10	10
		Fall	11 Sept 2009	10	7	9
		Overwintered	15 Jan 2010	7	ND	ND
	β-ocimene	Spring	26 May 2010	10	ND	ND
		Summer	18 July 2010	10	10	10
		Fall	11-14 Sept 2010	10	10	10
		Overwintered	7 Dec 2010	10	ND	ND

[a]Hormone treatment abbreviations: NT=Not Treated; JHA=Juvenile hormone analogue, methoprene. [b]Treatment not done.

Table 4-3. Experimental dates of EAG bioassays, nutrition regime and number of female *C. fraxinella* antennae tested when females were reproductively active after experiencing simulated winter conditions in the laboratory.

Host Volatile	Nutrition Regime	Dates of Experiment	N
(*E*,*E*)-α-farnesene	10% Sugar	9-10 December 2010	10
	Water		10
(*E*)-2-hexenal	10% Sugar	16-17 January 2010	10
	Water		10
Linalool	10% Sugar	16-17 January 2010	10
	Water		10
Methyl Salicylate	10% Sugar	16-17 January 2010	10
	Water		10
β-ocimene	10% Sugar	13 December 2010	10
	Water		9

Table 4-4. Impact of seasonality on male and female EAG response to a dose response of individual host volatiles. All doses of the individual volatiles were included in each model.

Sex	Host Volatile	Mean (+SE)* EAG response				F=***	P	HSD
		Spring	Summer	Fall	Overwintered			
Female	(*E*,*E*)-α-farnesene	0.49(0.02)a**	0.36(0.03)b	0.47(0.03)a	0.27(0.03)c	$_{3,216}$=33	<0.0001	0.02
	(*E*)-2-hexenal	0.40(0.03)b	0.39(0.03)b	0.40(0.03)b	0.55(0.05)a	$_{3,210}$=9	<0.0001	0.04
	Linalool	0.69(0.04)b	0.49(0.03)c	0.46(0.03)c	0.94(0.04)a	$_{3,228}$=64	<0.0001	0.03
	Methyl Salicylate	0.70(0.03)b	0.48(0.02)c	0.42(0.02)c	0.90(0.04)a	$_{3,228}$=110	<0.0001	0.02
	β-ocimene	0.52(0.03)a	0.34(0.03)bc	0.40(0.03)b	0.32(0.03)c	$_{3,234}$=18	<0.0001	0.02
Male	(*E*,*E*)-α-farnesene	0.68(0.01)a	0.48(0.03)b	0.36(0.02)c	0.44(0.03)b	$_{3,216}$=43	<0.0001	0.02
	(*E*)-2-hexenal	0.54(0.03)a	0.43(0.04)b	0.23(0.02)c	0.57(0.04)a	$_{3,210}$=50	<0.0001	0.02
	Linalool	0.52(0.03)b	0.49(0.03)b	0.36(0.03)c	0.67(0.04)a	$_{3,216}$=27	<0.0001	0.03
	Methyl Salicylate	0.84(0.03)a	0.49(0.03)b	0.36(0.02)c	0.74(0.03)a	$_{3,240}$=41	<0.0001	0.04
	β-ocimene	0.44(0.02)a	0.40(0.03)ab	0.34(0.03)ab	0.23(0.03)c	$_{3,234}$=26	<0.0001	0.02

*Means (+SE) are in mV that were back-transformed. **Different letters indicate significantly different EAG responses among seasons within the same row, based on results from HSD post-hoc test. ***F- statistics and P-values are from repeated measures ANOVAs conducted separately for each host volatile.

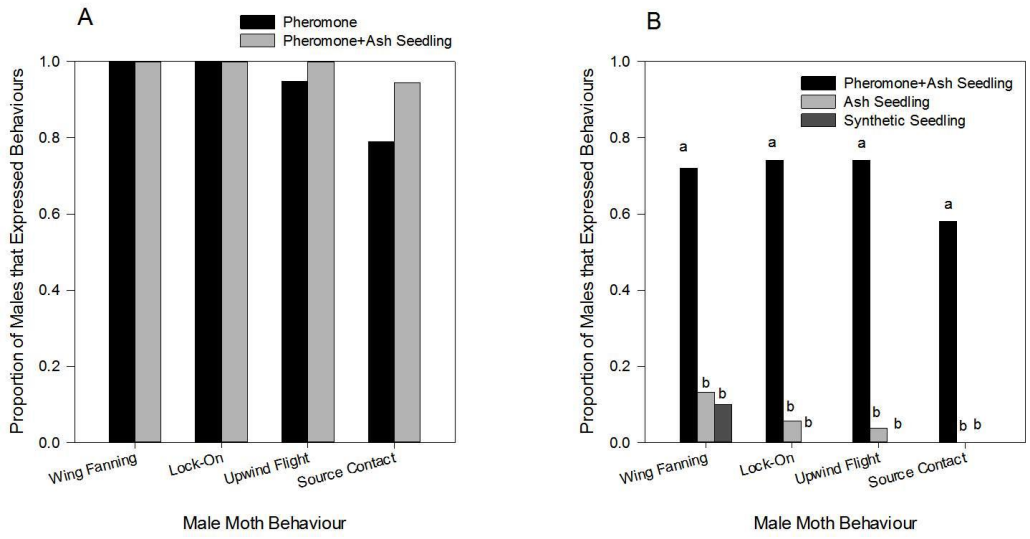


Fig. 4-1. Behavioural response of reproductively active male *C. fraxinella* in the spring to different semiochemical odour sources. (A) Odour source did not impact behavioural response: wing fanning (X^2_1=0, P=1), lock-on (X^2_1=0, P=1), upwind flight (X^2_1=1.36, P=0.2) and source contact (X^2_1=2.03, P=0.2). (B) Odour source did impact behavioural response: wing fanning (X^2_2=57.17, P<0.0001), lock-on (X^2_2=95.45, P<0.0001), upwind flight (X^2_2=99.37, P<0.0001) and source contact (X^2_2=80.55, P<0.0001). Different letters indicate significant differences between responses to odours sources within each behaviour (Tukey P≤0.05).

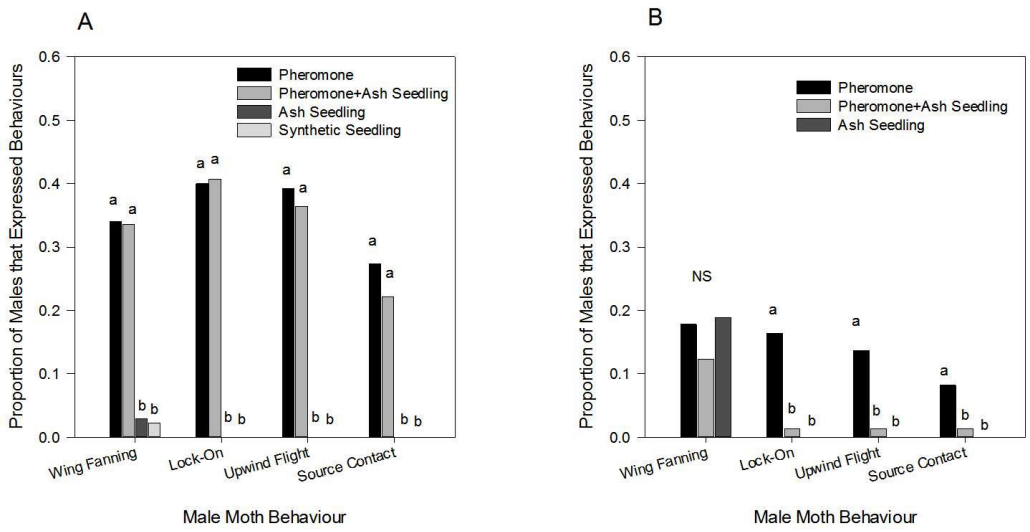


Fig. 4-2. Behavioural response of male *C. fraxinella* in reproductive diapause to different semiochemical odour sources in the (A) summer and (B) fall. (A) Odour source impacted male behavioural response in the summer: wing fanning (X^2_3=102.45, P<0.0001), lock-on (X^2_3=179.55, P<0.0001), upwind flight (X^2_3=166.35, P<0.0001) and source contact (X^2_3=103.32, P<0.0001). (B) In the fall, wing fanning was not impacted by odour source (X^2_2=1.38, P=0.5), but odour source did impact lock-on (X^2_2=22.95, P<0.0001), upwind flight (X^2_2=18.46, P<0.0001) and source contact (X^2_2=10, P<0.0001) behaviours. Different letters indicate significant differences between responses to odour sources within each behaviour (Tukey P≤0.05).

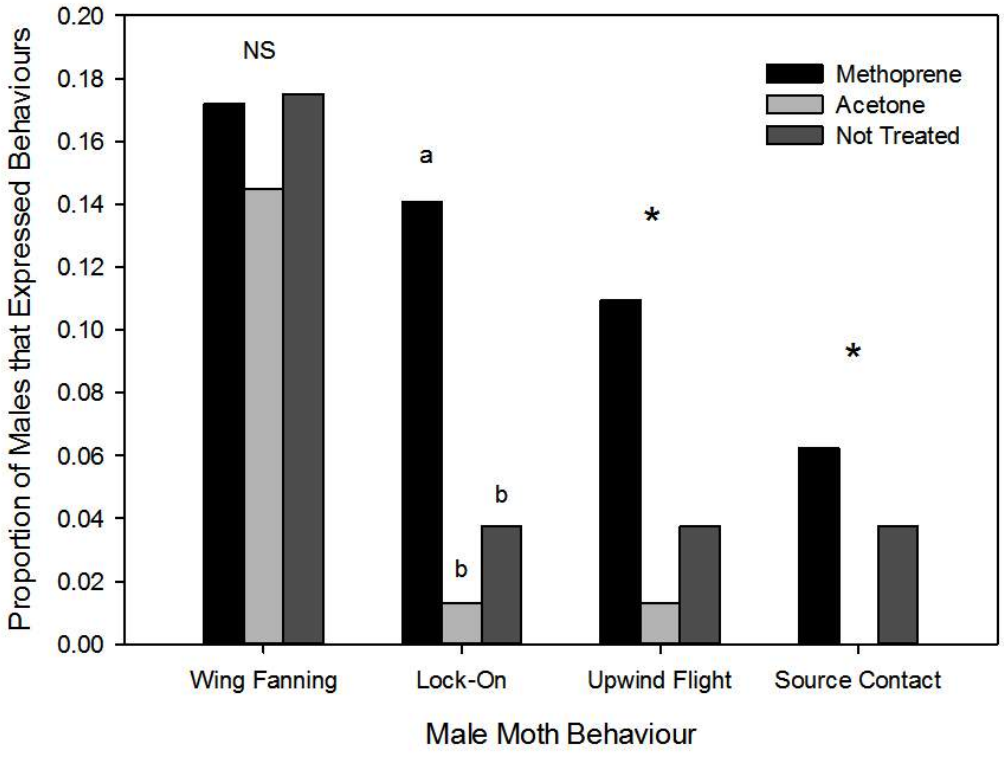


Fig. 4-3. Hormone treatment impacted male *C. fraxinella* response to different odour sources in the wind tunnel in the fall. Wing fanning was not impacted by hormone treatment (X^2_2=0.32, P=0.9). Hormone treatment did impact lock-on (X^2_2=11.46, P=0.003), upwind flight (X^2_2=7.35, P=0.03) and source contact (X^2_2=6.79, P=0.03) behaviours. For lock-on, different letters indicate significant differences between treatments (Tukey: P≤0.08). * Indicates a significant model for this behaviour but no significance was detected in the Tukey test.

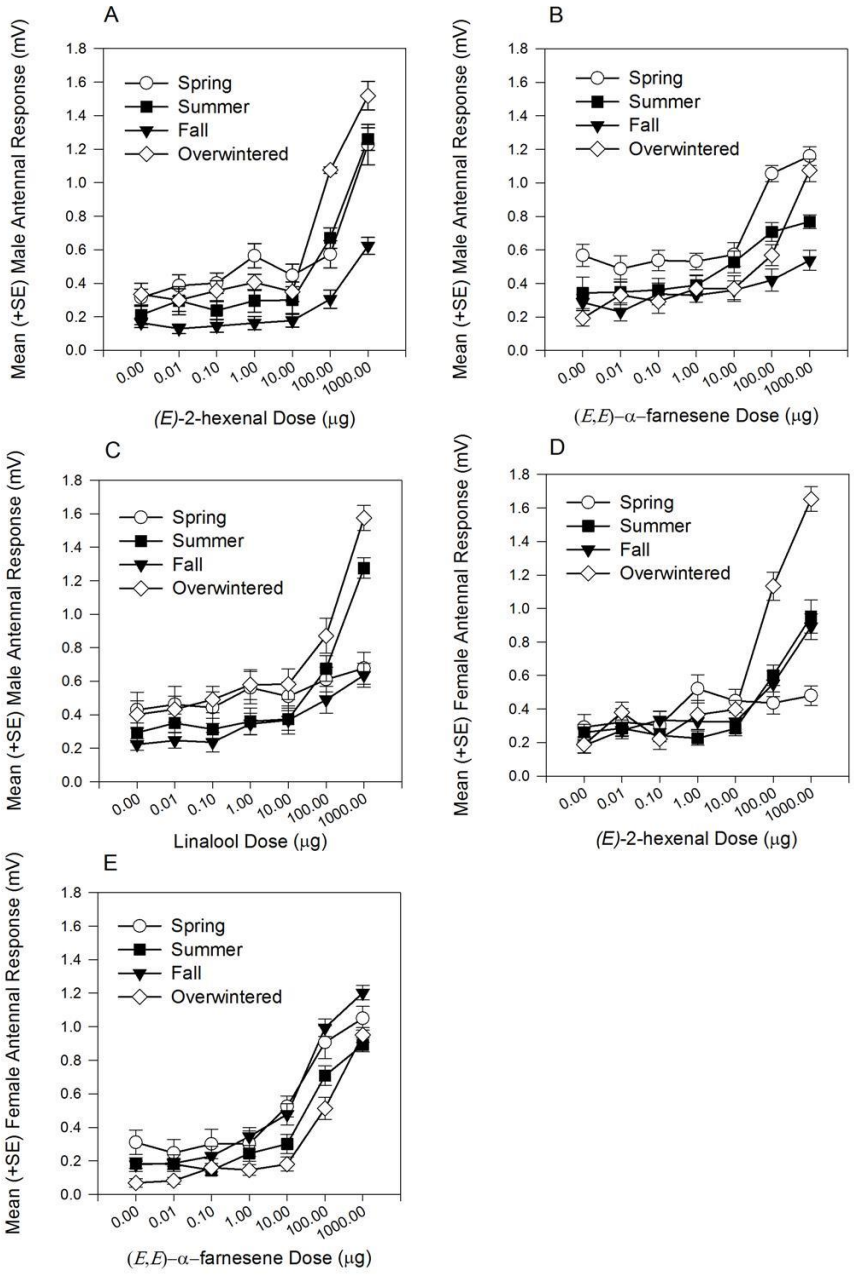


Fig. 4-4. Significant interactions between the season in which *C. fraxinella* moths were tested and host plant volatile dose impacted moth antennal responses to host plant volatiles. Male antennal response was impacted when tested to (A) (*E)*-2-hexenal ($F_{18,210}$=3.1, P<0.0001), (B) (*E,E*)-α-farnesene ($F_{18,216}$=2.9, P<0.0001), and (C) linalool ($F_{18,216}$=3.5, P<0.0001). Female antennal response was impacted

when tested to (D) (*E*)-2-hexenal ($F_{18,210}$=6.9, P<0.0001) and (E) (*E*,*E*)-α-farnesene ($F_{18,216}$=1.7, P=0.03).

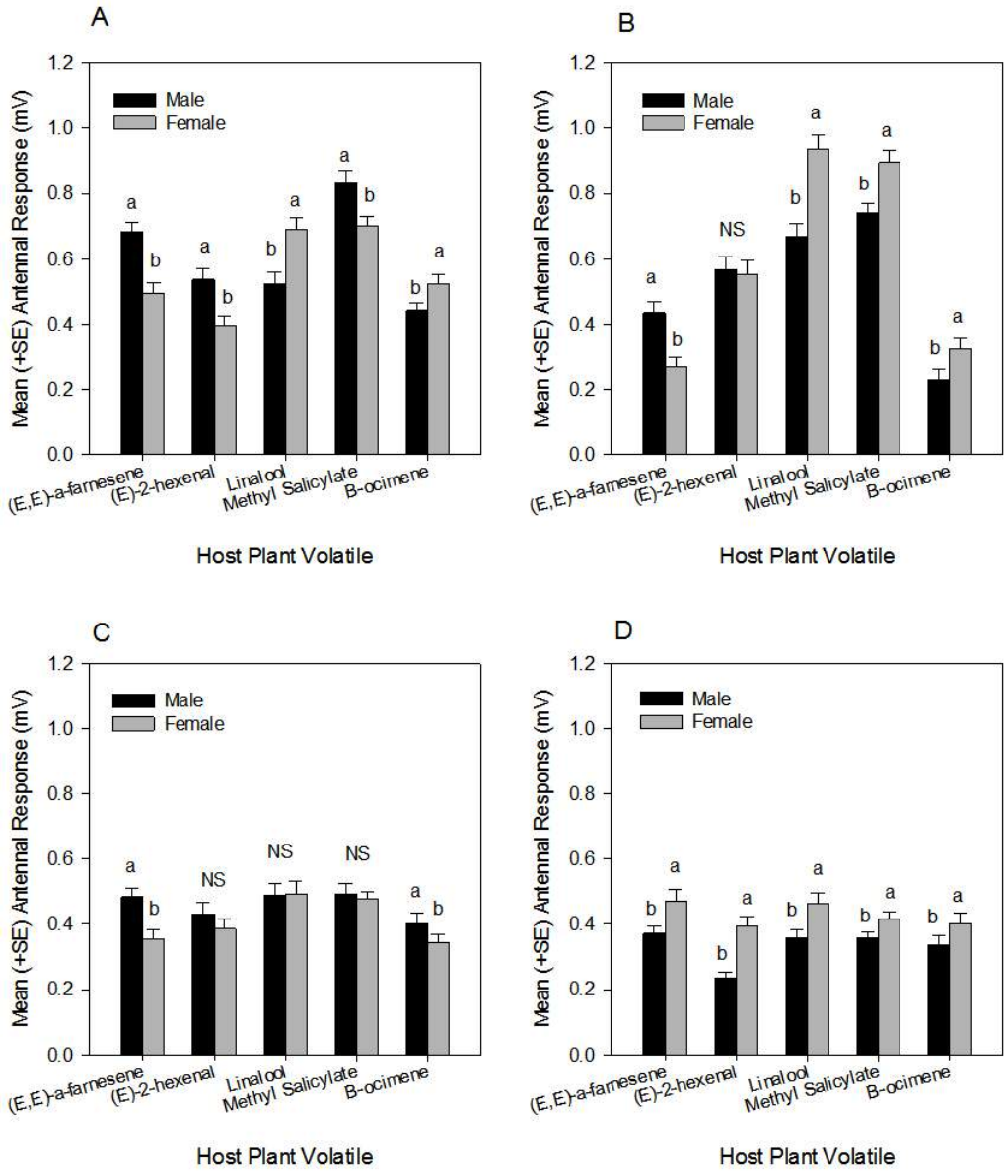


Fig. 4-5. Moth sex impacted *C. fraxinella* antennal response to host plant volatiles when moths were reproductively active in the (A) spring and (B) after experiencing winter conditions in the laboratory, and in reproductive diapause in the (C) summer and (D) fall. Antennal responses are the mean response of all antennae tested to all doses of the host volatile tested (0 μg-1000 μg). (A) In the spring, sex impacted antennal responses to all of the volatiles: (*E*,*E*)-α-farnesene ($F_{1,114}$=32.3, P<0.0001), (*E*)-2-hexenal ($F_{1,108}$=14.6, P=0.0002), linalool

($F_{1,108}$=13.0, P=0.0005), methyl salicylate ($F_{1,108}$=17.3, P<0.0001) and β-ocimene ($F_{1,114}$=8.1, P=0.005). (B) When tested after experiencing winter conditions in the laboratory, sex impacted antennal responses to (*E*,*E*)-α-farnesene ($F_{1,114}$=37.1, P<0.0001), linalool ($F_{1,102}$=35.5, P<0.0001), methyl salicylate ($F_{1,90}$=12.8, P=0.0006) and β-ocimene ($F_{1,114}$=8.9, P=0.004), but not to (*E*)-2-hexenal ($F_{1,90}$=0.15, P=0.7). (C) In the summer, sex impacted response to (*E*,*E*)-α-farnesene ($F_{1,108}$=27.5, P<0.0001) and β-ocimene ($F_{1,114}$=5.4, P=0.02), but not to (*E*)-2-hexenal ($F_{1,114}$=2.2, P=0.1), linalool ($F_{1,114}$=0.01, P=0.9) or methyl salicylate ($F_{1,114}$=0.3, P=0.6). (D) In the fall, sex impacted response to all of the host plant volatiles: (*E*,*E*)-α-farnesene ($F_{1,108}$=20.1, P<0.0001), (*E*)-2-hexenal ($F_{1,126}$=50.9, P<0.0001), linalool ($F_{1,114}$=14.8, P=0.0002), β-ocimene ($F_{1,114}$=7.2, P=0.009) and methyl salicylate ($F_{1,114}$=5.2, P=0.02). Different letters indicate significantly different antennal responses between sexes for the individual volatile tested.

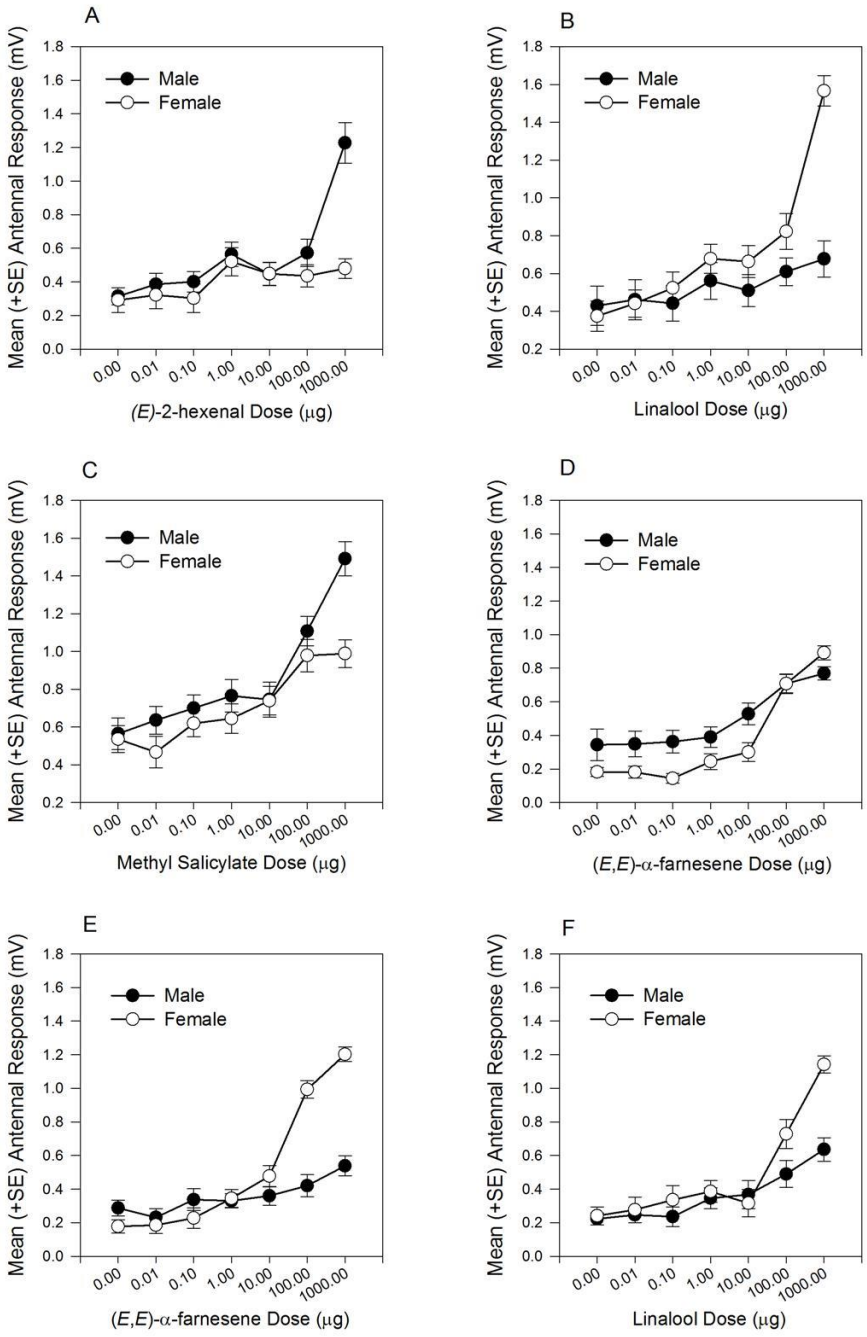


Fig. 4-6. Significant interactions between moth sex and host plant volatile dose impacted antennal response to host plant volatiles in the spring, summer and fall. In the spring, significant interactions impacted response to (A) (*E*)-2-hexenal ($F_{6,108}$=4.7, P=0.0003), (B) linalool ($F_{6,108}$=4.2, P=0.0009) and (C) methyl

salicylate ($F_{6,108}$=2.3, P=0.04). In the summer, significant interactions impacted response to (D) (*E*,*E*)-α-farnesene ($F_{6,108}$=3.8, P=0.002). In the fall, significant interactions impacted response to (E) (*E*,*E*)-α-farnesene ($F_{6,108}$=3.8, P<0.0001) and (F) linalool ($F_{6,114}$=4.0, P=0.001).

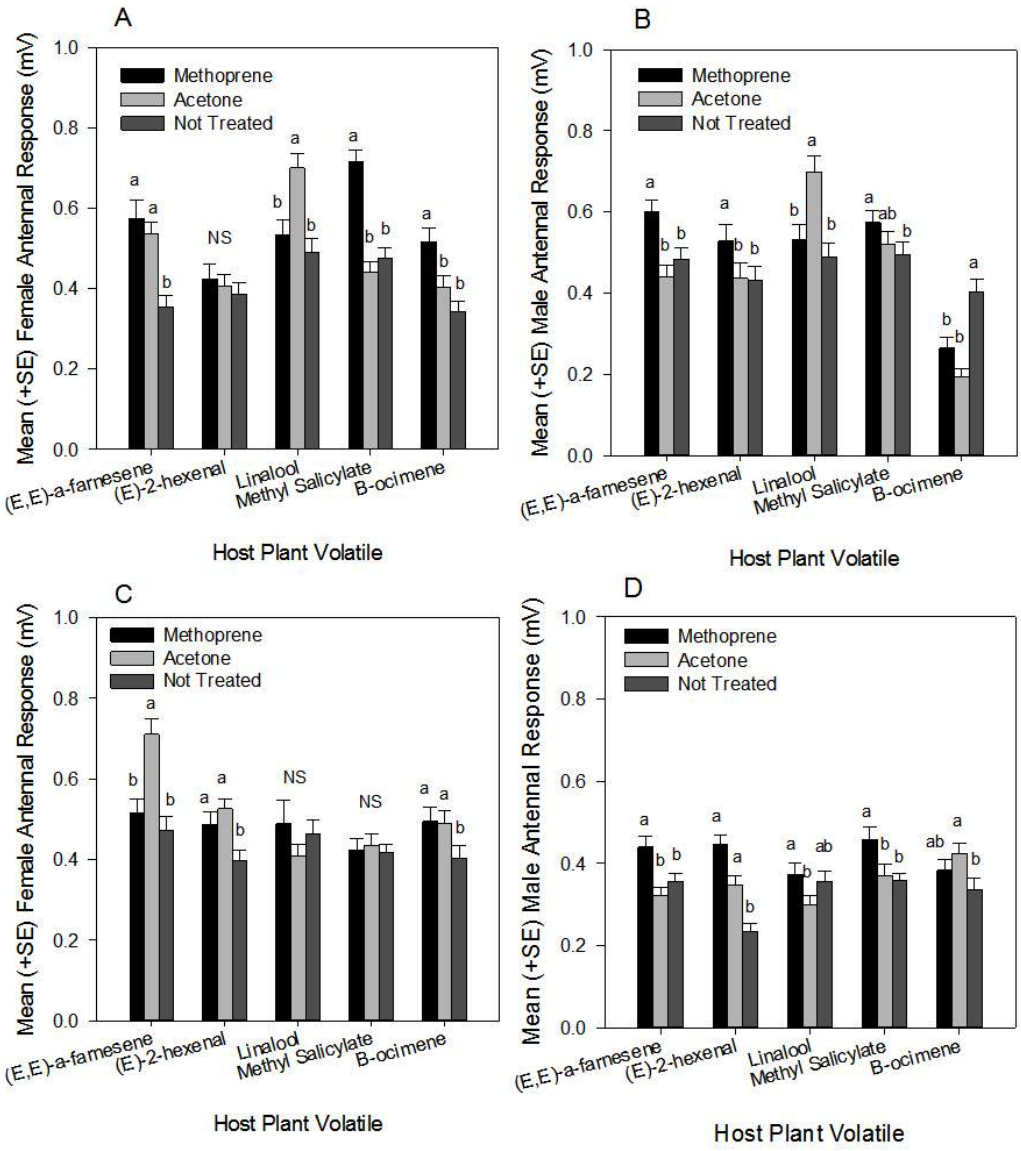


Fig. 4-7. Hormone treatment impacted *C. fraxinella* antennal response to host plant volatiles when moths were in reproductive diapause in the summer to (A) females (B) males, and in the fall to (C) females and (D) males. Antennal responses are the mean response of all antennae tested to all doses of the host volatile tested (0 μg-1000 μg). (A) Hormone treatment impacted female antennal response in the summer to (*E*,*E*)-α-farnesene ($F_{2,150}$=50.1, P<0.0001), linalool ($F_{2,174}$=22.9, P<0.0001), methyl salicylate ($F_{2,174}$=60.0, P<0.0001) and β-

ocimene ($F_{2,174}$=15.0, P<0.0001), but not (*E*)-2-hexenal ($F_{2,168}$=0.7, P=0.5). (B) Hormone treatment impacted male antennal response in the summer to (*E,E*)-α-farnesene ($F_{2,180}$=14.7, P<0.0001), (*E*)-2-hexenal ($F_{2,174}$=6.3, P=0.002), linalool ($F_{2,174}$=22.9, P<0.0001), methyl salicylate ($F_{2,174}$=3.5, P=0.03) and β-ocimene ($F_{2,192}$=21.6, P<0.0001). (C) Hormone treatment impacted female antennal response in the fall to (*E,E*)-α-farnesene ($F_{2,174}$=42.2, P<0.0001), (*E*)-2-hexenal ($F_{2,156}$=12.7, P<0.0001) and β-ocimene ($F_{2,174}$=7.0, P=0.001), but not to linalool ($F_{2,138}$=2.7, P=0.07) or methyl salicylate ($F_{2,168}$=0.2, P=0.8). (D) Hormone treatment impacted male antennal response in the fall to (*E,E*)-α-farnesene ($F_{2,162}$=13.2, P<0.0001), (*E*)-2-hexenal ($F_{2,156}$=12.7, P<0.0001), linalool ($F_{2,180}$=4.8, P=0.009), methyl salicylate ($F_{2,150}$=4.6, P=0.01) and β-ocimene ($F_{2,192}$=3.0, P=0.05). Different letters indicate significant differences between treatments for the individual volatile tested (HSD≤0.05).

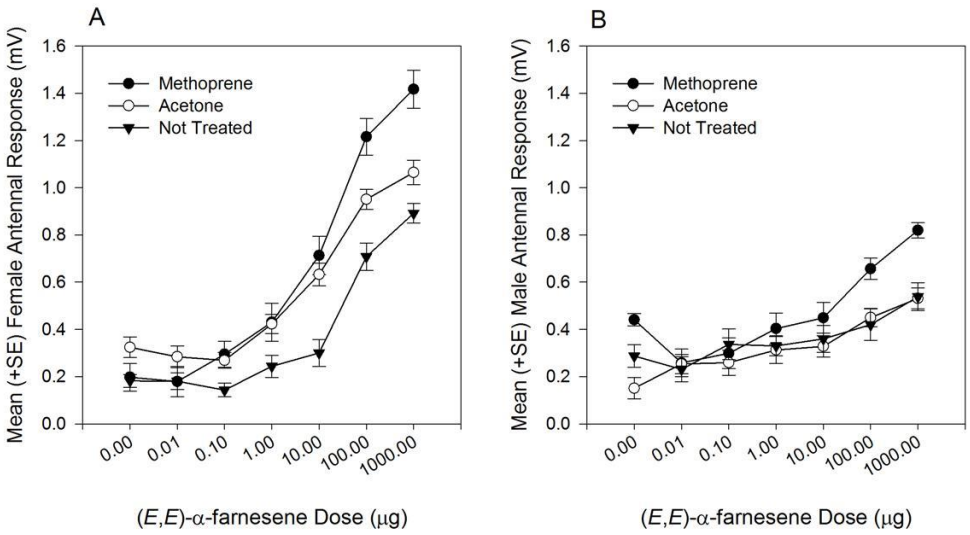


Fig. 4-8. Significant interactions between hormone treatment and (*E,E*)-α-farnesene dose impacted (A) female response in the summer ($F_{12,150}$=4.2, P<0.0001), and (B) male response in the fall ($F_{12,162}$=1.8, P=0.05).

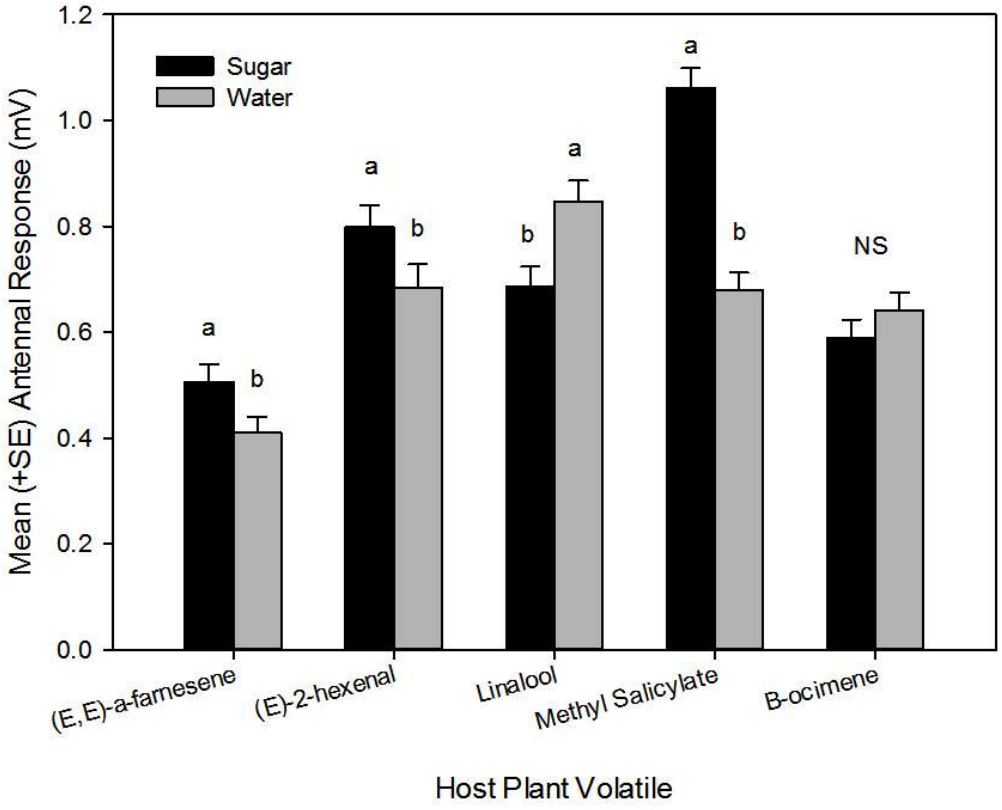


Fig. 4-9. The impact of nutritional status on female *C. fraxinella* antennal responses to host volatiles after experiencing winter conditions in the laboratory. Antennal responses are the mean response of all antennae tested to all doses of the host volatile tested (0 μg-1000 μg). Nutritional status impacted antennal response to (*E*,*E*)-α-farnesene ($F_{1,114}$=5.5, P=0.02), (*E*)-2-hexenal ($F_{1,114}$=6.2, P=0.02), methyl salicylate ($F_{1,114}$=79.9, P<0.0001) and linalool ($F_{1,114}$=12.3, P=0.0007). Nutritional status did not impact female response to β-ocimene ($F_{1,108}$=3.0, P=0.09). Different letters indicate significant differences between treatments for the individual volatile tested.

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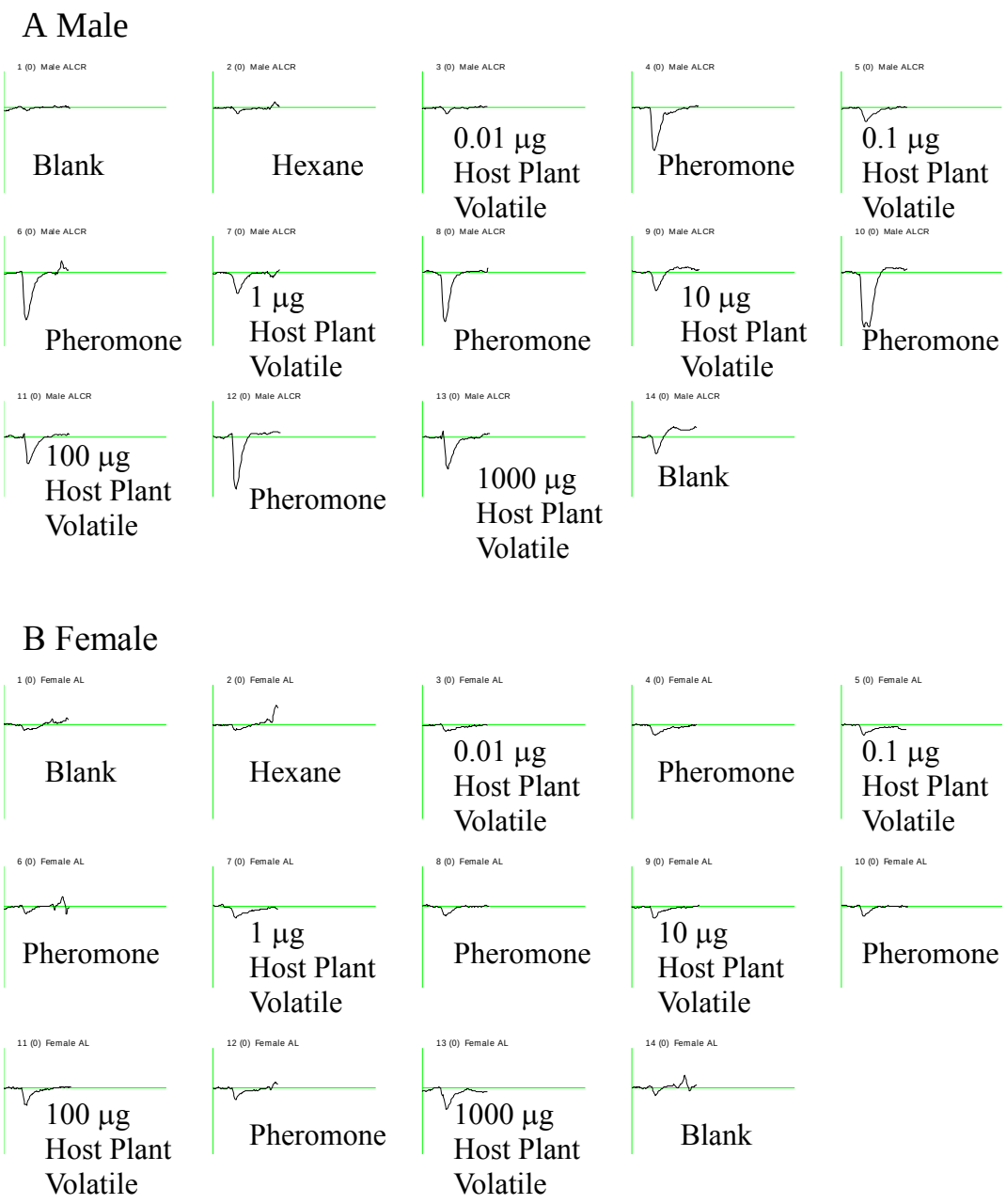
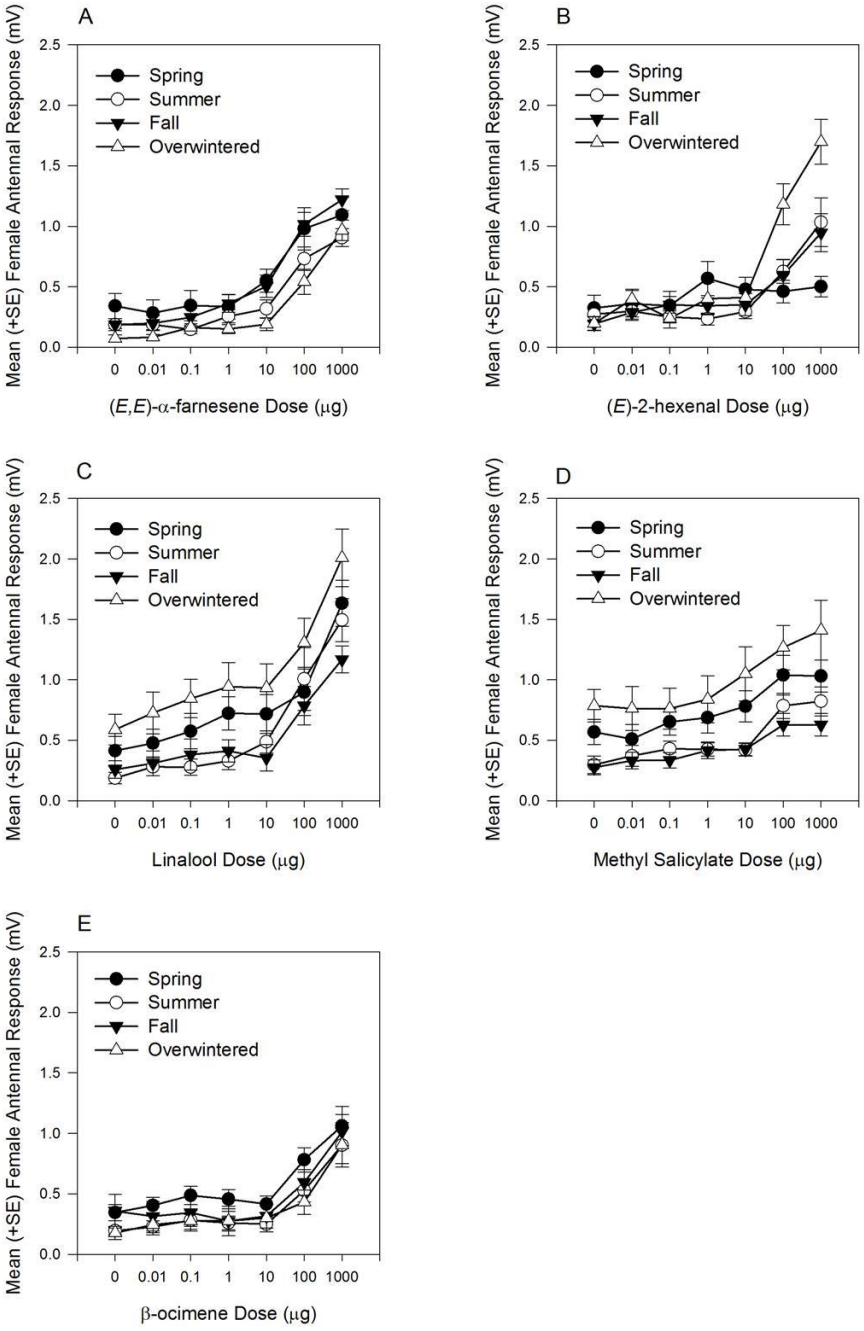
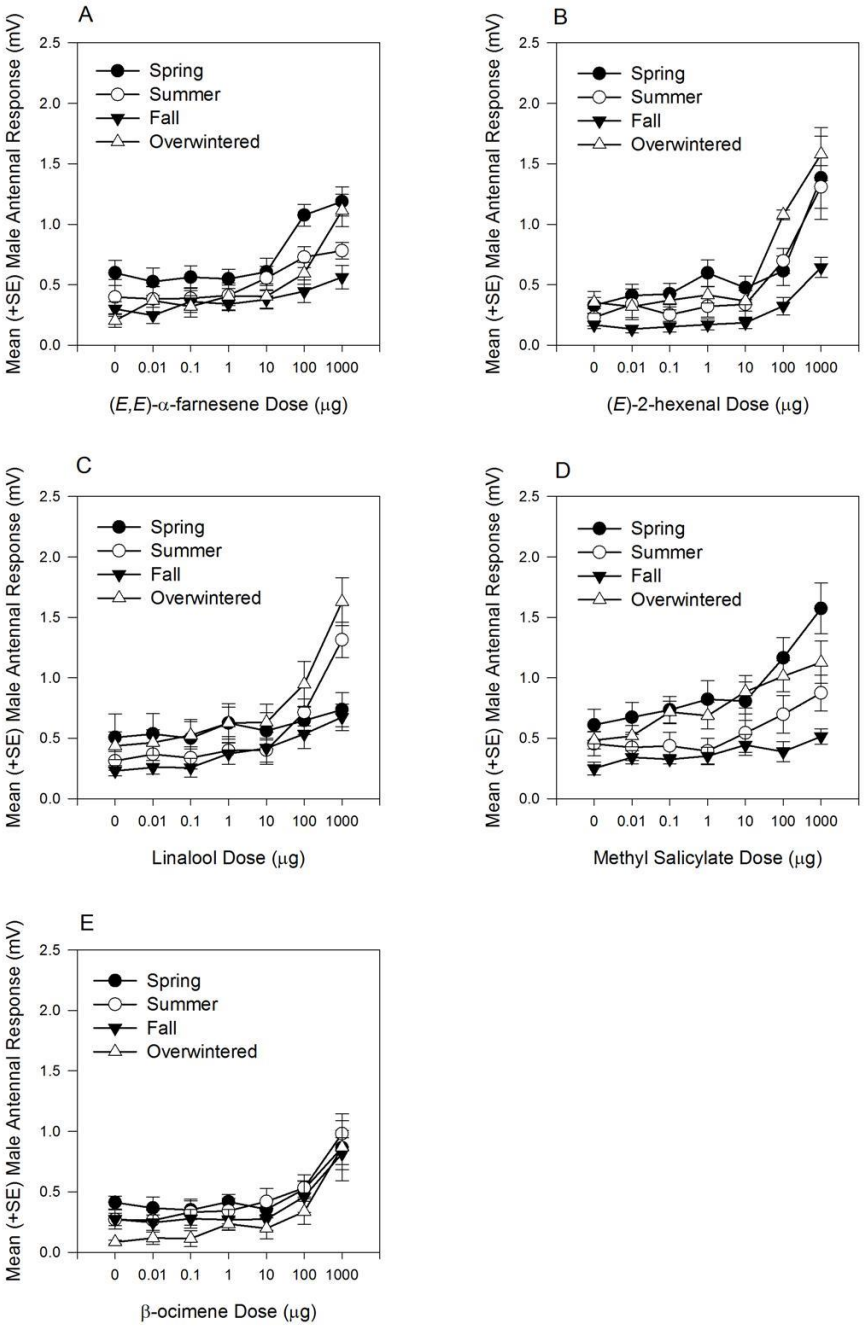


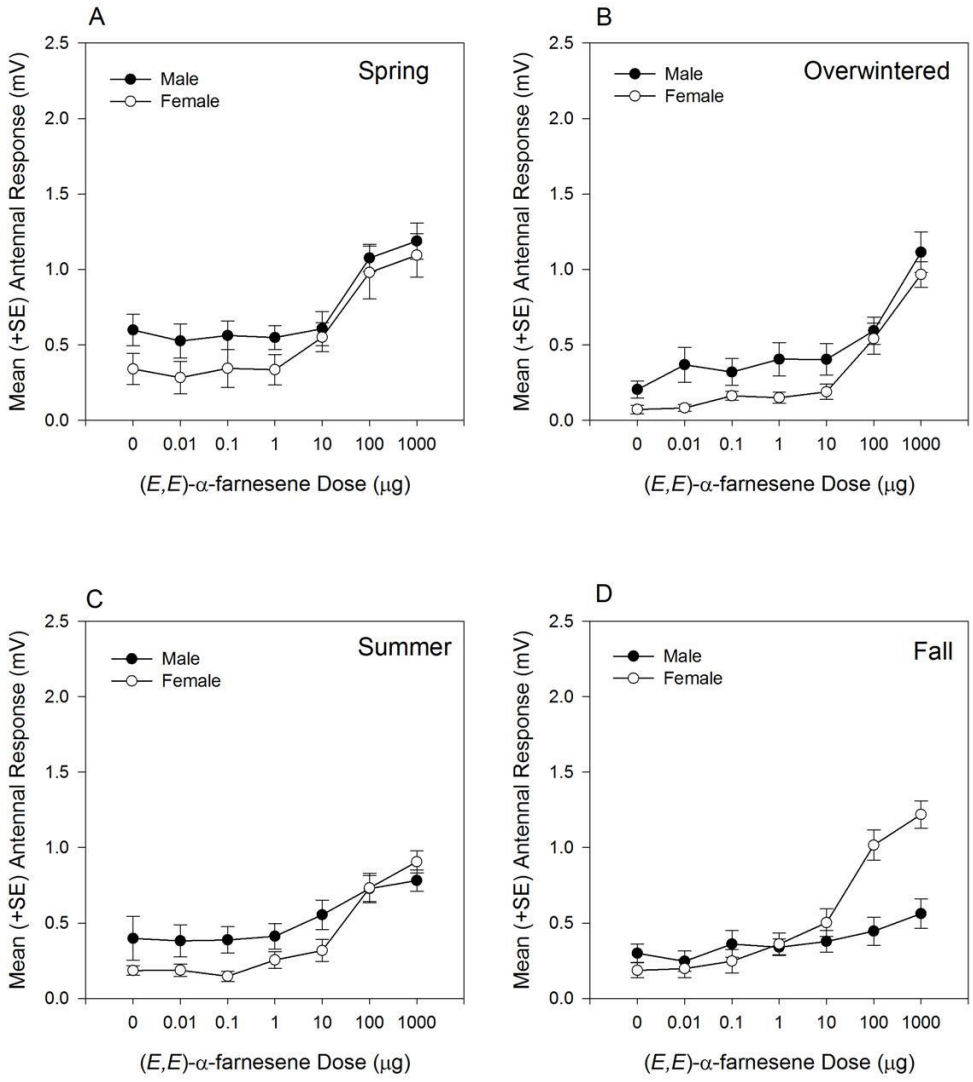
Appendix 4-1. Representative EAG traces from reproductively active, untreated, spring (A) male and (B) female *C. fraxinella* depicting the series of stimuli presented to each antenna. Male and female traces are from EAG responses to methyl salicylate tested on 2 May 2010.



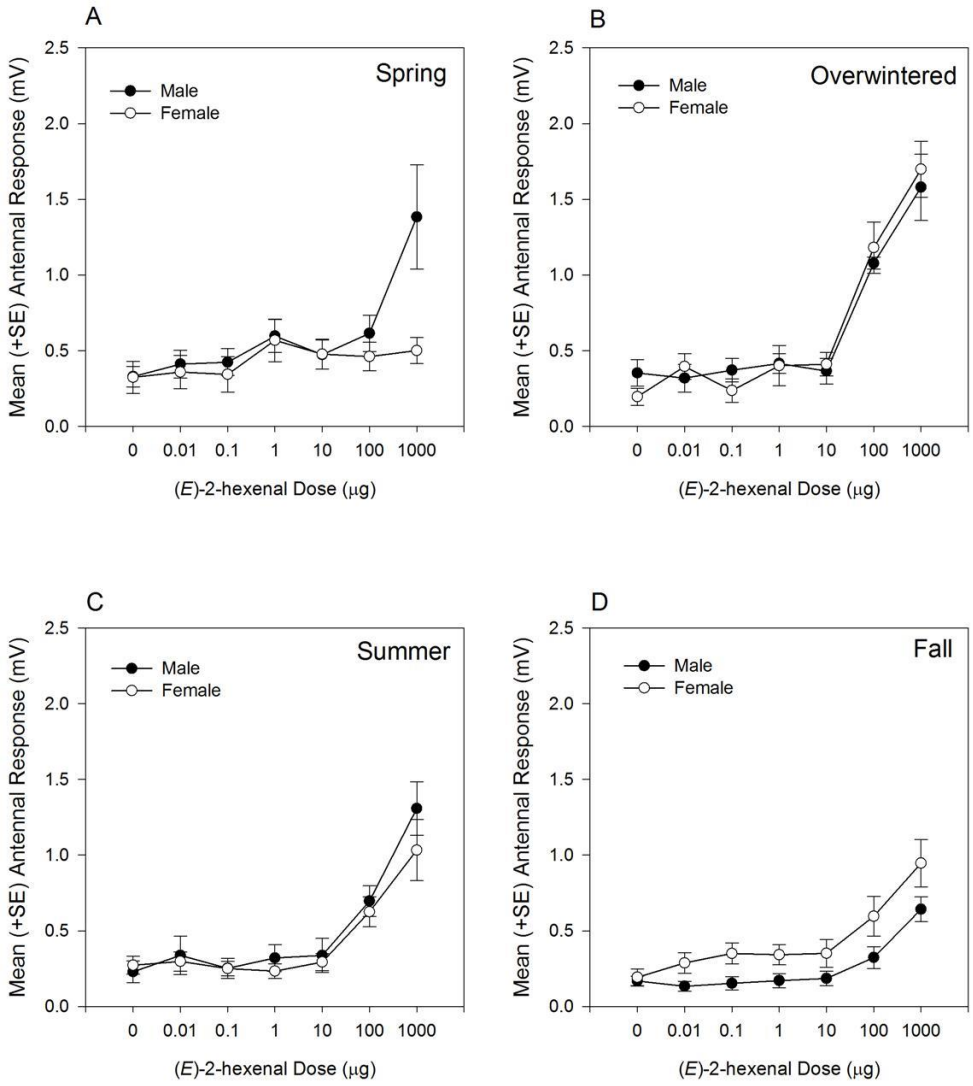
Appendix 4-2. Dose response curves of female *C. fraxinella* EAG response to individual ash host plant volatiles (A-E). Legend describes the different seasons in which females were tested, and all females were untreated. Graph depicts raw EAG responses.



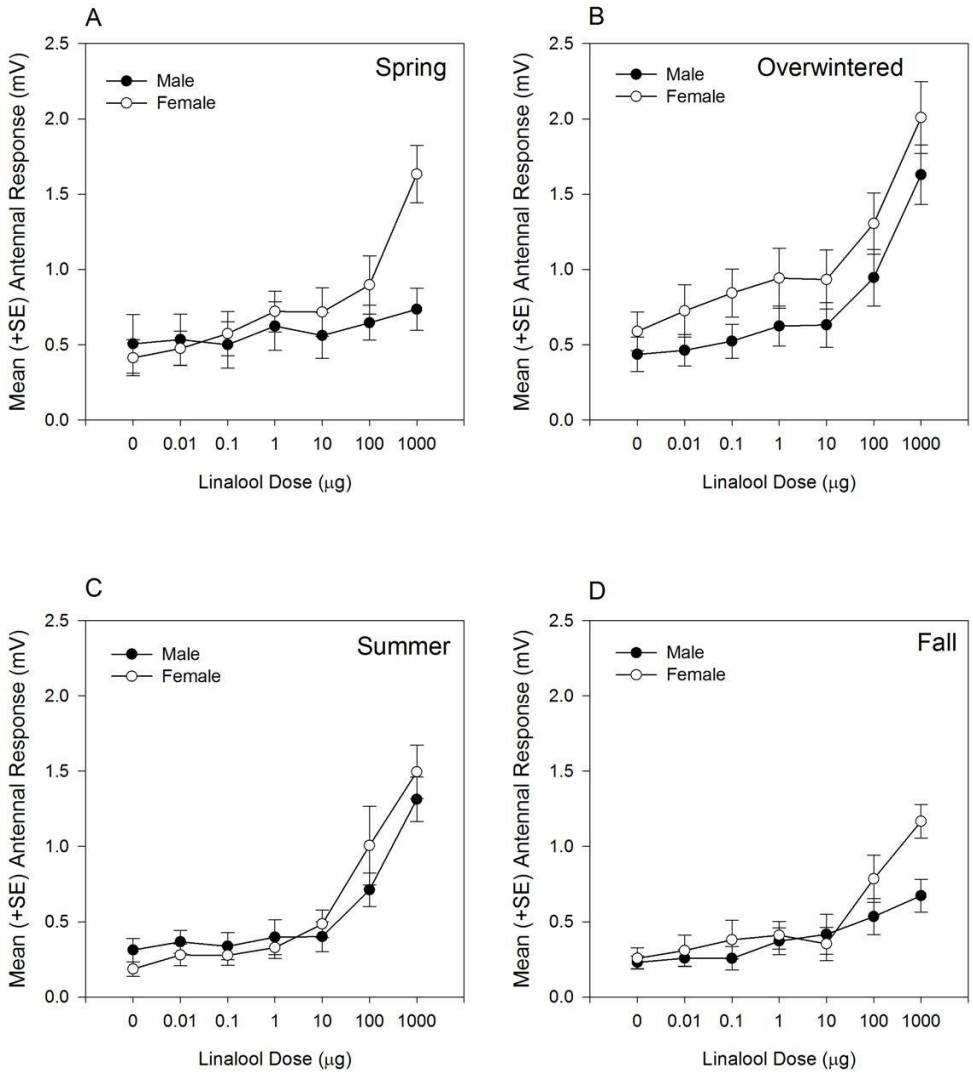
Appendix 4-3. Dose response curves of male *C. fraxinella* EAG response to individual ash host plant volatiles (A-E). Legend describes the different seasons in which males were tested, and all males were untreated. Graph depicts raw EAG responses.



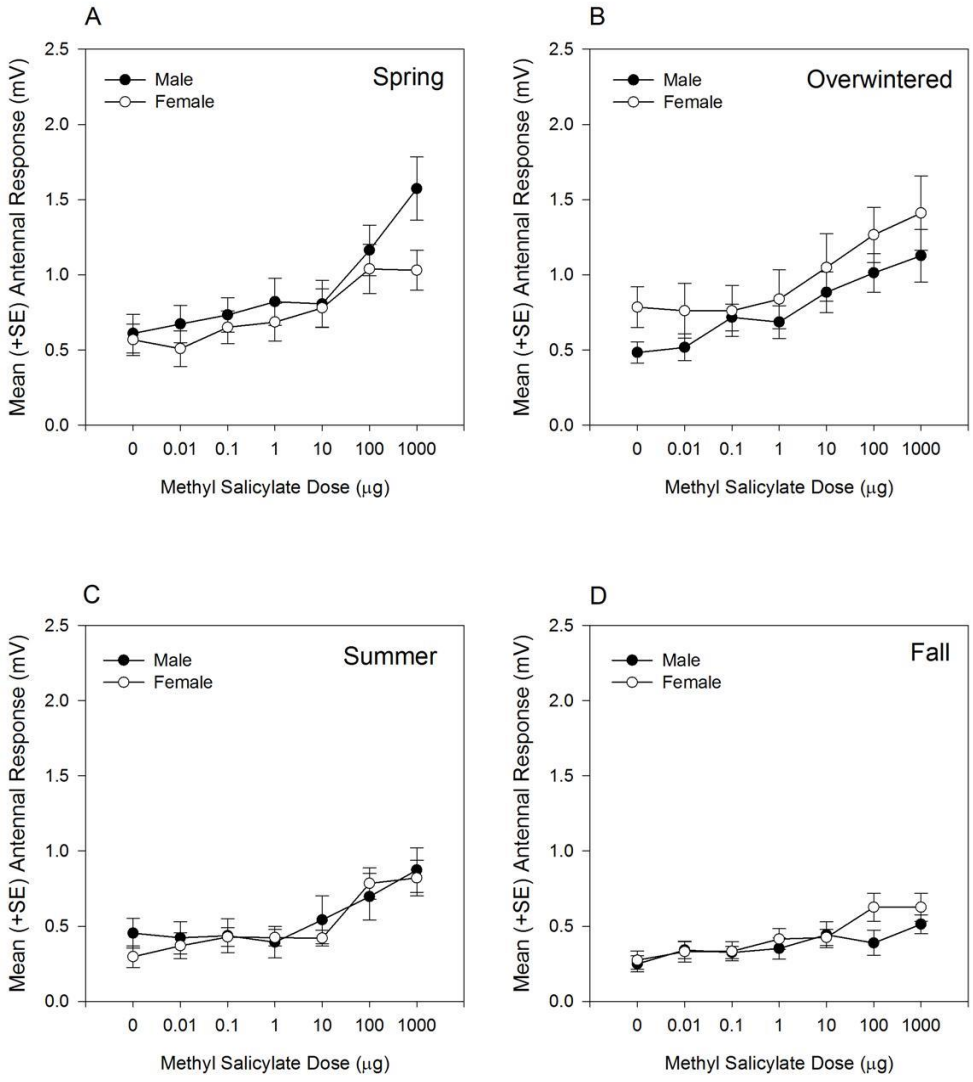
Appendix 4-4. Dose response curves comparing male and female *C. fraxinella* EAG response to (*E*,*E*)-α-farnesene. Moths were tested while reproductively active in (A) spring after overwintering in nature and (B) after overwintering under laboratory conditions, and while in reproductive diapause in the (C) summer and (D) fall. Graphs depict raw EAG data.



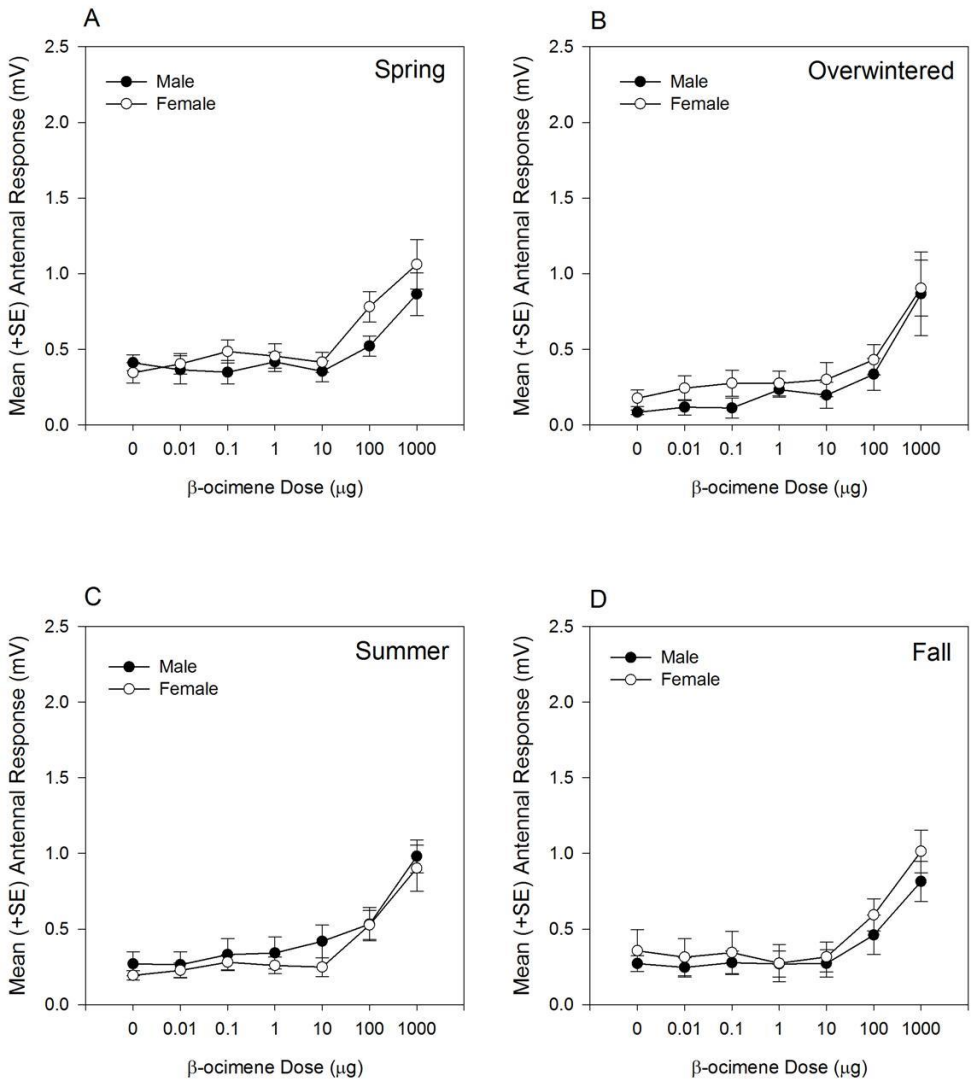
Appendix 4-5. Dose response curves comparing male and female *C. fraxinella* EAG response to (*E*)-2-hexenal. Moths were tested while reproductively active in (A) spring after overwintering in nature and (B) after overwintering under laboratory conditions, and while in reproductive diapause in the (C) summer and (D) fall. Graphs depict raw EAG data.



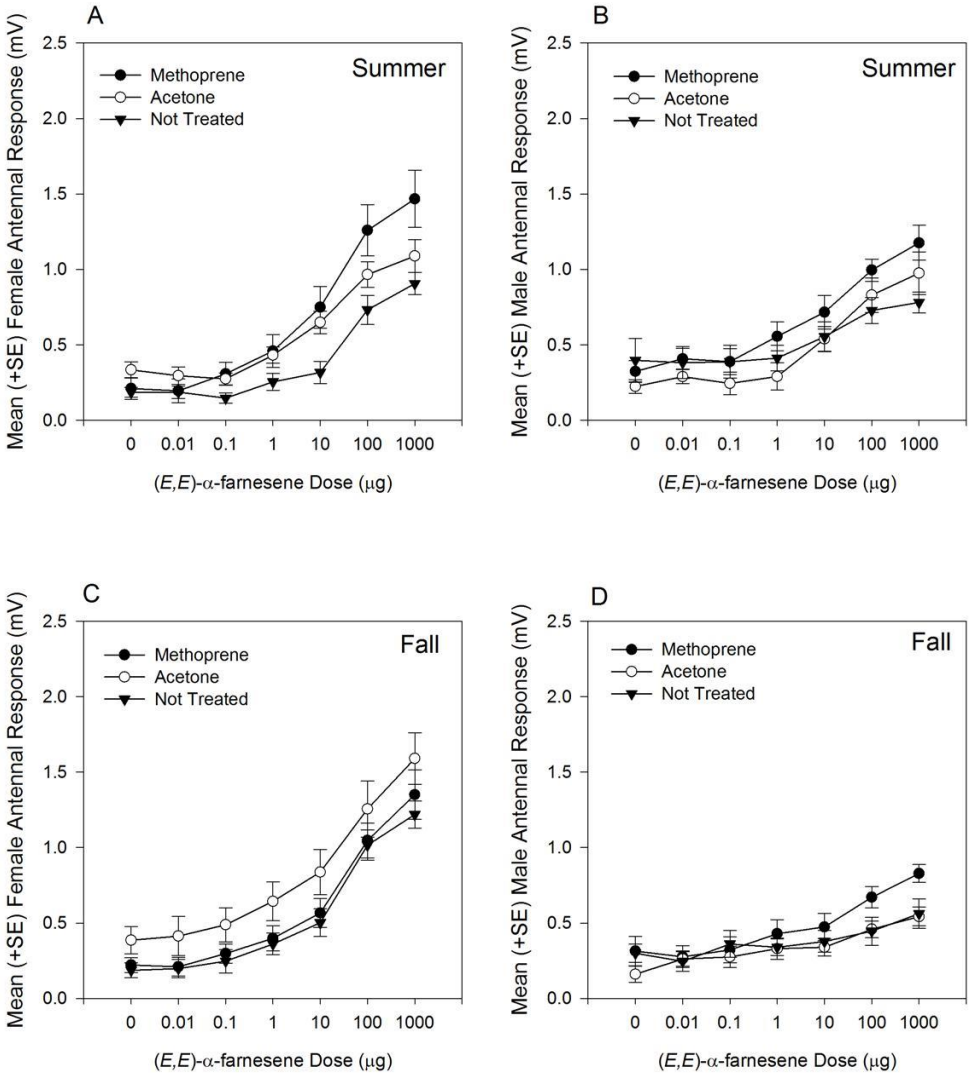
Appendix 4-6. Dose response curves comparing male and female *C. fraxinella* EAG response to linalool. Moths were tested while reproductively active in (A) spring after overwintering in nature and (B) after overwintering under laboratory conditions, and while in reproductive diapause in the (C) summer and (D) fall. Graphs depict raw EAG data.



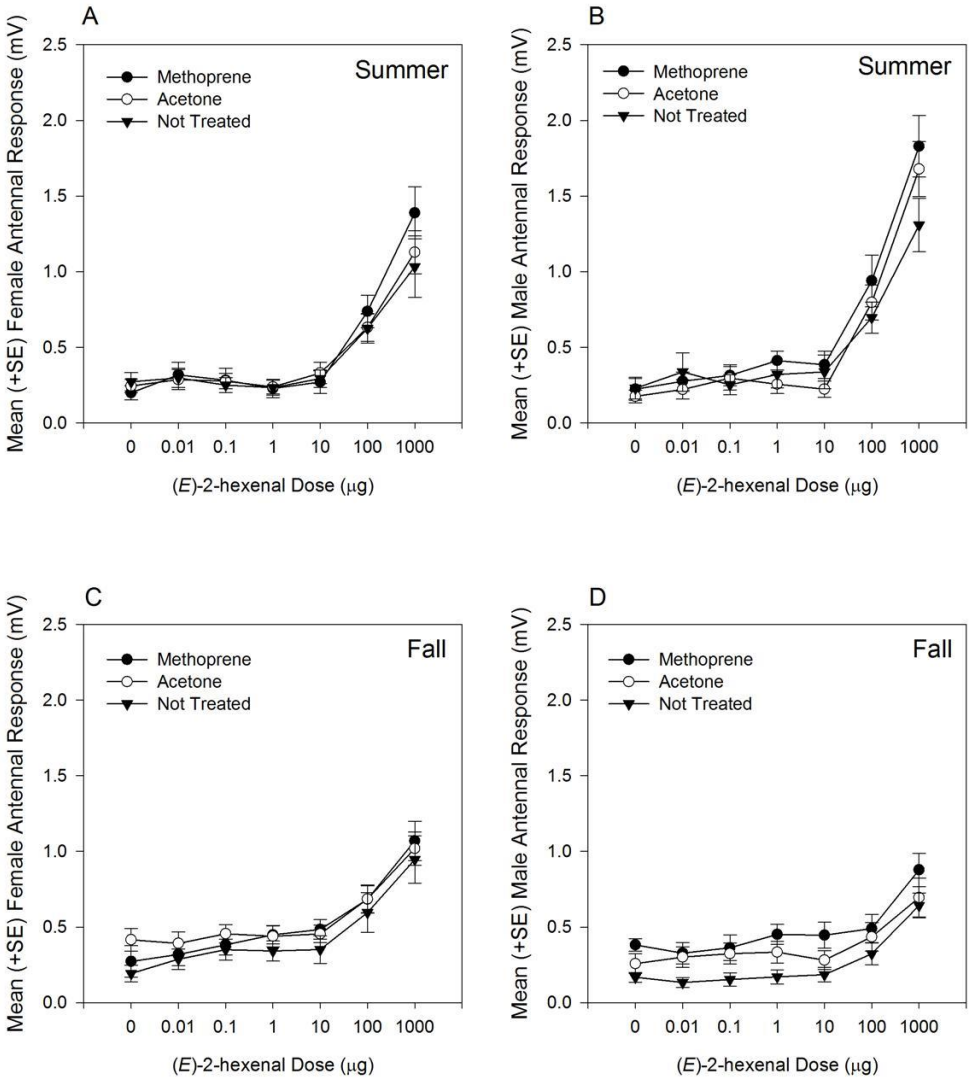
Appendix 4-7. Dose response curves comparing male and female *C. fraxinella* EAG response to methyl salicylate. Moths were tested while reproductively active in (A) spring after overwintering in nature and (B) after overwintering under laboratory conditions, and while in reproductive diapause in the (C) summer and (D) fall. Graphs depict raw EAG data.



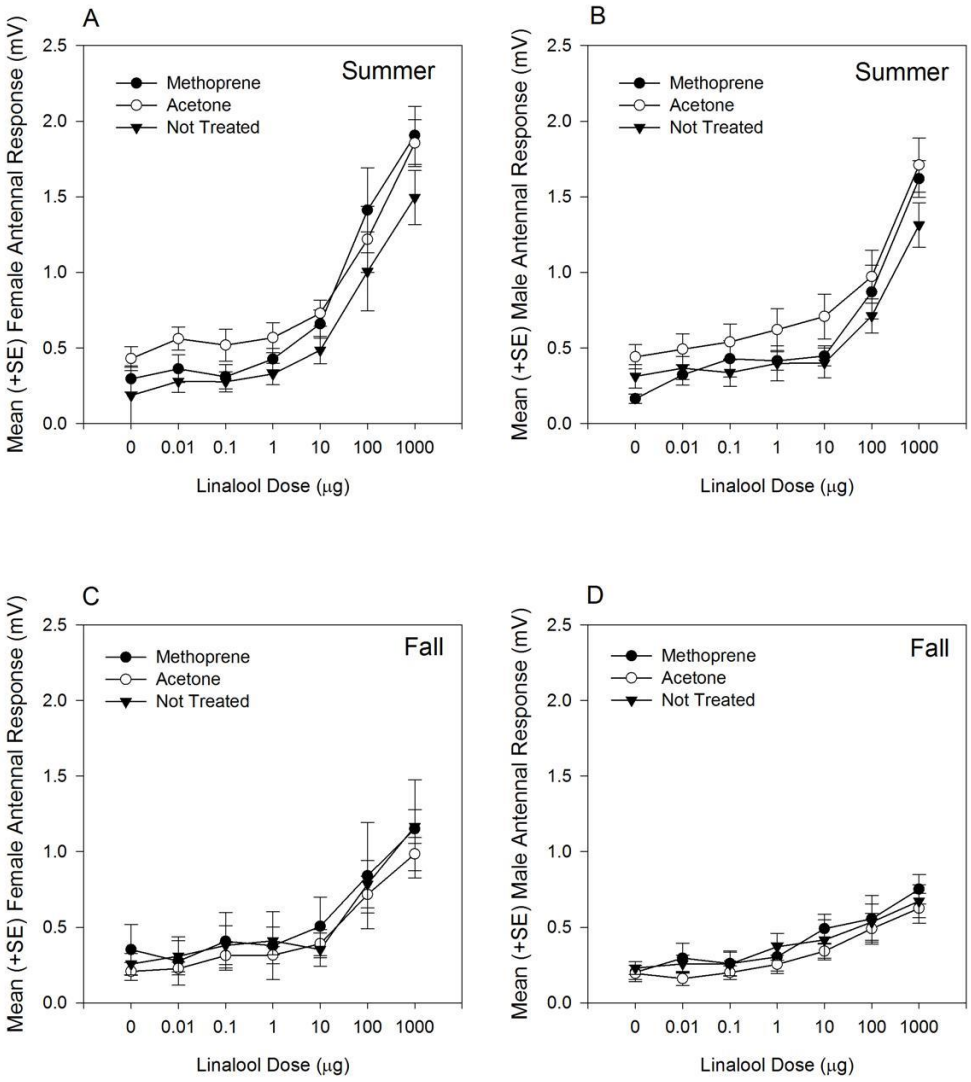
Appendix 4-8. Dose response curves comparing male and female *C. fraxinella* EAG response to β-ocimene. Moths were tested while reproductively active in (A) spring after overwintering in nature and (B) after overwintering under laboratory conditions, and while in reproductive diapause in the (C) summer and (D) fall. Graphs depict raw EAG data.



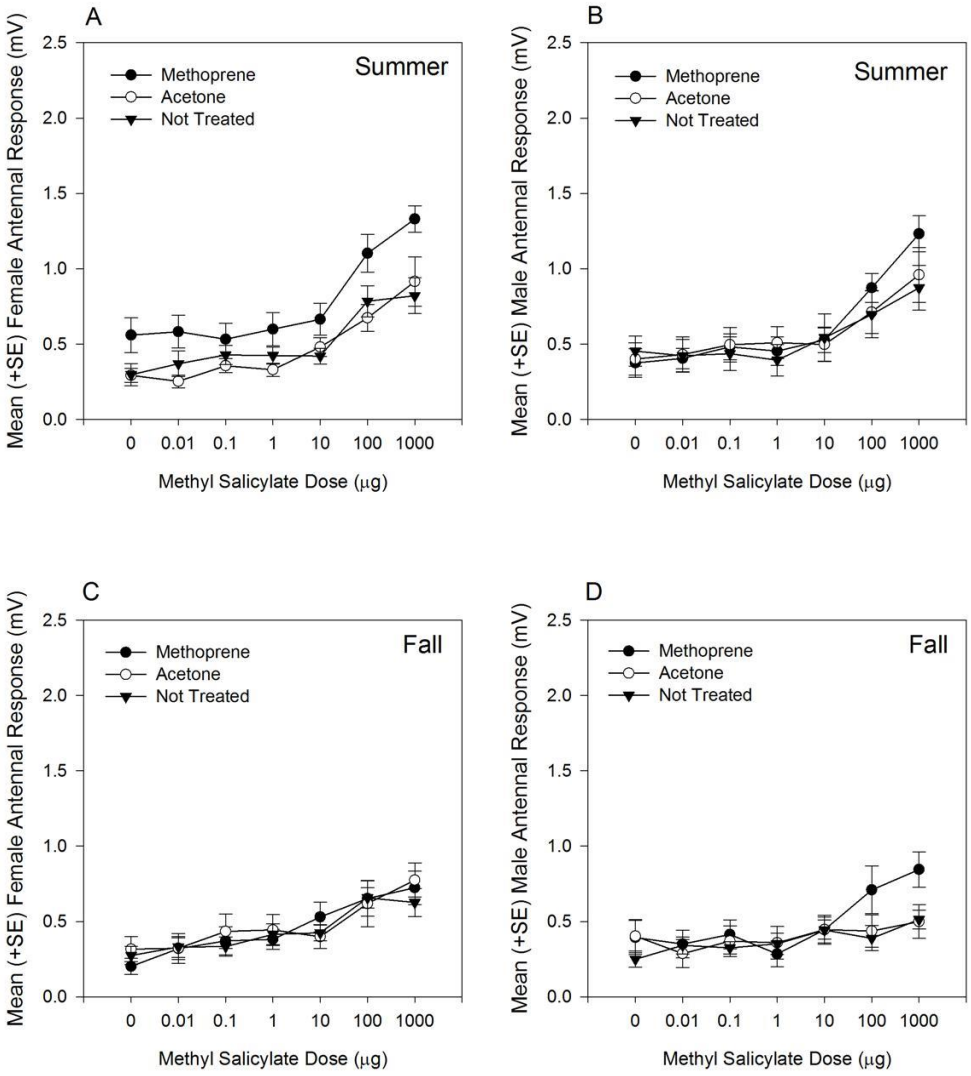
Appendix 4-9. Dose response curves of female and male *C. fraxinella* EAG responses to (*E*,*E*)-α-farnesene when moths were in reproductive diapause in the (A, B) summer and (C, D) fall. Moths were treated with the JHA methoprene, acetone or were untreated. Graphs depict raw EAG data.



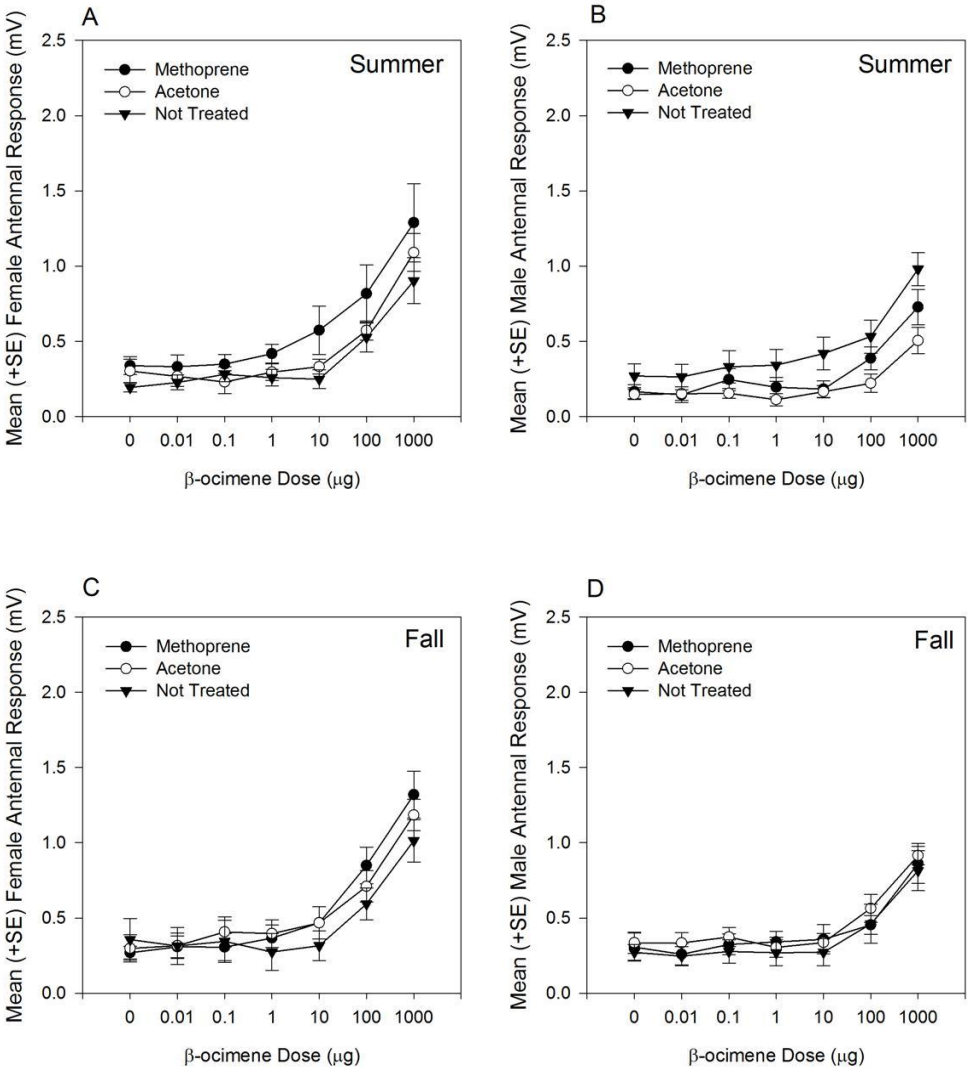
Appendix 4-10. Dose response curves of female and male *C. fraxinella* EAG responses to (*E*)-2-hexenal when moths were in reproductive diapause in the (A, B) summer and (C, D) fall. Moths were treated with the JHA methoprene, acetone or were untreated. Graphs depict raw EAG data.



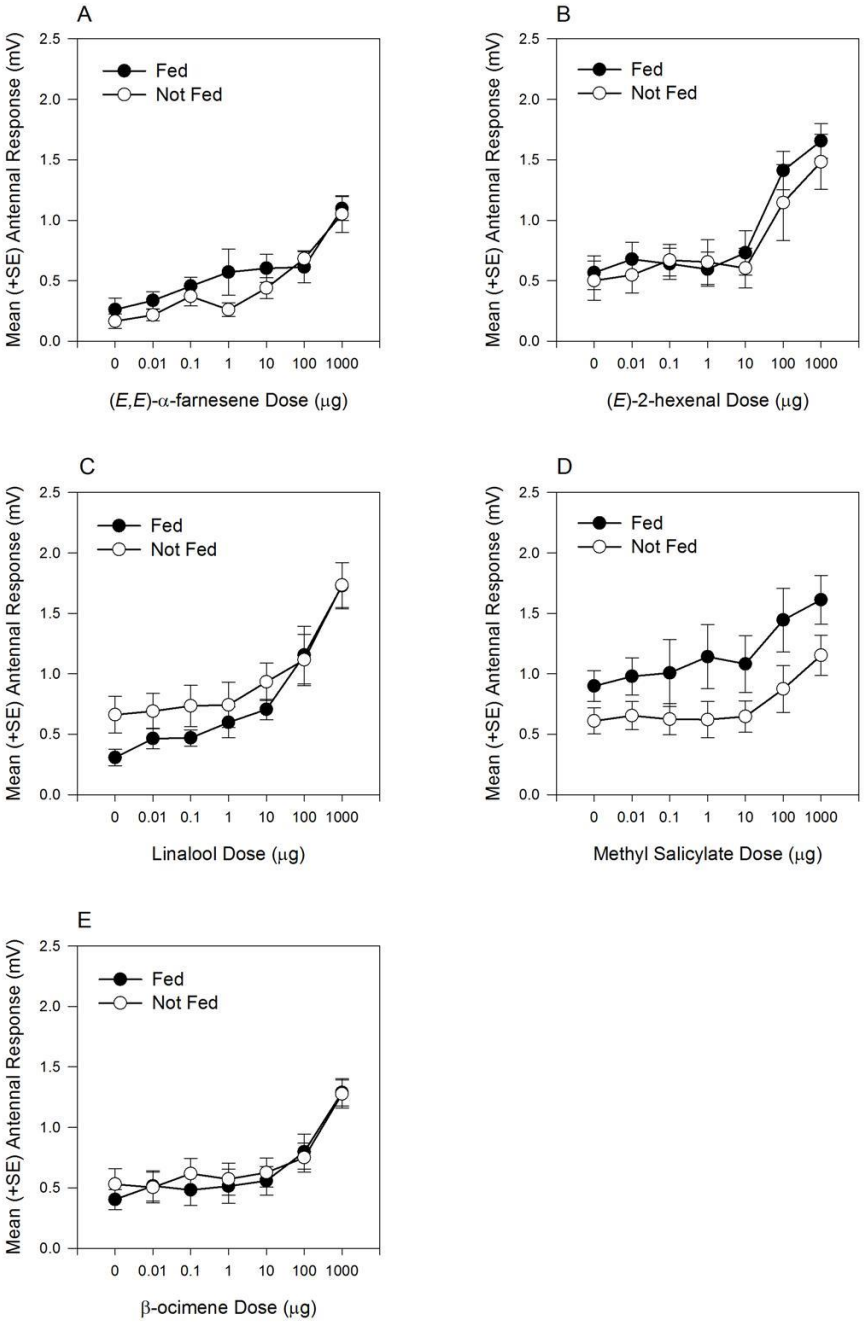
Appendix 4-11. Dose response curves of female and male *C. fraxinella* EAG responses to linalool when moths were in reproductive diapause in the (A, B) summer and (C, D) fall. Moths were treated with the JHA methoprene, acetone or were untreated. Graphs depict raw EAG data.



Appendix 4-12. Dose response curves of female and male *C. fraxinella* EAG responses to methyl salicylate when moths were in reproductive diapause in the (A, B) summer and (C, D) fall. Moths were treated with the JHA methoprene, acetone or were untreated. Graphs depict raw EAG data.



Appendix 4-13. Dose response curves of female and male *C. fraxinella* EAG responses to β-ocimene when moths were in reproductive diapause in the (A, B) summer and (C, D) fall. Moths were treated with the JHA methoprene, acetone or were untreated. Graphs depict raw EAG data.



Appendix 4-14. Dose response curves of female *C. fraxinella* EAG responses to individual ash host plant volatiles (A-E). Females overwintered under laboratory conditions and were provided either 10% sugar solution or water alone for one

week after removal from winter conditions prior to testing. Graphs depict raw EAG responses.

Chapter 5

Size and protein content of accessory glands in adult male *Caloptilia fraxinella* (Lepidoptera: Gracillariidae) in different physiological states

Introduction

Reproductive diapause is an adaptive strategy that prevents reproduction during unfavourable conditions and improves the survival of offspring by timing mating with favourable environmental conditions for offspring development (Denlinger et al. 2005; Macrae 2010; Pener 1992). Reproductive diapause in female insects primarily involves the arrestment of oocyte development and a lack of oviposition (Denlinger et al. 2005; Pener 1992). In male insects, reproductive diapause is diagnosed by an inability of males to locate and mate with a receptive female, and reduced sexual organs, including underdeveloped sex accessory glands (SAG) and sometimes, underdeveloped testes (Denlinger et al. 2005; Pener 1992).

The structure of male SAGs are extremely variable among insects, but are most commonly paired structures that occur as outpockets of the *vas deferens*, seminal vesicles or ejaculatory ducts, containing various types of secretory cells (Leopold 1976). Male SAGs secrete a complex mixture of proteins, carbohydrates and some lipids that make up the seminal fluid that surrounds and protects the spermatozoa, and also the foundations of the spermatophore, all to facilitate the transfer of sperm to females (Happ 1992; Leopold 1976). The spermatophore is a package of sperm and seminal fluids that is transferred from

males to females during mating in many insects (Gillott 2003; Happ 1992). Proteins are the most important constituents in male SAG secretions in terms of abundance and for both male and female reproductive success (Gillott 2003). Some proteins in the spermatophore and associated seminal fluid can significantly impact the reproductive behaviour of post-mated females (Leopold 1976; Gillott 2003) by decreasing female receptivity to re-mating or decreasing female attractiveness to males, and stimulating egg development and oviposition (Chapman et al. 2001; Dottorini et al. 2013; Gillott 2003; Kubli 2003; Leopold 1976; Shutt et al 2010; Wolfner, 1997). The spermatophore may also contain nutritional (Leopold 1976) or antimicrobial (Gillott 2003) components that are beneficial to the female. Male SAG size is an indication of reproductive capability and maturity in some insects, and the larger the SAGs (Baker et al. 2003; Bangham et al 2002) and the greater the content of SAG proteins (Braswell et al. 2006) the higher the fertilization success of males.

Juvenile hormone (JH) regulates insect growth and development at all life stages, and is an important gonadotropic hormone in many adult Lepidoptera (Ramaswamy et al. 1997; Minakuchi and Riddiford 2006). It is secreted by the *corpora allata* in the insect brain, and is the main hormone that controls induction or termination of reproductive diapause in insects (Denlinger et al. 2005; Kopper et al. 2001; Shiga et al. 2003). In some male Lepidoptera, the *corpora allata* lack the JH acid methyltransferase required to convert JH acid into JH, and so the *corpora allata* secrete JH acids rather than JH (Bhaskaran et al. 1988; Cusson et al. 1993; Ho et al. 1995; Peter et al. 1981). In several lepidopterans, including

Manduca sexta (Lepidoptera: Sphingidae), *Heliothis zea* (Lepidoptera: Noctuidae) and *Vanessa cardui* (Lepidoptera: Nymphalidae), male SAGs contain JH acid methyltransferase, which converts JH acids into JH (Bhaskharan et al. 1988). In *Heliothis virescens* (Lepidoptera: Noctuidae), JH is transferred from males to females during mating, where it stimulates yolk production and uptake into maturing oocytes and also stimulates the female *corpora allata* to synthesize endogenous JH (Gillott 2003; Park et al. 1998).

In lepidopterans that exhibit delayed mating, JH regulates several aspects of male reproductive activity, including SAG development (Gillott 1996; Gillott and Gaines 1992) and male sex pheromone response (Cusson et al. 1993; Duportets et al. 1996; Ramaswamy et al. 1997; Duportets et al. 1998; Denlinger 2002; Anton et al. 2007). Juvenile hormone impacts the growth and accumulation of secretions in SAGs and also the synthesis of specific proteins in SAGs of male insects (Gillott and Gaines 1992). Ecdysteroids are also found in the reproductive tract of adult male insects, and are known to regulate SAG development and differentiation (Duportets et al. 1998; Gillott and Gaines 1992; Hentze et al. 2013). Juvenile hormone and ecdysteroids function in tandem to ensure full development and activity of the male SAG (Gillott 1996). The focus in the current study is on the role of JH in male SAG development since most post-eclosion adult SAG development and production of protein secretions is controlled by JH (Gillott and Gaines 1992).

Juvenile hormone controls SAG development and protein synthesis and also reproductive diapause in several insect orders. In some butterflies, SAG

development and protein secretion is controlled by JH (Gillott and Gaines 1992; Herman 1975a,b; Herman and Bennett 1975; Herman and Dallmann 1981). Monarch butterflies, *Danaus plexippus* (Lepidoptera: Nymphalidae), experience a reproductive diapause prior to migration that is terminated by JH (Barker and Herman 1976). This reproductive diapause in males is marked by low titres of endogenous JH and reduced male SAGs in overwintering males (Herman 1975b). The growth of SAGs of male *D. plexippus* is dependent on JH, as allatectomized males have small SAGs that enlarge with JH analogue (JHA) treatment (Herman 1975a,b). Juvenile hormone is also required for the enlargement of SAGs post-eclosion in male *Nymphalis antiopa* (Lepidoptera: Nymphalidae) (Herman and Bennett 1975) and *V. cardui* (Herman and Dallmann 1981). Sex accessory gland development and reproductive activity are linked to increases in JH titre in *V. cardui* (Herman and Dallmann 1981). Newly-eclosed male *Agrotis ipsilon* (Lepidoptera: Noctuidae) do not respond to female sex pheromone, however treatment with a juvenile hormone analogue (JHA) induces behavioural response (Gadenne et al. 1993). The SAGs of male *A. ipsilon* enlarge and increase protein content for several days after adult eclosion, coinciding with JH acid biosynthesis and pheromone response (Duportets et al. 1998). In the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), JHA treatment with hydroprene increases the size and protein content of male SAGs (Parthasarathy et al. 2009). Males deprived of JH have low reproductive fitness due to low mating behaviour, poor sperm transfer, and low egg production of females that mate with JH-deprived males (Parthasarathy et al. 2009). In *Drosophila melanogaster*

(Diptera: Drosophilidae), JH deficiency lowers protein synthesis in male SAGs, while normal JH titre may promote mating behaviour in males (Wilson et al. 2003). In male firebugs *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae), males in reproductive diapause have small SAGs, whereas high JH titres result in increased protein content and SAG secretions in reproductively active males (Socha 2004). The protein content of SAGs increases with endogenous JH titre over a period of ten days until reproductive activity is induced in male cockroaches, *Blattella germanica* (Blattodea: Blattellidae) (Piulachs et al. 1992; Vilaplana et al. 1996). Juvenile hormone also controls protein secretion in male *B. germanica* SAGs that is necessary to form and pass a spermatophore from males to females (Vilaplana et al. 1996). Similarly, an increase in JH synthesis in male *Schistocera gregaria* (Orthoptera: Acrididae) is linked to enlargement of SAGs during a period of sexual maturation (Avruch and Tobe 1978).

Caloptilia fraxinella (Lepidoptera: Gracillariidae) (Ely) is a leafmining pest of ornamental ash trees (*Fraxinus spp.*). *Caloptilia fraxinella* is univoltine and spends the majority of its lifecycle, approximately nine months, as an adult (Pohl et al. 2004). Adults eclose in July, with the majority of the population in reproductive diapause (Evenden et al. 2007). Adults undergo a summer aestivation and overwinter away from their ash hosts in an unmated state. In the spring, adults emerge from overwintering locations in a reproductively active state, mate, and females oviposit on newly flushed ash leaflets (Pohl et al. 2004). Male response to the female-produced sex pheromone is plastic, and male

response is highest in the spring when moths are reproductively active (Evenden and Gries 2008).

Female *C. fraxinella* exhibit a full reproductive diapause, as newly-eclosed females in the summer do not mate, and all females that overwinter are virgin with no oocyte development (Evenden et al. 2007). Reproductively active females early in the spring readily mate and produce vitellogenic oocytes (Evenden et al. 2007). Both JH and adult nutrition are important in terminating reproductive diapause in female *C. fraxinella*. Females in reproductive diapause that are treated with a JHA and provided carbohydrate nutrients break diapause and develop vitellogenic oocytes and successfully mate with males (Evenden et al. 2007). Evidence for reproductive diapause in male *C. fraxinella* includes the fact that male adult moths overwinter (Evenden 2009) and their response to sex pheromone is plastic throughout the adult life stage (Evenden and Gries 2008). Only a small percentage of male *C. fraxinella* are able to behaviourally respond to female-produced sex pheromone in the summer and fall, while reproductively active males in the spring are highly responsive to pheromone (Lemmen and Evenden 2009). Juvenile hormone analogue treatment enhances male electrophysiological and behavioural response to pheromone when the males are in reproductive diapause in the fall, but not in the summer (Lemmen and Evenden 2009). To test the hypothesis that male *C. fraxinella* undergo an extended physiological reproductive diapause during the summer and fall, the current study assesses SAG size, SAG total protein concentration, and individual SAG proteins

at different times during the adult life stage. The role of JH and adult nutrition in the termination of male reproductive diapause is also examined.

Materials and Methods

Moth Collection

Male *C. fraxinella* presumed to be in reproductive diapause (Evenden et al., 2007) were reared from pupae collected from leaf rolls found on green ash, *Fraxinus pennsylvanica* at various locations in Edmonton, Alberta (53^{o} 34'N 113^{o} 31'W) in June-July 2008, 2009 and 2010. Individual leaf rolls were kept under long day/warm conditions (LD 16:8 h, 24^{o}C) in 30 ml transparent plastic cups grouped in transparent plastic bags with a damp paper towel to maintain humidity. Cups were checked at least twice weekly for adult eclosion, which occurred in July for all three years of the study. Adults were separated by sex and males were prepared for subsequent treatment and dissection.

Males early in reproductive diapause in the summer (Evenden et al. 2007) were maintained post-eclosion in 30 ml cups supplied with a 10% sugar solution through a dental wick. Males were maintained under summer-like conditions at LD 16:8 h, 24^{o}C for at least one week before JHA treatment, and again for one week post-JHA treatment. Males late in reproductive diapause in the fall were collected in the same manner as summer males, but were transferred outdoors after eclosion. Fall males were held in wooden cages with Nytex nylon mesh walls (H 80 cm x W 40 cm x D 40 cm) placed outdoors in Edmonton under a small shelter with exposure to natural temperature and photoperiod conditions, and supplied with a 10% sugar solution until one week before JHA treatment in

the fall. After treatment they were transferred indoors and maintained as described for summer males for at least one week prior to moth transfer to -20°C in preparation for SAG removal.

Reproductively active-overwintered males experienced an artificial winter under laboratory conditions, and were either reared from pupae and held outdoors until late-September, as described for fall males, or were collected free-flying in the fall at various sites in Edmonton, Alberta. Males were transferred indoors in late September and held under winter conditions of LD 0:24 h, 2°C for 2-3 months. Males were transferred to summer conditions and maintained as described for summer and fall males for at least one week prior to moth transfer to -20°C in preparation for SAG removal.

Reproductively active spring male *C. fraxinella* were collected as free-flying adults at various sites in Edmonton, Alberta in April-May 2008, 2009 and 2010 after overwintering outdoors under natural conditions. Males were held individually in 30 ml transparent cups and were maintained as in the other experiments at LD 16:8, 24°C for at least one week before transfer to -20°C. To determine if adult nutrition influenced SAG length or protein content of spring males, moths were provided with either a 10% sugar solution or water for one week before freezing.

JHA Treatment

Based on previous experiments (Evenden et al. 2007; Lemmen and Evenden 2009), male *C. fraxinella* that were in reproductive diapause in the summer and fall were treated with: (1) 1 μg of the JHA methoprene (94.3% pure,

Sigma-Aldrich, Oakville, Ontario, Canada) diluted in 1 µl of high-performance liquid chromatography (HPLC) grade acetone (Fisher Scientific, Ottawa, Ontario, Canada), (2) 1 µl acetone alone, or (3) were left untreated. Male moths were held using a gentle vacuum stream while treatments were applied exogenously to the ventral side of the abdomen. After treatment, males were transferred back to the appropriate experimental conditions for one week before transfer to -20^{o}C until dissection and SAG removal. Reproductively active wild-collected spring and laboratory-overwintered males were not subjected to JHA treatment.

Accessory Gland Dissection and Measurements

One week after treatment, male *C. fraxinella* were transferred to -20^{o}C for a minimum of two weeks before abdominal dissection and removal of individual SAGs. Moths were removed from cold storage approximately 15 minutes before dissection, and were then immersed in insect Ringer's solution. Males were held in place with blunt forceps, and fine-tipped forceps were used to gently pull the male claspers to remove the reproductive tract. The paired SAGs located along the reproductive tract (Appendix 5-1) were carefully measured using a micrometer attached to a dissection microscope (magnification x25).

The mean length of the two glands was used as the length of the SAG, and if one gland was damaged, only the measurement of the intact gland was used. Following removal and measurements, wet glands were transferred to a microfuge tube (1.5 mL) with either water or buffer solution and immediately placed in a -20^{o}C freezer until further analysis. As male SAG length is known to correlate with male body size (Bangham et al 2002), a body size estimate of forewing

length of each male moth was included as a covariate in the analysis. Forewings were removed and measured with either an ocular micrometer on a dissecting microscope (magnification x6) or were glued to paper, scanned, and measured using the program ImageJ (Schneider et al. 2012). The mean length of both forewings was used in the analysis, and if a wing was damaged or broken, only the length of the intact wing was used.

Sex accessory glands were removed from male *C. fraxinella* moths in spring (reproductively active), summer (reproductive diapause) and fall (reproductive diapause) and gland length was measured immediately. Moths from these populations were subjected to the following treatments: 1) spring fed, 2) spring unfed, 3) summer methoprene-treated, 4) summer acetone-treated, 5) summer untreated, 6) fall methoprene-treated, 7) fall acetone-treated and 8) fall untreated.

All statistical analyses were conducted in R v. 2.15.0 (R Development Core Team, 2012). A Shapiro-Wilk test determined that all SAG measurement data were normally distributed. A simple linear regression was performed to test whether there was a significant relationship between male forewing length and SAG length in the spring, summer and fall. To determine if nutrition or JHA treatment impacted SAG length, an ANOVA was conducted with treatment (nutrition or JHA) specified as an explanatory variable and wing length specified as a covariate to account for variation in male body size in each season. A separate ANOVA including season as the explanatory variable and wing length as the covariate was performed to test if SAG length changed with season.

Following a significant overall model, a Tukey-contrasts test was performed to determine significant differences between individual treatments.

BCA (bicinchoninic) Protein Assay

To determine the total protein concentration in male *C. fraxinella* SAGs from the variously treated moths at different times of the year, a BCA protein assay (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA, Product #23225) was performed in 2012. Moth treatments used in this experiment were classified as: 1) spring fed, 2) spring unfed, 3) overwintered, 4) summer methoprene-treated, 5) summer acetone-treated, 6) summer untreated, 7) fall methoprene-treated, 8) fall acetone-treated and 9) fall untreated. The protein buffer solution consisted of 10 ml of T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA, product #78510) with 100 μl of Protease Inhibitor Single-Use Cocktail and 100 μl of EDTA Solution (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA, Product #78430). Three pairs of male SAGs were placed in a microfuge tube (1.5 mL) with 20 μl of protein buffer solution immediately after removal from male moths. Glands were then frozen at -20^{o}C prior to use in the BCA assay. Sex accessory glands were removed from cold storage, allowed to thaw for 5 min at room temperature, and were then homogenized with a plastic pestle ten times. The pestle was rinsed 3 times with 20 μl of the buffer solution for a minimum volume of 80 μl in each tube. Gland solutions were centrifuged at 16 000 RCF for 5 min and supernatants collected. For the BCA assays, 50 μl of supernatant was used with Bovine Serum Albumin (BSA) as a standard. The absorbance of all samples

was measured with a spectrophotometer (Biochrom, Novaspec II) set at 562nm. A standard curve was prepared following the instructions in the protein assay kit, and used to determine the protein concentration in each of the SAG samples.

The SAG protein sample concentrations did not follow a normal distribution when tested with a Shapiro-Wilk test. Normality was achieved with a natural logarithm transformation of the data. A mixed model ANOVA was performed on the total protein concentrations with treatment as a fixed variable and replicate as a random factor.

SDS-PAGE Gels

The hypothesis that the protein content of male *C. fraxinella* SAGs differs with season and the physiological state of male *C. fraxinella* was tested in 2009. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to generate protein profiles of male SAGs.

After removal from male moths, one pair of SAGs was placed in a microfuge tube (1.5 mL) with water and frozen at -20^{o}C until use in SDS-PAGE gels. Glands were removed from cold storage and placed in a freeze dryer (VirTis Sentry 2.0) for approximately 24 h to remove all water from the glands. Following freeze drying, glands were homogenized with a plastic pestle 10 times in 10 μl of protein buffer solution. The pestle was rinsed twice with 5 μl of buffer solution, for a minimum volume of 20 μl. After 5 min centrifugation at 16 000 RCF, supernatant was collected for immediate use in SDS-PAGE gels. Acrylamide gels with a 4% w/v stacking gel and 12% w/v resolving gel were prepared, following the SDS-PAGE (Laemmli 1970) buffer system using a Bio-

Rad Mini Protean 3 apparatus (Bio-Rad, Richmond, CA, USA). Preparation of all solutions, buffers and gel formulations were conducted by following the instructions in the electrophoresis guide that accompanied the Bio-Rad apparatus. Briefly, 5 μl of 3X sample buffer with β-mercaptoethanol was added to 10μl of sample, and heated for 5 min at 95^{o}C before being loaded into each well.

Twelve gel replicates were run, and each gel contained SAG samples from the following seasons and treatments: 1) spring, 2) summer methoprene-treated, 3) summer acetone-treated, 4) summer untreated, 5) fall methoprene-treated, 6) fall acetone-treated and 7) fall untreated and one lane of 5 μl of prestained protein ladder, 11-250 kDa (Fermentas, Thermo Scientific, Ottawa, ON, PageRuler Plus Prestained Protein Ladder, #SM1811). Gels were run at 100 V for approximately 90 min until the stain front was near the bottom of the gel.

Gels were rinsed with 200 mL of mQH_2O for 15 min and then were stained with 20 mL of Imperial Protein Stain (Pierce, Rockford IL, USA, #24615) for 2 h. Stain was discarded, and gels were rinsed three times with 20 mL of mQH_2O, and were then placed in 200 mL of mQH_2O with a Kimwipe® (Kimberly-Clark) to destain overnight.

All gels contained protein bands (Appendix 5-2). Following a visual inspection of the gels, which revealed the presence of protein bands, gels were analyzed using AlphaView SA software (Cell Biosciences, Inc.). Molecular weights were estimated and the quantification of specific protein bands on the gels was performed using the known concentration of the protein ladder and analyzed with the Alphaview SA software.

The number of protein bands detected and counted for each season and treatment in each gel were compared using a GLM with a negative binomial error to account for data overdispersion. Season and treatment and gel replicate were all included as explanatory variables. The mass of specific protein bands did not achieve normality, and so a Kruskal-Wallis test was performed to examine the effect of season and treatment on the mass individual protein bands.

Results

Accessory Gland Dissection and Measurements

When male *C. fraxinella* were reproductively active in the spring, SAG length was positively correlated with forewing length (Fig. 5-1A). Sex accessory gland length was not correlated with forewing length when males were in reproductive diapause in the summer (Fig. 5-1B) or the fall (Fig. 5-1C). Sex accessory glands from reproductively active males in the spring were significantly longer than those from males in reproductive diapause in either the summer or fall (Fig. 5-2A). Although males in the summer and fall were both in reproductive diapause, summer males had longer SAGs than their fall counterparts (Fig. 5-2A). The nutritional status of reproductively active spring males did not impact SAG length (Fig. 5-2B). Juvenile hormone analogue treatment did not impact SAG length of males in reproductive diapause in the summer (Fig. 5-2C) or fall (Fig. 5-2D). There was a non-significant trend for accessory glands from methoprene-treated males in the fall to be longer than acetone-treated or untreated fall males (Fig. 5-2D).

BCA (bicinchoninic) Protein Assay

The season in which males were tested did not significantly affect the total concentration of protein in male SAGs (mixed effects model ANOVA: $F_{3,39}$=1.9, P=0.1). There was a marginally significant effect of JHA treatment on SAGs when males were in reproductive diapause in the fall, and methoprene-treated male SAGs contained more protein compared to the glands of control males (mixed effects model ANOVA: $F_{2,31}$=2.8, P=0.08) (Table 5-1). Juvenile hormone analogue treatment did not impact the protein concentration of SAGs of males in reproductive diapause in the summer (mixed effects model ANOVA: $F_{2,32}$=2.2, P=0.1) (Table 5-1). When reproductively active males were tested in the spring, nutritional status did not impact the total protein concentration in male SAGs (mixed effects model ANOVA: $F_{1,18}$=0.7, P=0.4) (Table 5-1).

SDS-PAGE Gels

Protein profiles were analyzed on 12 gels, each considered an independent biological replicate. SDS-PAGE of male *C. fraxinella* SAGs revealed a range of protein fractions with molecular weights ranging from 11 – 249 kDa. The protein profiles of the accessory glands were similar among samples for all seasons and treatments (GLM: $X_{6,0.05}$=5.1, P=0.5) (Table 5-2). The specific protein profiles of SAGs of each treatment that were consistently reproduced in at least two independent replicates were examined further.

The major protein bands detected and consistently reproduced were located ~ 55 kDa, 62 kDa, 66 kDa and 92 kDa (Fig. 5-3; Table 5-3). All four bands were present at all times of year and for all treatments (Table 5-3). There is a marginally significant effect of season and treatment on the mass of band 55

kDa (Kruskal-Wallis: $X_{6,0.05}$=11.2, P=0.08), however season and treatment did not impact the mass of protein band 62 kDa (Kruskal-Wallis: $X_{6,0.05}$=4.1, P=0.7), band 66 kDa (Kruskal-Wallis: $X_{6,0.05}$=4.0, P=0.7), or band 92 kDa (Kruskal-Wallis: $X_{6,0.05}$=8.0, P=0.2).

Discussion

The development of SAGs in adult male *C. fraxinella* is impacted by the reproductive physiological state of males. Male *C. fraxinella* SAG size is positively correlated with body size when males are reproductively active in the spring, but not when males are in reproductive diapause in the summer and fall. In the stalk-eyed fly, *Cyrtodiopsis dalmani* (Diptera: Diopsidae) (Baker et al. 2003) and *D. melanogaster* (Bangham et al. 2002) SAG size is correlated with body size, and also with male fitness, as larger males with larger SAGs have higher reproductive success than smaller males. It is not known whether larger male *C. fraxinella* are more successful at locating and mating with females, but SAG size is positively correlated with body size in reproductively active males who would be searching for mates.

While controlling for body size, reproductively active male *C. fraxinella* in the spring have longer SAGs compared to males in reproductive diapause in the summer and fall. The size of male SAGs is an indication of reproductive maturity, and many male insects that delay mating have a post-eclosion enlargement of male SAGs over the period of reproductive inactivity (Happ 1992; Herman 1975b; Herman and Bennett 1975; Herman and Dallmann 1981). Male *C. fraxinella* in reproductive diapause in the summer have longer SAGs than

males in reproductive diapause in the fall. This may be due to the conditions under which males were held. In the butterflies *D. plexippus, N. antiopa* and *V. cardui*, male SAGs are small after eclosion when the males are not reproductively active, but if males are held under summer-like conditions in the laboratory, the reproductive tract will start to enlarge and develop regardless of reproductive state (Herman 1975a,b; Herman and Bennett 1975; Herman and Dallmann 1981). If male *C. fraxinella* are held under warm conditions in the laboratory post-eclosion in the summer, they quickly become responsive to female sex pheromone over a period of weeks (Chapter 3). In this study, males were held indoors under summer conditions in the laboratory for at least one week prior to treatment and one week post-treatment until transfer to -20^{o}C. This two-week period may have stimulated the enlargement of the SAGs in males, resulting in the intermediate size observed in summer males. Alternatively, it is possible that a small portion of the population that eclose in July is reproductively active and some summer males may have larger accessory glands than fall males preparing to overwinter. Pairs of *C. fraxinella* have been observed to mate in the summer, and oviposition on secondary growth of ash in the summer has been reported (Wist, unpublished data). To test this further, males should be dissected and SAGs measured immediately upon eclosion and over a period of several weeks during which males are exposed to summer conditions.

A variety of specific proteins are detected in the SAGs of male *C. fraxinella*. The major protein bands detected are ~55 kDa, 62 kDa, 66 kDa and 92 kDa. In *A. ipsilon*, one main protein band was identified in male SAG at ~90 kDa

(Duportets et al. 1998), in *P. apterus* one main band was detected at ~53 kDa (Socha et al. 2004) and in *B. germanica* four main bands were detected in male SAG at ~65 kDa, 45 kDa and two bands at ~15 kDa (Vilaplana et al. 1996). In this study, protein profiles of male SAGs were similar in all seasons of testing and among glands of the variously treated males. When the mass of specific proteins was analyzed, season and treatments did not significantly impact the total mass of any of the individual protein bands that were consistently reproduced, and no clear patterns of protein mass change are evident.

Despite the difference in SAG size with season, the protein concentrations and the number of individual proteins in male *C. fraxinella* SAGs are similar in males across all seasons. Males have similar protein concentrations in the SAGs of reproductively active males that overwintered under natural outdoor conditions and that overwintered under artificial conditions in the laboratory, and also in males in reproductive diapause in the summer and the fall. In male insects that require a period of sexual maturation during the adult stage, the SAGs typically secrete and accumulate proteins during the pre-reproductive period. Male migratory grasshoppers, *Melanoplus sanguinipes* (Orthoptera: Acrididae), are sexually mature at 7 days old, and the protein content of SAGs increases over 14 days, at which point protein content is approximately 6 times higher in 14-day old virgin males compared to 1-day old males (Gillott and Friedel 1976). In the cockroach *Periplaneta americana* (Orthoptera: Blattidae), male sexual maturity is reached approximately 35 days after the imaginal molt (Blaine and Dixon 1973). During this period of sexual maturation, the size of the SAG tubules and the

number of individual protein bands do not change, but the protein content in male SAGs increases, which results in an increased weight of SAGs from 0.64 mg to 5.15 mg (Blaine and Dixon 1973). *Pyrrhocoris apterus* experiences a pre-reproductive diapause period, during which the amount of total proteins and the main 53 kDa protein in male SAG increases with male age (Socha 2004). The western tarnished plant bug, *Lygus hesperus* (Hemiptera: Miridae) experiences a temperature-dependent pre-reproductive period, during which male SAGs secrete and accumulate protein until reproductive maturity (Spurgeon and Cooper 2012). The protein content of SAGs of male *A. ipsilon* is low at adult eclosion when males are not reproductively active, and increases over a period of 4 days until adults are sexually mature (Duportets et al. 1998). The similarity in protein concentration of male *C. fraxinella* SAGs of males in different reproductive states is unusual. This could be due to the fact that males were held indoors under summer conditions for at least two weeks prior to freezing and SAG removal, so it is possible that the SAGs secreted similar amounts of proteins prior to measurement at the different times of year. Since JHA treatment increases SAG protein concentration and pheromone responsiveness of male *C. fraxinella* in reproductive diapause in the fall, increased SAG protein secretion may be a prerequisite to successful mating. It is possible that a difference in protein concentration based on male moth physiological state was missed due to testing male SAG protein concentration at only one time point during each physiological state. Sex accessory gland protein concentration and variety have not been previously tested in an insect with such a long adult life as *C. fraxinella*. Changes

in protein concentrations of male SAGs are typically observed over a period of days rather than months (Gillott 2003; Gillott and Gaines 1992).

Treatment with a JHA does not impact SAG size, but does impact the protein concentration in the SAGs of male *C. fraxinella*. Juvenile hormone analogue treatment does not impact SAG length when males are in reproductive diapause in the summer and the fall, likely because there is only one week between JHA treatment and the subsequent freezing of moths, which may not be sufficient for significant enlargement of the SAGs. However, there is a non-significant trend in the fall for methoprene-treated males to have longer SAGs than acetone-treated and untreated males. Treatment of male *C. fraxinella* in reproductive diapause in the fall with methoprene enhances the total concentration of protein in the SAGs compared to control males. Juvenile hormone controls SAG development and protein secretion and accumulation in SAGs in adult male insects in many species (Gillott 1996; Happ 1992). Juvenile hormone deprivation by allatectomy on newly-eclosed male *A. ipsilon* prevents the normal development of male SAGs, and total protein concentration remains low. Treatment with a JH or JHA partially restores the protein concentration in male SAGs (Duportets et al. 1998). Juvenile hormone deprivation by allatectomy also reduces the concentration and total number of proteins in SAGs of male *P. americana* (Blaine and Dixon 1973), *M. sanguinipes* (Gillott and Friedel 1976; Venkatesh and Gillott 1983) and *Rhodnius prolixus* (Hemiptera: Reduviidae) (Barker and Davey 1981; Gold and Davey 1989), and in most cases protein synthesis is restored with JH treatment. Maturation of male SAGs in monarch

butterflies, *D. plexippus*, requires active *corpora allata* or JH application to increase SAG size and weight (Herman 1975a,b). Juvenile hormone analogue treatment also enhances male *C. fraxinella* behavioural and electroantennogram responses to female sex pheromone in the fall, but not in the summer (Lemmen and Evenden, 2009), indicating that JH plays a major role in the termination of reproductive diapause in male *C. fraxinella*, and that the timing of JHA application is crucial to diapause termination. Male *C. fraxinella* are clearly in different physiological states in the summer and in the fall, and males are more susceptible to JHA treatment in the fall than in the summer. To my knowledge, this is the first time that JHA treatment has been shown to act differentially in the period of reproductive diapause at the level of protein concentration in male SAGs.

The development of male SAGs in *C. fraxinella* is similar to another long-lived moth in which this has been tested, *A. ipsilon*. As in *A. ipsilon* (Duportets et al. 1998), male SAGs of *C. fraxinella* enlarge post-adult eclosion. There is a non-significant trend for JHA treatment to enhance SAG length in the fall in male *C. fraxinella*. Further, JHA treatment enhances the protein content of male SAGs in both *A. ipsilon* (Duportets et al. 1998) and *C. fraxinella*. In *C. fraxinella* however, this only occurs when males are in reproductive diapause in the fall, and not in the summer. In both moths, SAG development coincides with male behavioural response, as males are not reproductively active when SAGs are immature. After JH treatment or when moths are reproductively active, male SAGs are fully

developed and males are fully responsive to female sex pheromone (Duportets at al. 1998).

Although a carbohydrate nutrition source is required for female *C. fraxinella* to break diapause and develop vitellogenic oocytes (Evenden et al. 2007), it is not necessary for males to develop functional SAGs. Access to a carbohydrate nutrition source in reproductively active males in the spring did not impact SAG length or total protein concentration in SAGs. Male *R. prolixus* deprived of a blood meal have very little protein accumulation in SAG during the first 20 days after the imaginal moult, while males provided a blood meal have an immediate increase in the protein concentration of SAG (Gold and Davey 1989). Restricting the amount of protein available to adult male *C. dalmanni* increased the length of time it took males to achieve sexual maturity and reduced the size of SAG and testes compared to males supplied with protein *ad libitum* (Baker 2003). Similar to *C. fraxinella*, in the rice bug, *Leptocorsia chinensis* (Hemiptera: Alydidae), females require a food source to resume ovarian development post-reproductive diapause, however, starved males are able to resume SAG development post-diapause without food (Tachibana and Watanabe 2007). A carbohydrate source is also not required for reproductively active male *C. fraxinella* to respond behaviourally to pheromone after overwintering (Chapter 3). Since *C. fraxinella* moths mate early in the spring, it may be advantageous for males to locate and mate with females immediately after emergence from overwintering, and males may acquire enough resources during the larval stage for full reproductive activity.

This study has confirmed that male *C. fraxinella* undergo a true reproductive diapause with underdeveloped SAGs during the period of reproductive diapause, along with reduced pheromone responsiveness to female sex pheromone (Lemmen and Evenden 2009). Juvenile hormone is important for regulation of reproductive diapause in *C. fraxinella*, as JHA treatment impacts the protein concentration of male SAGs along with male response to female sex pheromone when males are in reproductive diapause in the fall, but not in the summer. To further understand diapause regulation and the role of male SAG proteins in this long-lived moth, specific proteins could be identified and characterized with potential functions, as has been done in some other insects (Baer et al. 2009; Braswell et al. 2006; Chapman et al. 2001; Kubli 2003; Wolfner 1997). This would provide additional valuable information on the proteome of male SAGs of a long-lived moth that exhibits distinct periods of reproductive diapause and reproductive activity.

Table 5-1. Protein concentration in male *C. fraxinella* accessory glands at different times of the year (2012), as measured with a BCA protein assay.

Reproductive State	Season	Treatment[a]	N[b]	Mean (+SE) Protein Concentration[c] (μg/mL)	*F*[d]	*P*
Active	Spring	Fed	12	28 (4.2)	$_{1,18}$=0.7	0.4
		Unfed	9	30(5.2)		
	Overwintered	Fed	8	16(3.4)	N/A[e]	N/A
Diapause	Summer	JHA	12	28(3.7)	$_{2,32}$=2.2	0.1
		Acetone	12	30(2.2)		
		UT	12	27(7.1)		
	Fall	JHA	11	33(4.7)	$_{2,31}$=2.8	0.08
		Acetone	12	24(5.8)		
		UT	12	21(2.1)		

[a]Treatment abbreviations: JHA=Juvenile hormone analogue, methoprene; UT=Untreated. [b]N=number of three pooled sets of glands that were used for each BCA protein assay for each treatment and season. [c]The natural logarithm-transformed data used in the mixed model ANOVA were back-transformed to show the actual mean protein concentrations for each season and treatment. [d]F and P values are derived from a mixed model ANOVA including treatment and replicate specified as a random factor. [e]Analysis was not applicable.

Table 5-2. The mean number of protein bands detected in 12 SDS-PAGE gels (2009) on male *C. fraxinella* accessory glands.

Season	Treatment	Mean Number Protein Bands
Spring	None	13.2
Summer	Methoprene	15.8
	Acetone	14.6
	Untreated	13.4
Fall	Methoprene	13.2
	Acetone	14.1
	Untreated	16.2

Table 5-3. Major protein bands and their mass determined by SDS-PAGE gel analysis (2009) of male *C. fraxinella* accessory glands.

		Molecular Weight: Total Mass of Each Protein (μg), N							
Season	Treatment[a]	27	29	46	52	55	62	66	92
Spring	None	0.01,2	0.01,3	NP[b]	0.02,2	0.11,7	0.13,9	0.05,3	0.06,5
Summer	JHA	0.03,4	0.05,3	0.03,3	NP	0.20,7	0.14,10	0.04,6	0.02,6
	Acetone	0.01,2	0.03,3	NP	0.08,2	0.07,7	0.10,12	0.04,4	0.02,5
	UT	NP	NP	0.05,3	0.04,2	0.17,5	0.20,10	0.13,3	0.04,5
Fall	JHA	0.03,3	0.05,2	NP	NP	0.10,7	0.13,9	0.06,2	0.08,5
	Acetone	0.03,4	0.05,3	NP	NP	0.03,6	0.06,11	0.04,4	0.03,4
	UT	NP	0.02,3	NP	NP	0.06,9	0.12,11	0.04,2	0.02,5

[a]Treatment abbreviations: JHA=Juvenile hormone analogue, methoprene; UT=Untreated. [b]NP=Band was not present.

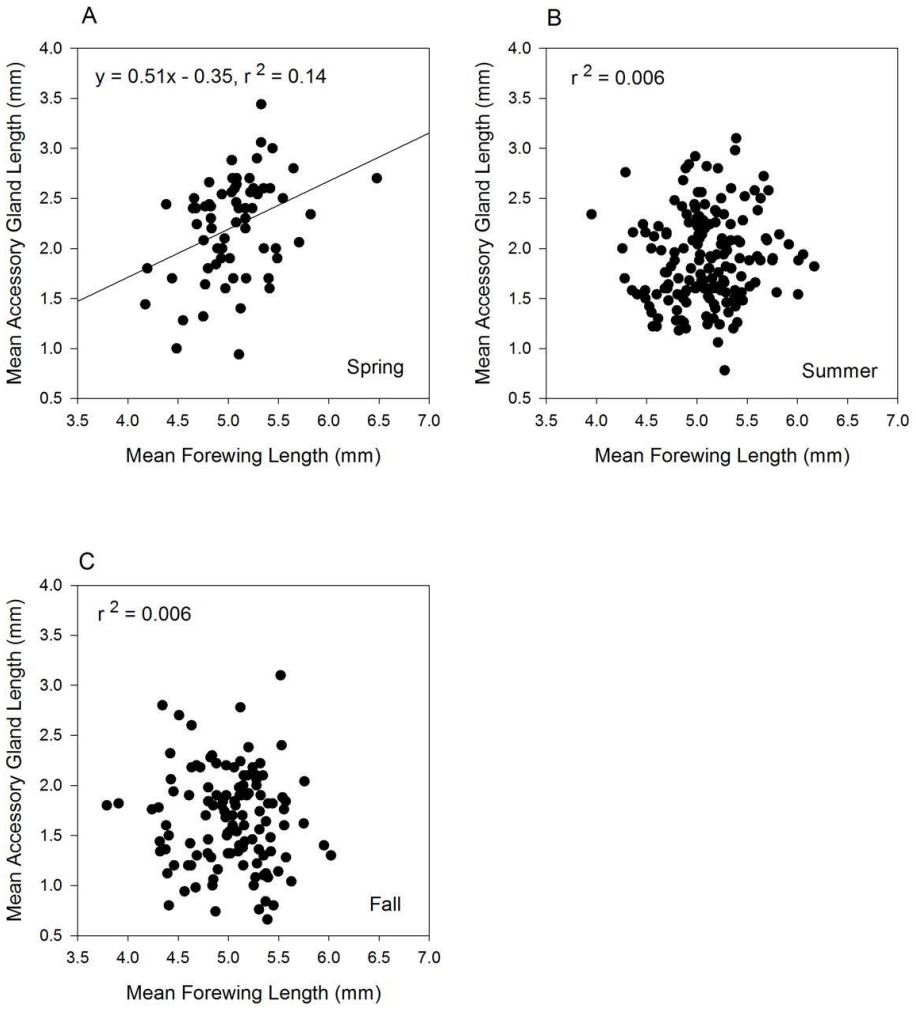


Fig. 5-1. Relationships between mean accessory gland length and mean forewing length when male *C. fraxinella* are (A) reproductively active in the spring ($F_{1,64}$=11.7, r^2=0.16, *P*=0.001), and in reproductive diapause in the (B) summer ($F_{1,164}$=0.98, r^2=0.006, *P*=0.3) and (C) fall ($F_{1,117}$=0.73, r^2=0.006, *P*=0.4).

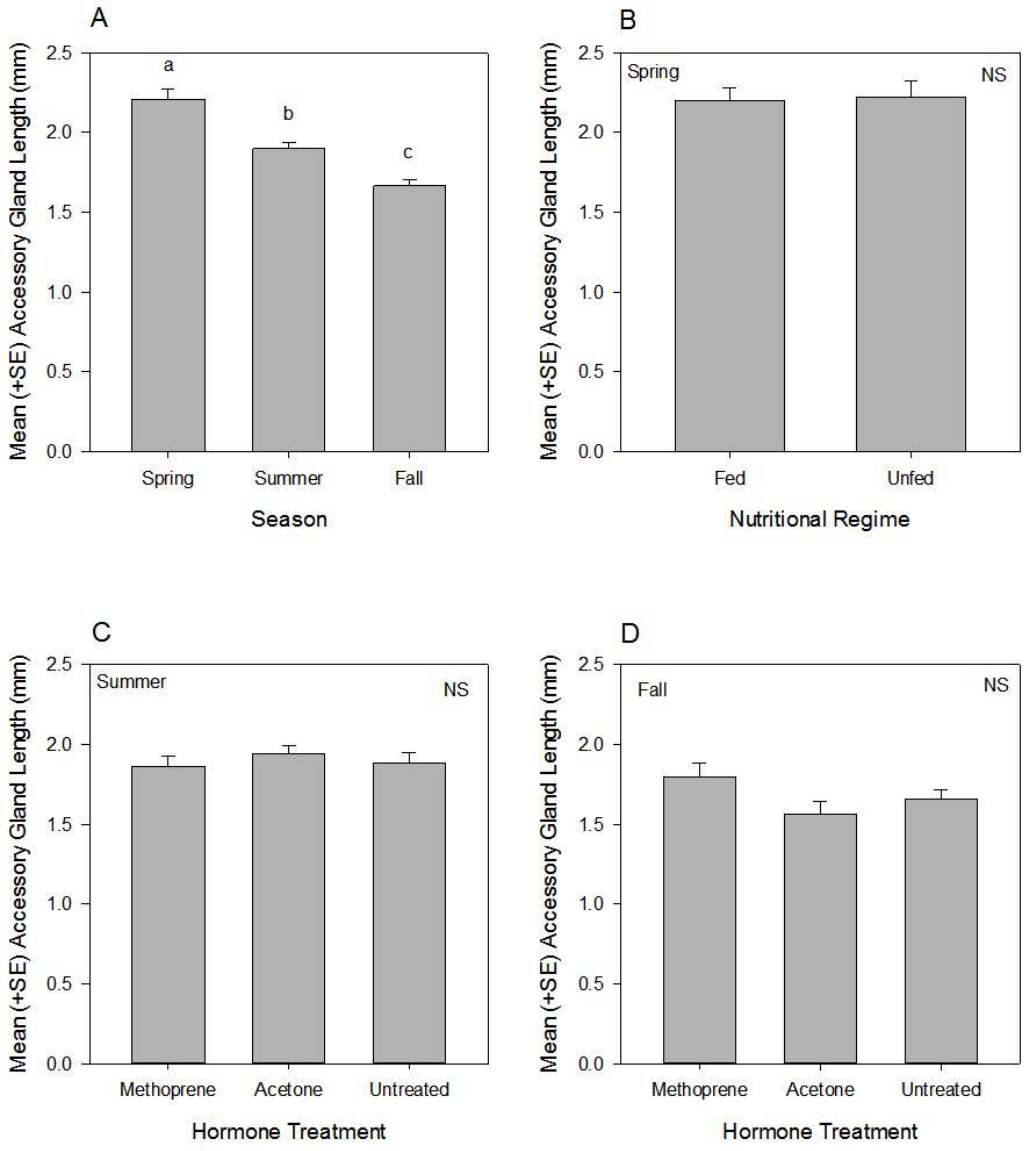


Fig. 5-2. Mean accessory gland lengths of male *C. fraxinella* (A) at different times of year (ANOVA: $F_{2,347}$=28.8, P<0.0001), and exposed to different (B) nutrient and (C and D) hormone regimes. (A) Different letters indicate significant differences between the seasons (Tukey-contrasts: P<0.001). (B) Nutrition did not impact accessory gland length of reproductively active males in the spring (ANOVA: $F_{1,63}$=0.007, P=0.9). Hormone treatment did not impact accessory

gland length of males in reproductive diapause in the (C) summer (ANOVA: $F_{2,162}$=0.09, P=0.6) or (D) fall (ANOVA: $F_{2,115}$=2.0, P=0.2).

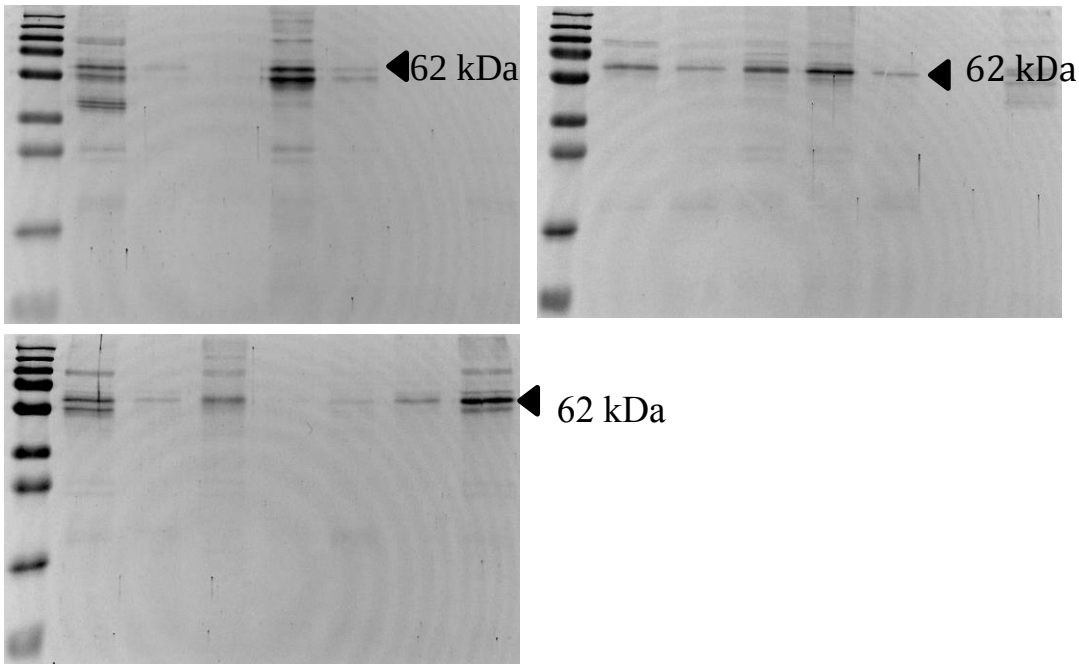


Fig. 5-3. Protein profiles of male *C. fraxinella* sex accessory glands from SDS-PAGE gels in 2009. Three gels are pictured as representative examples of all 12 gels. Abbreviations under the gels indicate the season and treatment of the individual moth in that lane. ML=molecular ladder; FM=fall methoprene-treated; FA=fall acetone-treated; FUT=fall untreated; SM=summer methoprene-treated; SA=summer acetone-treated; SUT=summer untreated; SP=spring untreated.

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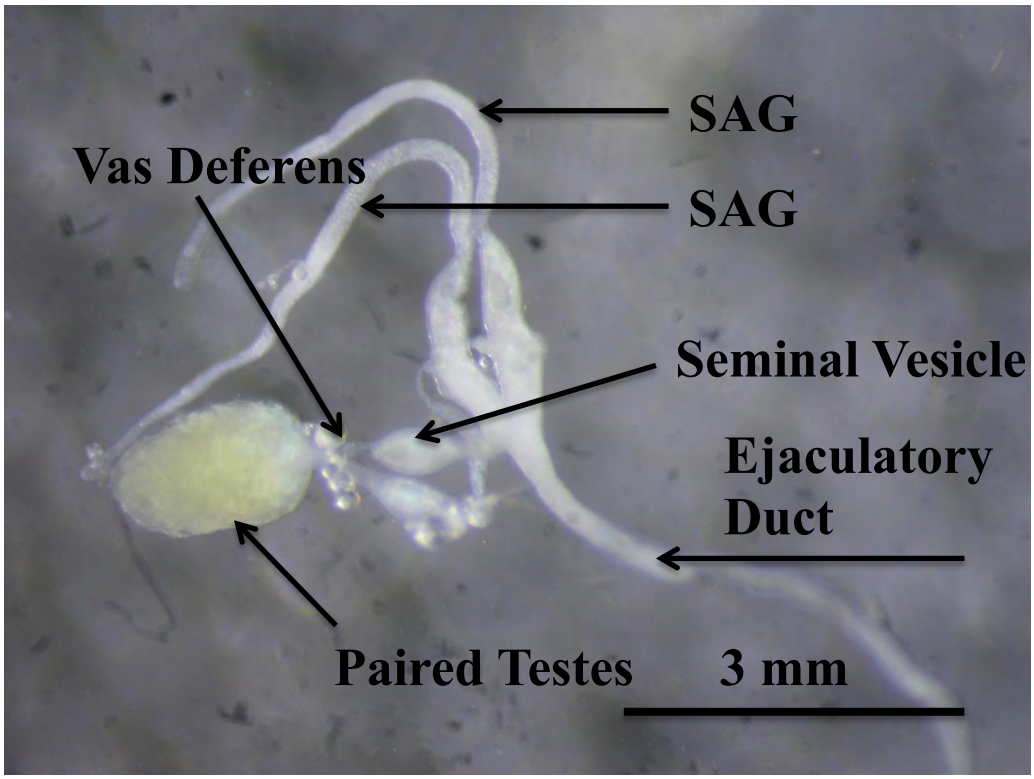
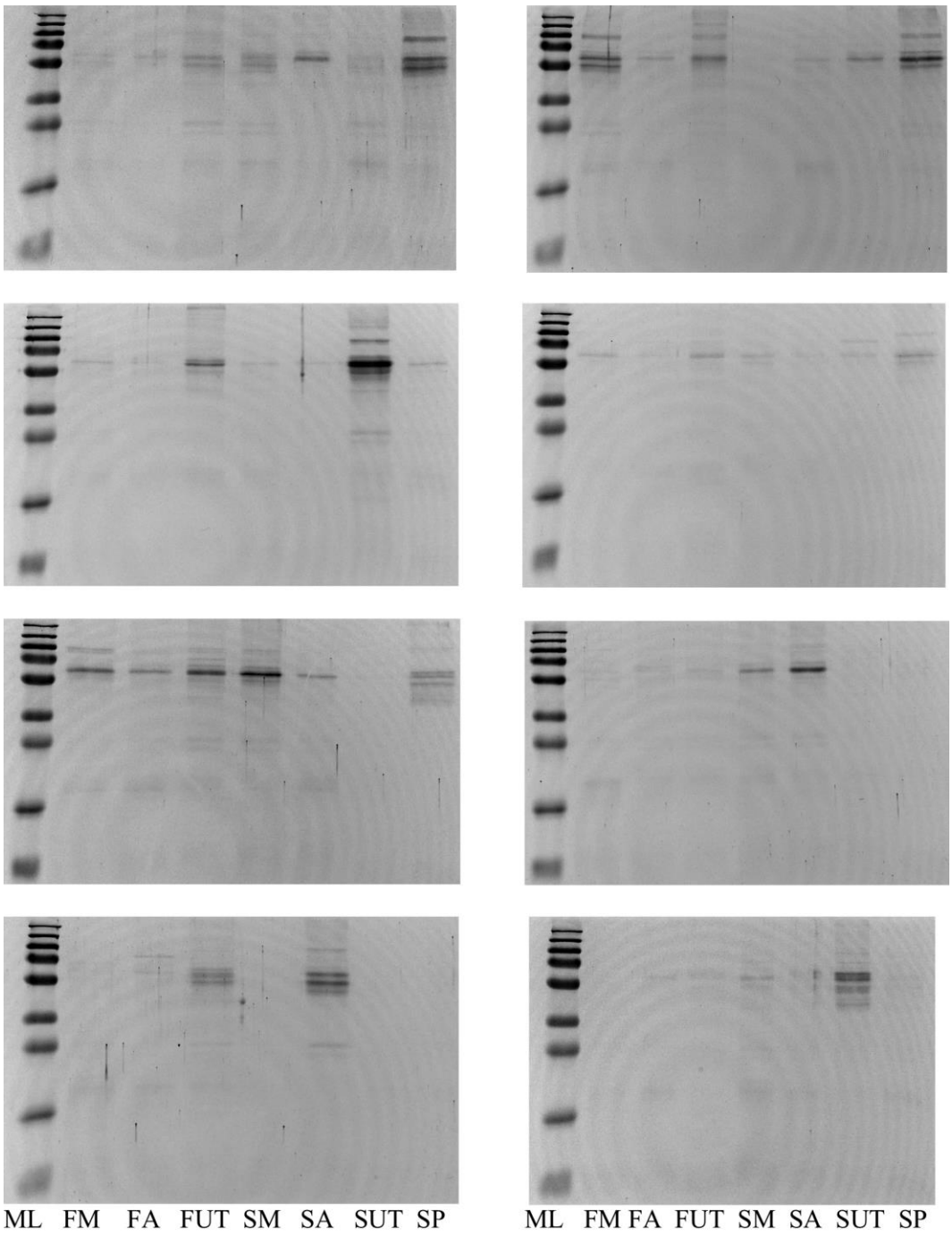


Appendix 5-1. Picture of male *C. fraxinella* sex accessory glands. Removed from males post-eclosion in July 2013. SAG=Sex accessory glands. Photo: Joelle K Lemmen.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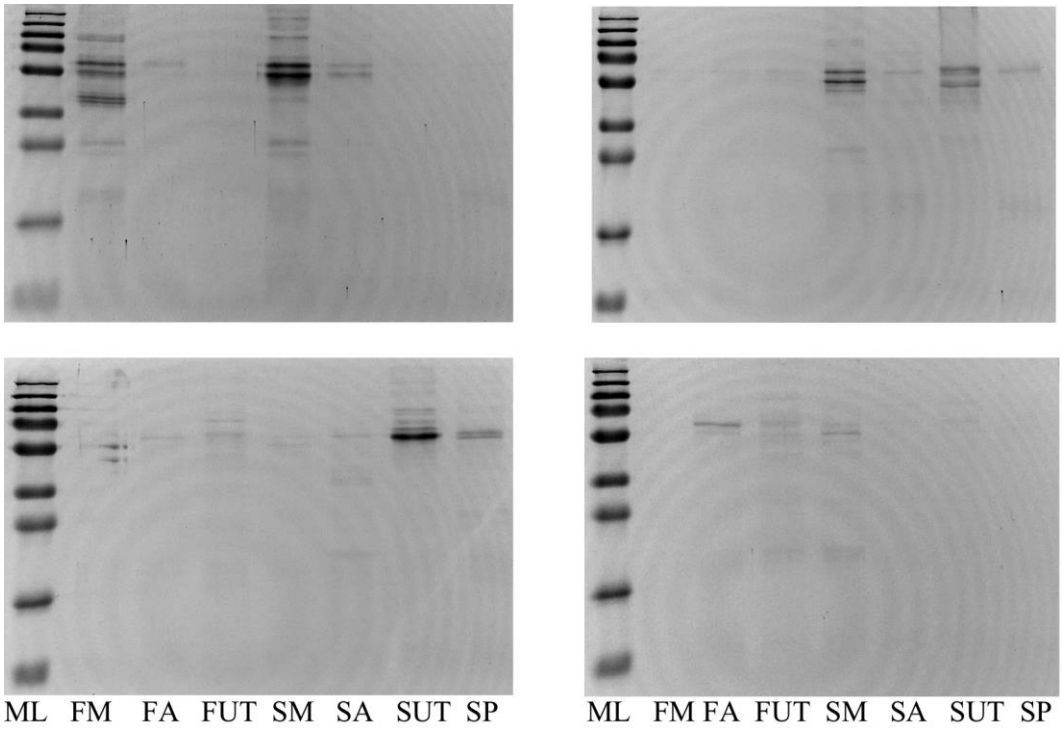
ML FM FA FUT SM SA SUT SP



Appendix 5-2. Images of all twelve gels from SDS-PAGE analysis in 2009 of proteins from sex accessory glands of male *C. fraxinella*. Abbreviations under the gels indicate the season and treatment of the individual moth in that lane. ML=molecular ladder; FM=fall methoprene-treated; FA=fall acetone-treated; FUT=fall untreated; SM=summer methoprene-treated; SA=summer acetone-treated; SUT=summer untreated; SP=spring untreated. Molecular Ladder (kDa) from top to bottom: 250; 130; 95; 72; 55; 36; 28; 17; 11.

Chapter 6

General Discussion and Conclusion

In this thesis, I studied pheromone response plasticity in a long-lived moth, *Caloptilia fraxinella*. My aim was to identify and examine mechanisms driving the pheromone response plasticity of male *C. fraxinella*, and to determine whether a similar plasticity exists in male and female *C. fraxinella* response to host volatiles. My final goal was to determine whether male pheromone response plasticity is part of a true reproductive diapause syndrome in male *C. fraxinella*.

Male *C. fraxinella* undergo a true reproductive diapause during the summer and fall, with reduced sex accessory glands (Chapter 5) and reduced pheromone responsiveness (Evenden and Gries 2008; Lemmen and Evenden 2009) during the period of reproductive diapause. Reproductive diapause in male insects is diagnosed mainly by the inability of males to mate successfully with receptive females (Denlinger et al. 2005, Pener 1992). This includes a lack of male response to female sexual signals and also the inability of a male to produce and pass on sperm to a female (Denlinger et al. 2005; Pener 1992). Reduced pheromone response in reproductively inactive male Lepidoptera is documented mainly in noctuid moths, including *Trichoplusia ni* (Shorey et al. 1968), *Heliothis zea* (Shorey et al. 1968), *Heliothis virescens* (Shorey et al. 1968), *Agrotis ipsilon* (Gadenne et al. 1993) and *Pseudaletia unipuncta* (Turgeon et al. 1983). In all of these moths, sex pheromone responsiveness is restored between approximately 2-5 days post-eclosion. Pheromone responsiveness is reduced for at least three months in male *C. fraxinella* during a reproductive diapause between July and

October (Chapter 3), which is a much longer period than has been observed in other Lepidoptera. The reduced size of male sex accessory glands (Chapter 5) during the summer and fall in male *C. fraxinella* further supports the hypothesis that males are in a state of reproductive diapause. Males with small accessory glands would not likely be able to produce a spermatophore during this time, as accessory gland growth needs to occur before males are reproductively active in the spring. Individuals with reduced or immature accessory glands are typically unable to produce a spermatophore or the necessary secretory proteins for successful spermatophore transfer to a female (Chen 1984; Leopold 1976). Adult maturation of male sex accessory glands during a period of reproductive inactivity occurs in several moths and butterflies, including *Danaus plexippus* (Nymphalidae) (Herman 1975), *Nymphalis antiopa* (Nymphalidae) (Herman and Bennett 1975), *Vanessa cardui* (Nymphalidae) (Herman and Dallmann 1981), and *A. ipsilon* (Duportets et al 1998), and also in different orders of insects including species of Coleoptera (Parthasarthy et al. 2009), Hemiptera (Socha 2004), Blattodea (Blaine and Dixon 1973; Piulachs et al. 1992; Vilaplana et al. 1996) and Orthoptera (Avruch and Tobe 1978; Gillott and Friedel 1976). In these cases, the length of time required for the sex accessory glands to develop is typically over a period of days or weeks (Blaine and Dixon 1973; Duportets et al. 1998; Gillott and Friedel 1976; Socha 2004), compared to approximately nine months prior to reproductive activity in *C. fraxinella*.

Based on behavioural (Chapters 2, 3), EAG (Chapters 2, 3) and sex accessory gland (Chapter 5) results, my work has shown that juvenile hormone

(JH) is the main endogenous factor responsible for termination of reproductive diapause and enhancement of pheromone response in male *C. fraxinella*. This finding is similar to other Lepidoptera that undergo a delay in mating and exhibit pheromone response plasticity (Denlinger et al. 2005; Kopper et al. 2001; Shiga et al. 2003). In most experiments conducted in this thesis (Chapters 2, 3), male pheromone response is enhanced with JHA treatment late in reproductive diapause in the fall, but not in early reproductive diapause in the summer. Juvenile hormone analogue treatment also increases the protein content of male sex accessory glands in the fall, but not in the summer (Chapter 5). Juvenile hormone is the main hormone involved in the termination of reproductive diapause in other insects, including adult maturation of male sex accessory glands (Gillott and Gaines 1992), and increased pheromone responsiveness by male Lepidoptera to female sex pheromone (Anton and Gadenne 1999; Gadenne et al. 1993). It is likely that JH impacts male *C. fraxinella* pheromone response and sex accessory gland development separately, as has been shown in *A. ipsilon* (Duportets et al. 1998). Juvenile hormone regulates male sex accessory gland development in many distantly related groups, and its absence halts or delays maturation of the glands (Gillott and Gaines 1992). Juvenile hormone is necessary for the increase in male pheromone responsiveness observed in reproductively active male *A. ipsilon* (Gadenne et al. 1993), however, to my knowledge *C. fraxinella* is the first documented species in which JHA treatment has a differential effect on reproductive activity, as measured by pheromone

responsiveness (Lemmen and Evenden 2009, Chapters 2, 3) and sex accessory gland development (Chapter 5), throughout the period of reproductive diapause.

Evidence from my most recent experiment (Chapter 3) suggests that male *C. fraxinella* physiology may be changing at the population level in its introduced range, as males are more responsive to pheromone after treatment with JHA earlier in reproductive diapause than has been previously reported (Lemmen and Evenden 2009). Mating and oviposition on newly flushed secondary growth at the base of ash trees has been observed during the summer (Tyler Wist, unpublished data), which indicates a possible first step in the development of multivoltinism in *C. fraxinella*. A second summer generation of *C. fraxinella* would likely be small, and constrained by access to fresh ash leaflets of secondary growth (Evenden 2009).

The biogenic amines octopamine, dopamine and serotonin do not play a role in the enhancement of male *C. fraxinella* response to pheromone when males are reproductively active or when they are in reproductive diapause, based on EAG and behavioural results (Chapter 2). This is contrary to other research on reproductively active moths in which octopamine treatment typically enhances male moth response to pheromone (Jarriault et al. 2009; Linn and Roelofs 1986; Linn et al. 1992; Linn et al. 1996; Pophof 2000; Pophof 2002). Treatment with octopamine, dopamine or serotonin actually lowers male *C. fraxinella* response to pheromone during reproductive diapause in the summer and fall. In male *A. ipsilon*, octopamine treatment has no impact on the pheromone response of newly-eclosed sexually immature males (Jarriault et al. 2009). It is essential that

males do not waste energy by attempting to locate and mate with refractory females (Pener 1992), and in *C. fraxinella*, biogenic amines may be important in maintenance of reduced pheromone responsiveness of males during reproductive diapause. Since there is already evidence in moths for the direct action of biogenic amines on male pheromone-specific olfactory neurons (Grosmaitre et al. 2001; Nickisch-Rosenegk et al. 1996, Pophof 2000), it is possible that in *C. fraxinella* biogenic amines reduce or inhibit olfactory neuron response to pheromone during the period of reproductive diapause.

Environmental conditions are important cues in the maintenance and termination of reproductive diapause in male *C. fraxinella*. Long day/warm conditions terminate reproductive diapause in male *C. fraxinella*. Pheromone-mediated behaviours in the wind tunnel are enhanced in males in reproductive diapause when they are held under long day/warm conditions compared to those held outdoors under natural conditions or indoors under short day/cool or long day/cool conditions during the same period (Chapters 2, 3). Photoperiod and temperature are the token stimuli that initiate, maintain and terminate reproductive diapause in many insect species (Kostal 2011). Short day conditions induce and maintain diapause and long day conditions terminate diapause or prevent individuals from entering diapause in species in many insect orders, including Coleoptera (Berkvens et al. 2008), Hymenoptera (Kipyatkov and Lopatina 2009), Orthoptera (Zhu et al. 2013), Hemiptera (Kostal et al. 2008; Morita and Numata 1997; Musolin and Ito 2008; Nakamura and Numata 2000; Numata 1992), and Lepidoptera (Barker and Herman 1976; Fujita et al. 2009; Pieloor and Seymour

2001; Pullin 1986). In male *C. fraxinella*, long day/warm conditions will terminate reproductive diapause after several weeks of exposure to these conditions, and JHA treatment will also terminate diapause in males. It is likely that diapause-terminating environmental conditions trigger activity in the *corpora allata* to produce JH or JH acids in male *C. fraxinella*, which terminates diapause and increases male response to female pheromone. This is similar to what occurs in monarch butterflies, *Danaus plexippus* (Lepidoptera: Nymphalidae), in which reproductive diapause is initiated and maintained by short day/cool conditions and can be terminated with long day/warm conditions or JH treatment (Barker and Herman 1976; Goehring and Oberhauser 2002; Herman 1981). Juvenile hormone also terminates reproductive diapause in female *C. fraxinella*, as females treated with JHA during reproductive diapause develop chorionated eggs (Evenden et al. 2007).

Male and female *C. fraxinella* EAG and male behavioural response to ash volatiles is plastic and depends on moth physiological state (Chapter 4). Both males and females have the highest EAG responses to individual host plant volatiles when they are reproductively active. It is expected that moth response to host volatiles be most acute when moths are reproductively active, since females search for oviposition locations and males search for mates during the spring (Pohl et al. 2004). Both females and males are present in ash trees while reproductively active in the spring (Evenden and Gries 2008; Pohl et al. 2004). It is also expected that moth response to host volatiles is lower during the period of reproductive diapause after eclosion in the summer and fall, as moths are not

present in ash trees in nature during this time (Evenden and Gries 2008; Pohl et al. 2004). After moth eclosion in the summer, adults in reproductive diapause typically leave their host and aestivate away from ash trees, and are observed conducting searching behaviours in the fall, presumably searching for overwintering locations away from ash trees (Evenden 2009; Pohl et al. 2004). Overwintering locations of *C. fraxinella* are unknown (Pohl et al. 2004), however they have been observed leaving wood and compost piles in communities close to ash trees in the spring (T. Wist and J. Lemmen, personal observations).

There is no synergism between an ash seedling and female sex pheromone that impacts male *C. fraxinella* behavioural response to the odours. Male response to female sex pheromone increases with the presence of host plant volatiles in some species of moths including *Lobesia botrana* (Lepidoptera: Tortricidae) (Von Arx et al. 2012), *Cydia pomonella* (Light et al. 1993; Yang et al. 2004), *Grapholita molesta* (Lepidoptera: Tortricidae) (Varela et al. 2011), *Spodoptera exigua* (Deng et al. 2004), *Helicoverpa zea* (Lepidoptera: Noctuidae) (Light et al. 1993), and *Eupoecilia ambiguella* (Lepidoptera: Tortricidae) (Schmidt-Busser et al. 2009). I expected the presence of an ash seedling to increase male *C. fraxinella* response to pheromone when they are reproductively active, since in nature males are known to fly to ash trees at this time of year (Evenden 2009). It seems in *C. fraxinella*, that male flight to host ash trees in the spring is driven mainly by response to pheromone rather than ash volatiles, despite the high response to host plant volatiles observed in EAG experiments during times of reproductive activity.

The role of JHA treatment in host plant volatile modulation differs between males and females. Unlike the role of JHA in male pheromone response, when males are in reproductive diapause, JHA treatment increases male response to several host plant volatiles in the summer, and to most of the volatiles in the fall. In females, JHA treatment increases response to some of the host plant volatiles in the summer, but not to any of the volatiles in the fall. It is possible that the increase in antennal response to host plant volatiles with JHA treatment in males is part of an overall enhancement of antennal response to odours that is also observed when fall males are treated with JHA and tested to pheromone (Lemmen and Evenden 2009). This seasonal effect of JHA treatment on female response to host plant volatiles is different from the effect of JHA treatment on female reproductive status. In female *C. fraxinella*, mating and oogenesis are dependent on JH, and females in reproductive diapause are more likely to break diapause, produce eggs and mate if treated with JHA and provided a carbohydrate nutrition source later in the period of reproductive diapause in the fall rather than earlier in the summer (Evenden et al. 2007). Juvenile hormone analogue treatment likely impacts female *C. fraxinella* EAG response to host plant volatiles and female reproductive state separately, as is likely the case in male response to pheromone and sex accessory gland development. The differential role of JHA on female host volatile response during the period of reproductive diapause further supports evidence that females are in distinct physiological states in the summer and fall (Evenden et al. 2007) similar to males (Lemmen and Evenden 2009; Chapters 2, 3, 4, 5).

It is clear that newly-eclosed *C. fraxinella* in the summer are in a different physiological stage of reproductive diapause compared to moths in the fall. A small portion of the *C. fraxinella* population do eclose in a reproductively active state, as a small number of moths will mate and females will develop eggs soon after eclosion in the summer under natural field conditions and when held under long day/warm conditions in the laboratory (Evenden et al. 2007). Male EAG and behavioural response to pheromone is also typically slightly higher in the summer compared with the fall (Chapter 2). In this study, I also found that male and female response to some host volatiles increases with JHA treatment early in reproductive diapause in the summer (Chapter 4), and male response to pheromone (Chapter 3) increases with exposure to long day/warm conditions or, in recent experiments JHA application in the summer. In the fall, moth physiology is quite different. All moths that overwinter are virgin (Evenden et al. 2007), untreated females do not mate or develop eggs (Evenden et al. 2007), and male response to pheromone is very low when males are maintained under natural conditions (Chapter 2,3,4). Also in the fall, male response to pheromone (Lemmen and Evenden 2009) and female oogenesis and receptivity to mating (Evenden et al. 2007) readily increase with JHA treatment. Moths early in reproductive diapause post-eclosion either have two populations, one small reproductively active portion and a larger portion in reproductive diapause, or newly-eclosed moths are in a lighter phase of diapause that could result in opportunistic mating if the right conditions occur. Some mating and oviposition has been observed in the summer, with females ovipositing on new ash leaflets at

the base of ash trees (T. Wist, personal communication). Moths in the fall have received overwintering cues such a naturally declining photoperiod and colder temperatures, and seem to be in a more complete state of reproductive diapause, during which JHA treatment has a greater impact on male and female reproductive development (Evenden et al. 2007; Chapter 5) and male pheromone response (Chapters 2, 3).

Many exciting and new conclusions can be reached from the work in this thesis. Not much work has been done on response to semiochemicals in a species of moth with an extended period of reproductive diapause that includes distinct physiological stages. This is the first study to demonstrate that JHA treatment impacts the peripheral nervous system in a female moth, and also the first to show that JHA treatment impacts antennal response of males and females to host plant volatiles (Chapter 4). This is also the first study to demonstrate that JH impacts male sex accessory gland development differentially during the period of reproductive diapause (Chapter 5). The reduced EAG and behavioural response of males to pheromone with biogenic amine treatment has not been documented before during a period of reproductive diapause, and may be the first evidence that biogenic amines are important in maintaining low pheromone responsiveness during reproductive diapause (Chapter 2). Finally, the finding that in recent experiments JHA treatment impacts male pheromone response earlier in period of reproductive diapause than has been previously documented (Lemmen and Evenden 2009) supports the hypothesis that the *C. fraxinella* population is

changing, and this may be the first step in the development of a small second generation in this species in its expanded range (Chapter 3).

Future Directions

The life history of *C. fraxinella*, with a nine-month reproductive diapause, allowed me to further our understanding of moth olfactory plasticity and physiology in a species that experiences an extended reproductive diapause. The data in my thesis provide information that confirms the important roles of temperature, photoperiod and JH in diapause maintenance and termination in male *C. fraxinella*. These results also illustrate the importance of replicating natural conditions prior to experimentation.

There is an opportunity to further examine the mechanisms of factors controlling reproductive diapause in this species. It is not known whether the *corpora allata* of male *C. fraxinella* produce JH or JH acids, and the mechanisms by which JHAs act on male sex accessory gland development and pheromone response are not known. It is also not known whether long day/warm diapause terminating environmental conditions triggers male *corpora allata* to produce endogenous JH or JH acids, or whether it stimulates a different neuromodulator or allatotropic factor, which in turn stimulates the *corpora allata*. We know that JHA enhances male EAG response to pheromone, but we don't know whether it has an additional effect on the central nervous system. Intracellular recordings of male antennal lobe neurons could confirm whether male pheromone response is also modulated at the level of the central nervous system, as it is in *A. ipsilon* (Anton and Gadenne 1999; Gadenne and Anton 2000).

The plasticity of male and female *C. fraxinella* to host plant volatiles is an area in which much more research can be conducted. Electroantennogram responses of males and females can be tested to a complete blend of ash host volatiles. This would determine if moth response to the actual signal released from the tree is also highest during the period of reproductive activity in a manner similar to EAG response to individual host plant volatiles (Chapter 4). It would also be valuable to conduct intracellular recordings on male and female *C. fraxinella* responses to host volatiles, to see whether EAG response directly translates into behavioural response, or whether there is central nervous system modulation of host plant volatiles on moth behaviour.

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