Semiochemical-Mediated Interactions of an Invasive Leaf miner, *Caloptilia fraxinella* (Lepidoptera: Gracillariidae), on a Non-Native Host, *Fraxinus* spp. (Oleaceae) and its Native Parasitoid, *Apanteles polychrosidis* (Hymenoptera: Braconidae)

by

Tyler Jonathan Wist

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Abstract

The ash leaf-coneroller, *Caloptilia fraxinella* (Ely) (Lepidoptera: Gracillariidae) is an introduced moth to the urban forest of Western Canada that specializes on ash (*Fraxinus* spp.) (Oleaceae). It uses green ash, *F. pensylvannica* Marsh. var. *subintegerrima* (Vahl) Fern*,* and black ash, *F. nigra* Marsh in its expanded range but prefers black over green ash for oviposition. The oviposition preference for black ash was not linked to enhanced performance of offspring on black ash as green ash supported higher larval survival and faster development than black ash. Younger leaflets are preferred by female moths over older leaflets and green ash elicits more host location flight by gravid females than black ash in wind tunnel experiments.

Caloptilia fraxinella adults are attracted to volatile organic compounds (VOCs) released from ash seedlings. The antennae of mated female *C. fraxinella* consistently detect six VOCs released from black ash and green ash, four of which are common to both species. Synthetic copies of these VOCs elicited as much oriented upwind flight in mated female *C. fraxinella* as green and black ash seedlings but did not elicit contact with the VOC lure. Lures do not attract more female *C. fraxinella* than unbaited control traps in field experiments. These experiments lay the foundation for further development of semiochemical lures based on host VOCs to monitor and potentially control populations of *C. fraxinella*.

Apanteles polychrosidis Vierek (Hymenoptera: Braconidae), is identified here as the dominant parasitoid of *C. fraxinella* in its expanded range. It exhibits protandrous adult emergence and adults mate soon after females emerge. The remainder of the parasitoid complex affecting *C. fraxinella* in Edmonton is, in order of parasitism rate, *Diadegma* Förster (near *D. fenestrale* (Holmgren)) (Hymenoptera: Ichneumonidae), three *Sympiesis* Förster species (Hymenoptera: Eulophidae) (*S. sericeicornis* (Nees von Esenbeck)*,* one near *S. viridula* (Thomson) and one unknown *Sympiesis* species, and *Pteromalus phycidis* (Ashmead)

(Hymenoptera: Pteromalidae). Of these parasitoids, only *A. polychrosidis* is present in numbers high enough to control populations of *C. fraxinella.* Parasitism rate by *A. polychrosidis* of *C. fraxinella* on black ash is higher than on green. Parasitism is positively dependent on *C. fraxinella* density on black ash but is negatively density dependent on green ash. *Apanteles polychrosidis* exhibits differential attraction to the VOCs of each ash species which may mediate the differential parasitism response. In olfactometer experiments, *A. polychrosidis* use host location cues induced by feeding damage on black ash but not on green ash. Thirteen ash VOCs are detected by the antennae of *A. polychrosidis*.

Preface

Chapter four of this thesis has been published as Wist T.J. and Evenden M.E. 2013. Parasitoid complex and bionomics of *Apanteles polychrosidis* on the ash leaf cone-roller. The Canadian Entomologist 145: 416-429. I was responsible for the data collection and analysis as well as the manuscript composition. Maya Evenden was the supervisory author and was involved with concept formation and manuscript editing.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

GC-EAD: Gas chromatographic-electoantennographic detection

GLV: Green leaf volatiles

HIPV: Herbivore-induced plant volatiles

Or.flight: Oriented upwind flight

ORN: Olfactory receptor neurons

IPM: Integrated pest management

FID: Flame ionization detector

GLMM: Generalized linear mixed effects model

SEM: Scanning electron microscopy

VOC: Volatile organic compound

Chapter 1: Introduction

The terms specialist and generalist describe the host ranges of herbivorous insects with specialists feeding on one or a few closely related host plants (mono and oligophages) and generalists (polyphages) feeding on many host species. Insect herbivores that specialize are more common than generalists (Bernays and Graham 1988). The host plant is usually chosen by the adult female and the preference hierarchy of the female determines the host range, especially when larvae are non-dispersive (Faeth 1985; Auerbach and Simberloff 1989).

Some generalist species with a diverse range of host plants use volatile organic compounds (VOCs) that are common among their hosts in host location for oviposition (Tasin et al. 2010). Common VOCs among plants also allow insects with narrow host ranges to adopt new host plants which are chemically similar to the old hosts (Agosta 2006, Erbilgin et al. 2014). Many VOCs are the by-products of primary metabolism of the plant. Volatile organic compounds are also derived from secondary metabolites that function to defend the plant against herbivory (Fraenkel 1959), environmental stress and invasion by micro-organisms (Schoonhoven et al. 2005). Several sensory cues from plants are integrated by insects when locating a host plant (Ramaswamy 1988) but chemical cues such as the ratio of attractive and repellent VOCs (Bruce et al. 2005) and stimulatory and deterrent surface chemicals are the dominant cues in host plant recognition (Renwick and Chew 1994).

Herbivore host plant preference and offspring performance

The “Mother-knows-best” (Gripenberg et al. 2010), preference-performance (Jaenike 1978), and “the optimal-oviposition” hypotheses (Thompson 1988) are all names for the same prediction, that the oviposition preference of a female insect should be positively linked to the performance of her offspring. Females should prefer to oviposit on or in host plants that maximize the fitness of their offspring (Jaenike 1978; Thompson 1988). A positive preference-performance linkage is especially important for leaf-mining insects with non-motile larvae in which host location is entirely dependent upon the oviposition choice of the female

(Faeth 1985; Auerbach and Simberloff 1989). When oviposition preference matches offspring performance the choice of the female confers fitness benefits to her and her offspring. A poor host plant choice by the female though, can significantly reduce or negate their fitness (Kagata and Ohgushi 2001). Therefore, positive linkages between preference and performance are expected and frequently occur; but negative linkages occur with a similar frequency (see references in Thompson 1988; Thompson and Pellmyr 1991; Mayhew 1997; Gripenberg et al. 2010). Offspring performance in the Lepidoptera is commonly measured with larval survival, larval development and pupal mass (Kagata and Ohgushi 2001; Yamasaki and Fujisaki 2010; Videla et al. 2012; Zhang et al. 2012). Survival of offspring is a good indicator of the performance of larvae on a given host plant (Kagata and Ohgushi 2001) while slow development suggests that host defensive compounds or inferior nutrition affect growth and increase the chance that a predator or parasitoid encounters the larvae (The slow growth-high mortality hypothesis, Benrey and Denno 1997). High pupal weight can indicate a high nutritional value of the plant and in most moths, large pupae lead to large adults with increased reproductive capacity (Lill and Marquis 2001).

Odour mediated host location by herbivorous insects

Plants release VOCs through regular respiration. Herbivorous insects have specific receptors to detect these plant VOCs (Visser 1986). A group of compounds called green leaf volatiles (GLVs) are conserved across plant families (Visser et al. 1979; Tressel et al. 1981) and are present in the headspace of most green (chlorophyllous) plants. Green leaf volatiles are comprised of six carbon alcohols, aldehydes and acetates produced by oxidation of leaf lipids (Visser et al. 1979). Other classes of plant-released VOCs that attract insects are the phenyl-propanoids, products of the shikimate pathway and isoprenoids (terpenoids) produced via the mevalonate and non-mevalonate pathways (Paré and Tumlinson 1997; Rohdich et al. 2002; Bruce et al. 2005). The entirety of the VOC blend and often its single components have been tested for electrophysiological, olfactometer and field activity on insects that exploit these plants as hosts (Visser and Avé 1978; Visser et al. 1979; Dickens 1984; Dickens 1989; Hansson et al. 1999; Nikonov et al. 2001). Often, individual olfactory receptor neurons (ORNs), contained in sensillae on the antennae of insects, are highly specific for one volatile molecule or class of molecules and can respond to small concentrations (~10 pg) of these odorants (Hansson et al. 1999). Individual ORNs usually respond solely to a pheromone or to a host plant VOC in single cell recordings (Dickens 1989) which indicate a specific role in olfaction for each receptor type. Some sensillae can house several ORNs that respond to different host plant VOCs (Pophof et al. 2005). Individual sensilla auricillica can respond to plant VOCs (Anderson et al. 2000) and pheromones (Ebbinghaus et al. 1998; Ansebo et. al. 2005).

Host location by lepidopterans

Host location by lepidopterans for oviposition can be divided into a sequence of three searching steps often mediated by plant-produced VOCs (Ramaswamy 1988; Liu et al. 2006): host-habitat location, host location and host acceptance. Host habitat location occurs over a long range whereby the female follows a stimulus or stimuli in order to find the host plant. Host location by moths that is mediated by VOCs is often studied in wind tunnel (Tasin et al. 2006a; b) and olfactometer (Pivnick et al. 1994) bioassays. One of the model systems for VOC-mediated flight in moths is *Manduca sexta* (Sphingidae) females attracted to the odour of fresh tobacco leaves in a wind tunnel (Willis and Arbas 1991). Volatile organic compounds travel in odour plumes from plants and these plumes are made up of isolated pockets of odorants (Schoonhoven et al. 1998). As these packets do not provide an odour gradient, plant volatiles often do not provide directional information to searching moths. Moth orientation to chemical cues is upwind with the aid of visual cues (optomotor anemotaxis) (Traynier 1968; Kennedy and Marsh 1974; Finch 1980). Female *M. sexta* fly upwind in response to tobacco leaves and slow down if they fly without contact with an odour pocket. In the event of losing an odour trail, moths fly across the wind current and "cast" backwards with the wind to regain contact with the odour pockets in the plume (Willis and Arbas 1991). Upon encounter with another odour pocket, moths resume their upwind flight towards the source. Field assays of VOC-mediated host location occur under natural conditions and most commonly test attractiveness of synthetic VOC lures to the target insect (Knight and Light, 2001; 2005; Cha et al. 2008; Knight el al. 2011; Lu et al. 2012).

The prevailing hypothesis, termed "the ratio-specific hypothesis" in host plant location in insects is that females recognize host plants through detection of specific VOCs that occur in the proper ratio representing their host plant (Bruce et al. 2005; Bruce & Pickett, 2011). In contrast, the "species-specific volatile" hypothesis (Fraenkel 1959) assumes that a single specific VOC identifies a plant as a host to the searching female. There are instances in which the "species-specific volatile" hypothesis does describe moth attraction to host plants. A single VOC, allyl isothiocyanate, is sufficient to identify cruciferous plants as hosts for the diamondback moth, *Plutella xylostella* (Plutellidae) (Han et al. 2001). Codling moth, *Cydia pommonella*, (Tortricidae) is attracted to a single VOC released by ripe pears, the pear ester (ethyl, (*E*,*Z*)-2,4-decadienoate) (Knight and Light 2004) which is used to bait traps to monitor codling moth populations against a background of odorants produced by apple, pear and walnut tree hosts. More commonly, moths use a blend of common plant VOCs to identify host plants (Bruce et al. 2005). Indeed, most insects require a blend of 3-10 VOCs to recognize host plants (Bruce and Pickett 2011). The grape berry moth, *Paralobesia viteana* (Clemens) (Lepidoptera: Tortricidae) uses a ratio of common plant VOCs to locate its host and when the ratio is altered to that released by an apple tree, most of the attraction to synthetic VOC lures is lost (Cha et al. 2011). The grapevine moth, *Lobesia botrana* (Dennis et Schiffermüller) (Lepidoptera: Tortricidae), is attracted by a blend of VOCs specific to its preferred host, *Daphne gnidium* (Thymelaeaceae) and a subset of those VOCs which are released from another host (Tasin et al. 2010). These results suggest that a plastic response to the identity and ratios of plant VOCs allows for shifts onto new host plants. The ratio of VOCs of the host plant is variable with phenology and so plasticity in response to VOCs permits host location at opportune times for host colonization (Najar-Rodriguez et al. 2010). Plasticity of response to varying ratios of common VOCs allows a specialist herbivore to recognize closely related host plants and could result in a preference hierarchy of hosts within the narrow host plant range.

Host plant location for oviposition can require response to other non-chemical cues and often a multi-modal response is necessary (Ramaswamy 1988; Reed et al. 1988; Renwick and Chew 1994; Smallegange et al. 2006; Teles Pontes et al. 2010). Visual cues can guide the flying insect as it approaches an oviposition substrate (Bernays and Chapman 1994). The tomato fruit borer, *Neoleucinodes elegantis* (Guenée) (Lepidoptera: Crambidae) cannot pinpoint oviposition substrates in complete darkness, even in the presence of VOC cues, thus visual cues are necessary to properly place eggs (Teles Pontes et al. 2010). Blinded peach tree borers, *Synanthedon pictipes* (G&R) (Lepidoptera: Sessidae) rarely choose the most appropriate oviposition host and substrate compared to sighted conspecifics (Reed et al. 1988). Vision is less important than VOC cues in host location by the crepuscular moth, *Plutella xylostella* (L.)*,* (Lepidoptera: Plutellidae) and moths can find their preferred oviposition host even under red-light conditions (Couty et al. 2006). Vision is not always integrated with VOC cues for host location and the geometrid, *Cidaria albulata* seems only to use VOC cues to locate hosts (Douwes 1968).

Leaf-mining microlepidopterans orient to VOCs released by their hosts (Hanson, 1995). For example, female potato moths *Phthorimaea operculella*, (Gelechiidae), miners on potato leaves (Solanaceae: *Solanum* spp) are attracted to host VOCs from potato plants in field (Foot 1979; Fenemore 1988) and laboratory olfactometer studies (Goldson and Emberson 1977). The navel orangeworm, *Amyelois transitella* (Pyralidae) is attracted over long distances to VOC of its host plants (Phelan and Baker 1987, Phelan et al. 1991). Host location is mediated by plant VOCs in tortricid micro-moths studied to date (Coracini et al. 2004; Tasin et al. 2006a; 2006b; Cha et al. 2008; Tasin et al. 2010). Host volatiles are likely the main source of attraction for oviposition on plants for all microlepidopterans.

A limited number of studies evaluate the VOC-mediated host plant location for oviposition by microlepidopteran leafminers. The tomato leaf miner, *Tuta absoluta*, (Meyrick) (Lepidoptera: Gelechiidae) uses small variations in the identity and ratio of VOCs to locate the most suitable host plant for offspring development (Proffit et al. 2011). Leaf mining by larvae of the horse chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić (Gracillariidae) significantly changes the VOCs of horse chestnut *Aesculus hippocastanum* Sap. (Hippocastanaceae) leaves. Oviposition and the early mining stages (1st-3rd instar) stimulate production of alcohols, aldehydes, phenolics, and terpenes while later mining stages (4th and 5th instar), and the ensuing reduction in green leaf tissue, release more tridecane, furanoid, and aldehydes, such as decanal and (*E,E*)-2,4-hexadienal (Johne et al. 2006). Leaf mining-induced changes in GLVs are detectable by moths and four of the single compounds released in early larval infestation elicit oviposition. In contrast, two of the compounds released during late stage larval mining deter oviposition. A change in the identity and ratio of VOCs signals to gravid females that the leaf is no longer suitable for larval development (Johne et al. 2006). Female *Stilbosis juvantis* (Cosmopterigidae), an oak (*Quercus* spp.) leaf miner, also preferentially oviposit on undamaged leaves rather than damaged leaves (Faeth 1985). Changes in VOCs that signal maturation of leaves are reduced emissions of methanol and terpene (Bracho-Nunez et al. 2011).

The final step in choosing a host plant is host acceptance followed by oviposition if the host plant is suitable. Moths use both tactile and chemical cues either alone or together to accept or reject a host. Chemical cues are sensed by female moths through physical contact with the substrate of sensory structures present on the antennae, tarsi, and ovipositor. Females of the citrus leaf miner, *Phyllocnistis citrella*, (Lepidoptera: Gracillariidae) search leaves of the lemon tree, *Citrus limon*, (Rutaceae) by walking linearly with antennae touching the leaf surface. When moving from leaf to leaf within a canopy, female moths predominantly fly upwards and thus search the lower canopy first and then make their way to the upper canopy (Liu et al. 2006). The limabean pod borer, *Etiella zinckenella,* (Lepidoptera: Pyralidae) searches soybean pod surfaces with bent abdomen and antennae in contact with the pod substrate (Hattori 1988). This moth lays single eggs after contact with sepals or trichomes which suggests either a tactile oviposition acceptance cue or a contact chemical cue released from the trichomes. Potato moths, *P. operculella*, use a complex of amino acids and specifically L-glutamic acid, which is found in high concentrations in potato leaves, in oviposition host acceptance (Meisner et al. 1974). The aspen blotch miner, *Phyllonorycter salicifoliella* (Lepidoptera: Gracillariidae), discriminates between three host species of *Populus* and oviposits less frequently on *P. balsamifera* due to a higher level of phenolic glycosides in the leaves compared to its primary host, *P. tremuloides* (Auerbach and Alberts 1992). Differential oviposition by this gracillariid moth indicates that females use contact chemoreception to discriminate among host plants at close range.

Host acceptance by female leaf miners also involves positioning of the egg on the leaf. Leaves are not homogeneous units but vary significantly, especially between their upper and lower surfaces (Reavey and Gaston, 1991). Many leaves do not have stomata on the upper surface to reduce water loss by evapo-transpiration. This situation results in two-tiers of parenchyma with palisade mesophyll beneath the adaxial (upper) leaf surface and the lower spongy mesophyll adjacent to the abaxial (lower) leaf surface, which contains air pockets of high humidity connected with stomatal pores. The different areas within the leaf represent separate environments to leaf-inhabiting insects such as leaf miners. Oviposition preference can also occur within a leaf as the position of offspring can influence larval survival. The surface texture between the adaxial and abaxial leaf surfaces is often extremely different which introduces the possibility of tactile acceptance cues for oviposition. Visual cues are almost certainly involved in identification of the leaflet surfaces (Van Leerdam et al. 1984). Leaf-mining moths from diverse families prefer to oviposit on hirsute leaf surfaces (Fenmore, 1978; 1980; Kan and Waku 1985; Gupta and Thorsteinson 1960) as opposed to smooth leaf surfaces (Hagley et al. 1980). Leaf trichomes may protect leaf-mining offspring from natural enemies (Sugiura 2011). Contact with surface trichomes and sepals stimulate oviposition in the lima bean pod borer with no apparent chemoreceptive cues necessary (Hattori 1988).

Tritrophic interactions

Host location by insects in the second, third and higher trophic levels is often mediated by volatile organic compounds (Dudareva et al. 2006). Plants can signal the presence of feeding herbivores to herbivore natural enemies such as predators and parasitoids through VOC emissions (reviewed by Price et al. 1980, Paré and Tumlinson 1999; De Moraes 2000; Dudareva et al. 2006; Turlings and Ton, 2006; Unsicker et al. 2009). This “signalling” by damaged plants has been referred to as a “call” to the third trophic level in the form of induced, indirect plant defence. In this relationship, the parasitoid responds by orientation to herbivore-induced plant volatiles (HIPV) that signal the presence of an herbivore host. This indirect defensive strategy of plants is called the “body-guard” hypothesis (Price et al. 1980) because herbivore damage to the plant induces a change in the plant volatile profile that “calls” the herbivore’s natural enemies to assist the plant. Herbivore induced plant volatiles (Dicke et al. 1990; Turlings et al.1990; 1991a,b; De Moraes et al. 1998) can even occur as a response to oviposition (Fatouros et al. 2012). Natural enemies of herbivores may have been pre-adapted to use HIPVs to orient to plants where their hosts could be found. One theory suggests that parasitoids of herbivores are descendants of parasites of the plants themselves and that use of larval hosts present on plants is a derived trait (Vinson 1976). Thus, attraction to plant VOCs as a method of locating plants would have occurred prior to the development of the parasitoid habit in these wasps. The plant may benefit as herbivores that feed on it are killed but few studies have measured increases in plant fitness as a result of HIPV production. Recent work suggests that hyperparasitoids (parasitoids of parasitoids) also orient to HIPVs, calling into question the "call for help" or "body guard" hypothesis (Kaplan 2012).

Chemically-mediated host location by parasitoids

To find hosts, parasitoids follow a strategy divided into several stages (Flanders 1953; Lewis et al. 1976; Salt 1935). The first stage is inactivity in which an innate releaser is triggered when the parasitoid is physiologically ready to oviposit. The parasitoid enters the habitat-searching stage where it orients over long distances via plant VOCs to the host's area. The parasitoid enters the search and attack stage in the vicinity of hosts and this is mediated by short range cues associated with the host insect. Search and attack is further divided into substeps such as approach to host, host examination and oviposition and these are mediated by physical, visual and chemical stimuli.

Habitat location by parasitoids from a distance is primarily mediated by attractive VOC cues (Visser 1986; Vet and Dicke 1992; Mumm and Dicke 2010). Parasitoids locate the habitat of their host by orientation to a plant that typically harbours host larvae. Volatile organic compounds from plants are the most likely long range orientation cues due to the large volatile signal presented by plants compared to the small size of the parasitoid's herbivorous host. Volatile cues from host insects such as kairomones found in herbivore frass are used less by parasitoids than plant cues in long range detection of hosts because the signal is smaller and less volatile than that presented by the damaged plant (Turlings et al. 1991). However, long range orientation to kairomones from the host insect does occur, for example, *Microplitis croceipes* (Hymenoptera: Braconidae) is attracted to frass and larvae of *Heliothis virescens,* a pest of cotton (Elzen et al. 1987).

Herbivore-induced plant volatiles are grouped into three main classes of compounds; green leaf volatiles derived from fatty acid synthesis, phenyl-propanoids, and terpenoids

(Arimura et al. 2009). Herbivore-induced plant volatiles attract herbivore natural enemies such as predatory mites (Dicke and Sabelis 1988), generalist predators such as lady bugs (Coleoptera: Coccinellidae) (Reddy 2002) and parasitoid wasps (Turlings et al. 1990; Whitman and Eller 1990) but have also been shown to attract hyperparasitoids that parasitize parasitoids (Kaplan 2012). Changes in the plant VOC profile induced by herbivory can be quantitative in which the ratios but not the identities of the VOCs change or qualitative in which VOCs distinct from those in the undamaged VOC profile are released (Dicke et al. 2003). Herbivore-induced plant volatiles that attract *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) are induced in maize plants by injection of the salivary exudate from a stem borer larva (Potting et al. 1995), which indicates that the elicitor of the HIPV response is present in the oral secretion of the herbivore. The HIPVs released by the plant can differ with the herbivore that induces them for attraction of the appropriate parasitoid (Dicke et al. 1990a,b; Dicke 1999). In many of the tritrophic interactions in which HIPVs are identified, the primary parasitoids are *Cotesia* or *Apanteles* species (Hymenoptera: Braconidae) (Mumm and Dicke 2010) which were within the same genus until they were taxonomically split (Mason 1981).

While HIPVs are important for parasitoid attraction, parasitoids may also be attracted to constitutively available VOCs from undamaged plants. For example, naïve *Microplitis croceipes* are attracted to undamaged cotton, *Gossypium hirsutum,* in a wind tunnel (Elzen et al. 1987). Odours from undamaged *Zea mays*, *Sorghum bicolor,* and napier grass, *Pennisetum purpureum*, attract two *Cotesia* spp. (Ngi-song et al. 1996). In some cases, mechanical damage to plant tissue alone is sufficient to attract parasitoids. For example, *C. glomerata* (Hymenoptera: Braconidae) is more attracted to artificially damaged leaves than to larvae or frass but damage by an herbivorous insect is still most attractive to the parasitoid (Steinberg et al. 1993). *Cotesia glomerata* and *C. rubecula* are highly attracted by odours emanating from herbivore-damaged plants, whereas odours from larval frass or the larvae themselves are much less attractive (Nealis 1986; Steinberg et al. 1993). *Cotesia flavipes* is attracted by the odour of maize stems damaged by the stem borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), and the scent of stem borer frass (Potting et al. 1995; Ngi-song et al. 1996). In this system, HIPVs are produced not just at the site of damage but are induced as a systemic response in other plant tissues, as uninfested leaves from plants with infested stalks attract parasitoids (Potting et al. 1995).

Systemic induction of volatile production occurs in several plants including tomato (Farag and Paré 2002), *Arabidopsis thaliana* (Van Hulten et al. 2006), and corn (Turlings and Tumlinson 1992) and can occur in response to mechanical damage alone (Mithöfer et al. 2005; Connor et al. 2007). Release of HIPVs can also prime neighbouring conspecific plants not currently under herbivore attack (Arimura et al. 2002; Engelberth et al. 2004) and plants of other species (Kessler et al. 2006) to release HIPVs by activation of defence genes (Arimura et al. 2000; Pickett and Poppy 2001) and the jasmonate pathway (Thaler 1999). Herbivore-induced plant volatiles can be exploited for use in pest management to attract beneficial insects in the field (James 2003a; James 2003b; James 2005; James and Grasswitz 2005; Turlings and Ton 2006). Methyl salicylate, methyl jasmonate, and (*Z*)-3-hexenyl acetate attract encyrtid and mymarid parasitoid wasps to grape crops (James and Grasswitz 2005).

Long range host-location to HIPVs by parasitoids of leaf miners occurs in many tritrophic systems (Sugimoto et al. 1988a; Dicke and Minkenberg 1991; Petitt et al. 1992; Keller and Horne 1993; Lengwiler et al. 1994; Finidori-Logli et al. 1996; Dutton et al. 2000). *Cotesia plutellae* (Hymenoptera: Braconidae) is attracted to three six carbon GLVs, *(Z)*-3-Hexenyl acetae, *(E)*-2-Hexenal, and *(Z)*-3-Hexen-1-ol (Reddy et al. 2002), released from cabbage (Reddy and Guerrero 2000), fed upon by diamondback moth. The herbivore- induced blend of volatiles can be very complex such as in lima bean where 27 different compounds were induced by herbivory by spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae) and beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) (deBoer et al. 2008).

Host-location to plant VOCs by parasitoids of gracillariid leaf miners on deciduous trees has been demonstrated in one instance (Lengwiler et al. 1994; Dutton et al. 2000). *Apanteles* cf. *circumscriptus* (Nees von Esenback) (Hymenoptera: Braconidae) is more attracted to apple trees, *Malus domestica* (Rosaceae), damaged by the spotted tentiform leaf miner, *Phyllonorycter blancardella,* Fabricius (Lepidoptera: Gracillariidae) than undamaged trees (Lengwiler et al. 1994; Dorn et al. 1999; Dutton et al. 2000). Apple leaves infested with leaf miners attract more female *A.* cf. *circumscriptus* than undamaged leaves in a Y-tube olfactometer (Lengwiler et al. 1994) and the VOC cue was plant-derived (Dutton et al. 2000).

VOC-mediated differential parasitism

Some parasitoids differentially parasitize their herbivore hosts depending upon the host plant of the herbivore. Differential parasitism readily occurs when generalist parasitoids forage more on the plant as a niche, than specifically targeting their herbivore host (Matthews 1974). Differential parasitism is linked to semiochemical differences among the food plant of the herbivore (Benrey et al. 1997; Liu and Jiang 2003) and herbivore-damaged plants recruit more parasitoids than undamaged plants (Turlings et al. 1990; Rose et al. 1998). Different host plants can respond to feeding from the same herbivore with different HIPVs (Turlings et al. 1995; Dicke and van Loon 2000; Shiojiri et al. 2010). Chemically mediated differential parasitism is well documented (Barbosa et al. 2001; Liu and Jiang 2003; Lou et al. 2006; Poelman et al. 2009; Degen et al. 2012) and can be linked to the parasitoid's response to the density of the herbivore on different host plants (Lessells 1985; Stiling 1987; Romeis et al. 1997; Fernandez-Arhex and Corley 2003). Herbivore density may be broadcast to parasitoids by the intensity of the HIPV signal released (Geervliet et al. 1998; Shiojiri et al. 2010; Girling et al. 2011) and this signal is dependent on the host plant of the herbivore (Shiojiri et al. 2010).

The tritrophic system

In this thesis, the semiochemical-mediated tritrophic interactions of an specialist, herbivorous leaf-mining moth, the ash-leaf coneroller, *Caloptilia fraxinella* (Ely) (Lepidoptera: Gracillariidae) and a parasitoid wasp, *Apanteles polychrosidis* Vierek (Hymenoptera: Braconidae) are explored on two species of ash trees. This tritrophic interaction occurs in Edmonton, AB (Pohl et al. 2004), Saskatoon, Saskatchewan (personal observation), and Drumheller, AB (personal observation), and other western Canadian prairie cities where *C. fraxinella* exists such as Calgary, AB and Regina, SK.

First trophic level: Ash trees (Fraxinus spp.)

Ash trees, *Fraxinus* spp. (Oleaceae) are a significant part of the urban forest of Western Canadian cities. However, these ash trees are planted outside of their native ranges (Farrar 1998). The western limit of black ash in Canada is Southern Manitoba while green ash is found further West into Southern Saskatchewan (Farrar 1998). Black ash and green ash are popular choices for urban forests as boulevard and park trees. For example, green ash comprises ~40% and black ash 2% of the canopy in Edmonton, AB (Saunders et al. 2004), and the City of Saskatoon, SK has nearly 19,000 ash trees total (~20% of the tree inventory) (Jeff Boone, City of Saskatoon Pest Management: personal communication). The most numerous ash species in the urban forest are black ash, *Fraxinus nigra* Marsh., and green ash, *F. pennsylvanica* Marsh. var. *subintegerrima* (Vahl) Fern. with Manchurian ash, *F. mandshurica* Rupr. and hybrids of black and Manchurian ash (vars. “Northern Gem” and “Northern Treasure”) also planted. The ash leaf is odd pinnately compound usually with 5-7 (green ash) and 9-13 (black ash) distinct leaflets.

Volatile profiles of green ash (Markovic et al. 1996, de Groot et al. 2008) and Manchurian ash, *F. mandshurica* (Rodriguez-Saona et al. 2006) have been identified. Four green leaf volatiles (GLVs) released by Manchurian and green ash: hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenol, and (*E*)-2-hexenol, are electrophysiologically active and attractive to the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Rodriguez-Saona et al. 2006; de Groot et al. 2008). Seven VOCs were identified from the headspace of green ash leaves; *trans*-ocimene, linalool, methyl salicylate and *cis*-3 hexenyl acetate, (*E*) 4,8-dimethyl 1, 3, 7 nonatriene, α-farnesene, *trans*-nerolidol (Markovic et al. 1996) with several additional GLVs found in subsequent studies (Rodriguez-Saona et al. 2006; de Groot et al. 2008).

Second trophic level: Caloptilia fraxinella

Caloptilia fraxinella is a leaf-mining moth that has invaded horticultural ash plantings of *Fraxinus* spp. throughout the western Prairie Provinces (Pohl et al. 2004), and reduces the aesthetic value of these trees. This species is native to Canada and exists at low levels within the native range of its two hosts black ash, and green ash, which are native to Canada. Prior to 1998, *C. fraxinella* was recorded only from southern Québec and Ontario (Pohl et al. 2004). Host plants of *C. fraxinella* in urban municipalities are ornamental green ash, black ash, Manchurian ash and white ash (*F. americana* L.) (Pohl et al. 2004). In 1999, *C. fraxinella* was first found in Edmonton, AB (Pohl et al. 2004), and Saskatoon, SK in 1998 (Wist, unpublished, June 1998) and has since become ubiquitous on ash trees in the urban forest of all of the Prairie municipalities where *Fraxinus* species occur. *Caloptilia fraxinella* populations have increased for the past twelve years and now infest most ash trees citywide in Edmonton. Damage to ash trees caused by *C. fraxinella* is considered cosmetic and therefore, no control measures have been undertaken by municipalities to target this invasive species (Pohl et al. 2004).

The ash leaf coneroller is a microlepidopteran moth with both a leaf mining and leaf rolling habit that damages host plants. The moth itself has a long adult stage (nine months) due to a summer aestivation and obligate winter reproductive diapause (Evenden et al. 2007). Adult moths emerge from overwintering sites in early spring, and mate before ash leaves flush on trees (Evenden et al. 2007). Eggs are laid singly (Fig. 1-1A, B, F) but in clutches (Chapter

2) of up to 30 eggs (Pohl et al. 2004) on the adaxial surface of newly flushed leaflets of horticultural ash. Prior to hatching, the black head capsule of young larvae can be seen within the chorion of their egg (Fig. 1-1B). After hatching, first instar larvae sometimes enter the leaflet tissue after a short migration across the leaflet (Fig. 1-1F) or more commonly, beside their shed chorion (Fig. 1-1D). First and second instar larvae consume the upper palisade mesophyl layer and remain visible beneath the cuticle of the adaxial leaflet surface (Fig. 1-1D,C,E,F). First and second instar larvae mine in a serpentine pattern (Fig. 1-1G,H). Serpentine mines often intersect (Fig. 1-1E,G) and more than one larva can occur in the same mine (Pohl et al. 2004). The second instar larvae mine to the leaflet edge where they form blotch mines on the leaflet margins (Pohl et al. 2004) when they stop mining in a forward direction and consume tissue around them (Fig 1-1H). The edges of a heavily infested leaflet often curl inwards (Fig. 1-1G,H) when third instar larvae spin a “mat” of silk within the blotch mine.

Fourth instar larvae exit the mined leaflet (Evenden 2009) and either walk to a new leaflet or release a silken line to “balloon” to adjacent leaflets. Once on a new leaflet, the larva rolls the tip of the leaflet upwards until it forms a characteristic upside-down pyramidal cone (Fig. 1-1K), then seals the edge of the cone to the base of the lamina with silk. Within the leaflet cone the larvae feed and pass their fourth and fifth instars (Fig 1-1I,J). They deposit frass in the lower portion (tip of the pyramid) of the leaflet cone (Fig. 1-1I) which harbours fungi and often leads to unsightly decay of the leaflet during the summer months. This sanitation feature may be an anti-predator defence because some parasitoids use VOCs from frass to find their hosts (Elzen et al. 1987; Takabayashi & Takahashi 1989).

Before pupation, larvae chew an exit window through the parenchyma in the base of the pyramid (upper leaflet surface) (Fig. 1-2A) leaving the upper cuticle intact. The cocoon that harbours the pupa (Fig. 1-2D, upper) is spindle shaped, straw coloured and is anchored within the base of the pyramidal leaflet-cone by thin silk strands attached to each end of the cocoon (Pohl et al. 2004). Three pupae can occur per leaflet roll (Fig. 1-2A,B) (Pohl et al. 2004) and up to four may occur (personal observation) in trees with high *C. fraxinella* densities. At eclosion, the cocoon and pupal case are left behind (Fig. 1-2B,C) and sometimes the pupal case is left to extrude through the exit window (Pohl et al. 2004). Larvae pass through five larval instars before pupation based on the number of shed head capsules found inside mines and leaflet rolls (Pohl et al. 2004; Evenden 2009). The adult moth at rest sits in a characteristic gracillariid tripod pose with its body held at a 45° angle (Fig. 1-2F). The moth has a 12.0 mm wingspan and banding patterns of white, grey, brown, black and orange scales on their forewings (Pohl et al. 2004) (Fig. 1-2F). The cottony ash psylid, *Psyllopsis discrepans* (Homoptera: Psyllidae) uses the leaflet rolls of *C. fraxinella* on black ash as shelter (Fig. 1-2C) for their second generation nymphs (Wist: unpublished).

Third trophic level: Parasitoids, with a focus on **Apanteles polychrosidis**

Several parasitoids have been reared from *C. fraxinella*: *A. polychrosidis, Pteromalus phycidis* (Ashmead) (Hymenoptera: Pteromalidae), as well as *Zagrammosoma americanum* Girault and *Elachertus argissa* (Walker) (Hymenoptera: Eulophidae) (Pohl et at. 2004). *Apanteles* species (Hymenoptera: Braconidae), herein described to species as *Apanteles polychrosidis* Vierek (Hymenoptera: Braconidae) (Fig 1-2. G) is the dominant parasitoid in Edmonton (Pohl et al. 2004) and their interaction has been named the *Caloptilia/Apanteles* complex (Pommen, 2003). Little is known about the biology of this parasitoid wasp and how it exploits *C. fraxinella.* This knowledge gap provides an opportunity to describe the bionomics of the parasitoid and its interaction with this host including its host location and exploitation behaviour. *Apanteles polychrosidis* is reported to use larval hosts from the family Tortricidae (Lepidoptera) with an free feeding life habit (Seaman et al.1990; Biddinger et al. 1994; Li et al. 1999; LaGasa et al. 2000; Cossentine et al. 2004, 2005; Fernández-Triana and Huber 2010) and has not previously been documented to use hosts concealed in leaf mines prior to its association with *C. fraxinella* described here. *Apanteles polychrosidis* overwinters in larvae of obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), emerges in the spring to parasitize the summer generation, then overwinters again in the fall generation of *C. rosaceana* in orchards of British Columbia, Canada (Cossentine et al. 2004). With two generations, *Apanteles polychrosidis* is bivoltine (Cossentine et al. 2004; Cossentine 2008). *Apanteles polychrosidis* is widely distributed in the Nearctic Region (Fernández-Triana and Huber 2010). Other *Apanteles* species overwinter as pre-pupae or first and second instar larvae within overwintering lepidopteran larvae (Shaw and Huddleston 1991) but the overwintering host of *A. polychrosidis* in Edmonton is unknown.

The rate of *A. polychrosidis* parasitism of *C. fraxinella* in Edmonton was 26% in 2001 and dropped to 9% in 2002, and 2% in 2003 (Pohl et al. 2004). However, the rate of parasitism started to rise in 2006 (Wist, unpublished) and by 2007, the parasitism rate in Edmonton was roughly estimated at 75% from two sites (Chris Saunders, City of Edmonton Pest Management, personal communication) and sometimes as high as 80% (Dr. Joelle Lemmen, University of Alberta, personal communication.). Apparently this parasitoid has the potential to greatly increase its population density which will reduce the overall number of *C. fraxinella* moths that emerge from leaflet rolls, and may result in a reduction in *C. fraxinella* population density and damage the following season. Peak *A. polychrosidis* adult activity occurs in mid-July in Edmonton and corresponds emerging adults (Fownes 2002, Pommen 2003).

Apanteles polychrosidis females are found on ash trees while *C. fraxinella* are in their fourth instar and dispersing from leaflet mines to leaflet rolls (Wist and Evenden 2013). The initial hypothesis that host use may occur during their first to third larval instars while *C. fraxinella* larvae are in their leaflet mining phase was not supported. The leaflet mining larvae are visible through the thin cuticle of the ash leaflet (Fig. 1-1 C,D,E,H) and could also be vulnerable to attack by *A. polychrosidis* and other parasitoids at this stage based on a combination of lack of mobility due to confinement and little protection by the thin plant cuticle. The least likely stage for attack is the leaf-roller stage when the tightly bound leaflet provides a barrier to all but the most persistent and large predators, such as yellow jacket wasps (personal observation). Larvae are most likely already parasitized by their fourth larval instar when they roll leaflets in preparation for pupation (Fownes 2002; Pommen 2002) and with the significant increase in number of female *A. polychrosidis* at the fourth instar of its host (Wist and Evenden 2013) it is likely that the majority of oviposition occurs while *C. fraxinella* is exposed. *Apanteles polychrosidis* emerges from its host inside the cone-rolled leaflet and chews its way out of the leaflet roll. *Apanteles polychrosidis* parasitism is distinguishable by the side exit hole made by the adult upon eclosion (Fig. 1-2 E) and by the absence of an exit window excavated by *C. fraxinella* larvae prior to pupation on the pyramidal base of the leaflet roll. Inside the parasitized leaflet roll is a morphologically different cocoon (Fig. 1-2 D bottom) than that of *C. fraxinella* (Fig. 1-2 D top) and these do not exist in leaflet rolls together which confirms that *C. fraxinella* is killed by the emergence of *A. polychrosidis*.

Thesis overview and objectives

This thesis investigates the semiochemical communication among three levels in this tritrophic system. It begins with exploring the role of semiochemicals in host plant location by female *C. fraxinella* (Plate 1a). Semiochemicals are identified that are electrophysiologically active to *C. fraxinella* from both black ash and green ash. A blend of synthetic semiochemicals is tested for attractiveness to female *C. fraxinella* in a wind tunnel and in the field. The host plant preference of *C. fraxinella* is identified and the performance of larvae on two ash species is determined and compared to the oviposition preference of females (Plate 1b). The parasitoid community that has developed on *C. fraxinella* in its new range and their host use is investigated with special emphasis on *A. polychrosidis* (Plate 1c). The relationship of the parasitism rate of *A. polychrosidis* to the density of *C. fraxinella* is explored in the final data chapter (Plate 1d). Host plant VOCs from undamaged, mechanically damaged and leaf mined leaflets of black ash and green ash are tested against a clean air control to investigate the VOC signals used by *A. polychrosidis* to locate hosts. In addition, VOCs that are electrophysiologically active to *A. polychrosidis* are identified.

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Plate 1-1. Graphical overview of thesis. a) Chapter two examines the volatile organic compounds (VOCs) involved in host location for oviposition by female *Caloptilia fraxinella*. b) In Chapter three the oviposition and host location preference of female *C. fraxinella* for black ash and green ash is determined. Then the performance of larvae on each ash species is examined and compared with the preference of female moths in a test of the preference–performance hypothesis. c) Chapter four identifies the parasitoid complex, their rates of parasitism and the phenology of their use of *Caloptilia fraxinella* larvae to identify the dominant parasitoid. d) In chapter five the relationship of the parasitism rate of *Apanteles polychrosidis* to the density of *C. fraxinella* is explored and the VOC cues used in host location are investigated.

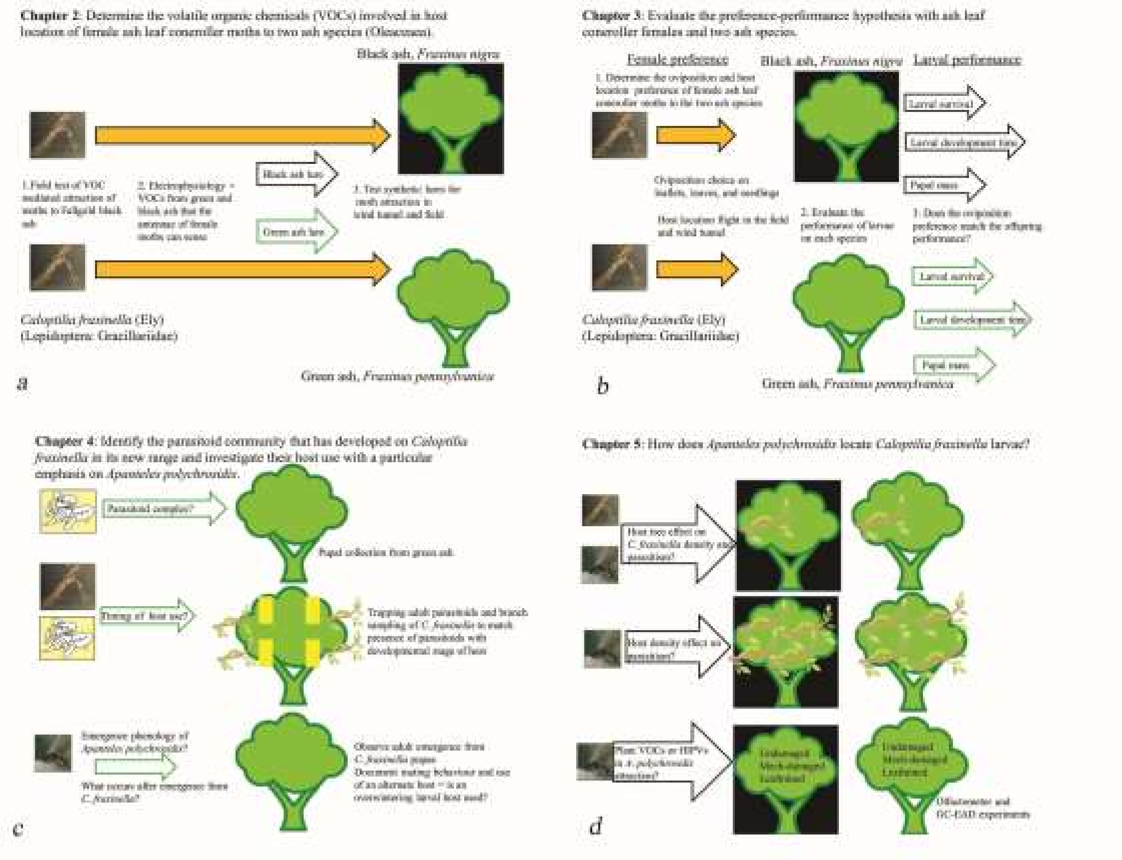
Chapter 2: Determine the volatile organic chemicals (VOCs) involved in host location of female ash leaf coneroller moths to two ash species (Oleaceae).
Black ash, Fraxinus nigra
1. Field test of VOC mediated attraction of moths to Fallgold black ash
2. Electrophysiology – VOCs from green and black ash that the antennae of female moths can sense
Black ash lure
Green ash lure
3. Test synthetic lures for moth attraction in wind tunnel and field
Caloptilia fraxinella (Ely)
(Lepidoptera: Gracillariidae)
Green ash, Fraxinus pennsylvanica
a
Chapter 3: Evaluate the preference-performance hypothesis with ash leaf coneroller females and two ash species.
Female preference
Black ash, Fraxinus nigra
Larval performance
1. Determine the oviposition and host location preference of female ash leaf coneroller moths to the two ash species
Larval survival
Larval development time
Pupal mass
Oviposition choice on leaflets, leaves, and seedlings
Host location flight in the field and wind tunnel
2. Evaluate the performance of larvae on each species
3. Does the oviposition preference match the offspring performance?
Larval survival
Larval development time
Pupal mass
Caloptilia fraxinella (Ely)
(Lepidoptera: Gracillariidae)
Green ash, Fraxinus pennsylvanica
b
Chapter 4: Identify the parasitoid community that has developed on Caloptilia fraxinella in its new range and investigate their host use with a particular emphasis on Apanteles polychrosidis.
Parasitoid complex?
Pupal collection from green ash
Timing of host use?
Trapping adult parasitoids and branch sampling of C. fraxinella to match presence of parasitoids with developmental stage of host
Emergence phenology of Apanteles polychrosidis?
What occurs after emergence from C. fraxinella?
Observe adult emergence from C. fraxinella pupae
Document mating behaviour and use of an alternate host – is an overwintering larval host used?
c
Chapter 5: How does Apanteles polychrosidis locate Caloptilia fraxinella larvae?
Host tree effect on C. fraxinella density and parasitism?
Host density effect on parasitism?
Plant VOCs or HIPVs in A. polychrosidis attraction?
Undamaged
Mech-damaged
Leafmined
Undamaged
Mech-damaged
Leafmined
Olfactometer and GC-EAD experiments
d

Figure 1-1. Egg and larval portion of the life cycle of the ash leaf coneroller *Caloptilia fraxinella* **A.** Eggs of *C. fraxinella* on the adaxial surface of a leaflet of *F. nigra*. **B.** Eggs ready to hatch with first instar larvae visible within the chorion. Scale = 1 mm C. first instar larva (magnified from D, black arrowhead) Notice the black head capsule. **D.** Two first instar larvae at commencement of serpentine mining. Larva at black arrowhead magnified in **C.** Note the two empty chorions (*ch*) and the entrance wound (white arrowhead) where the second larva entered the leaflet. **E.** Serpentine mines on a leaflet of *F. nigra* with one larva (arrowhead). Scale = 1mm. **F.** Eleven eggs, three first instar larvae beginning mines (black arrowheads) and one first instar larva (white arrowhead) within a mine. Note the empty chorion some distance from the point of larval entry, which suggests that the larva travelled before entering the leaflet. **G.** Serpentine leaflet mines on the adaxial surface of two opposite *F. pennsylvanica* leaflets. Black arrowheads indicate where larvae have begun to "blotch" mine leaflet edges. The leaflet on the right has begun to curl inwards towards the midrib due to silk deposition by third instar larvae. **H.** Heavy mining damage on a *F. pennsylvanica* leaflet. Blotch mining at the leaflet margins has separated the cutical from the mesophyl (shiny areas). Leaf curl was severe so that the leaf had to be held open to photograph. Black arrowheads are visible larvae. **I.** Cone-rolled leaflet opened to show fourth instar larva. Note accumulation of frass in the lower portion of the cone (arrowhead). **J.** Fourth instar larva within cone-rolled leaflet. **K.** *Fraxinus nigra* leaflets rolled into pyramidal cones by *C. fraxinella*. Note leaflet deformation (arrowhead) where the larva has tied the lower leaflet margin with silk to complete the base of the cone.

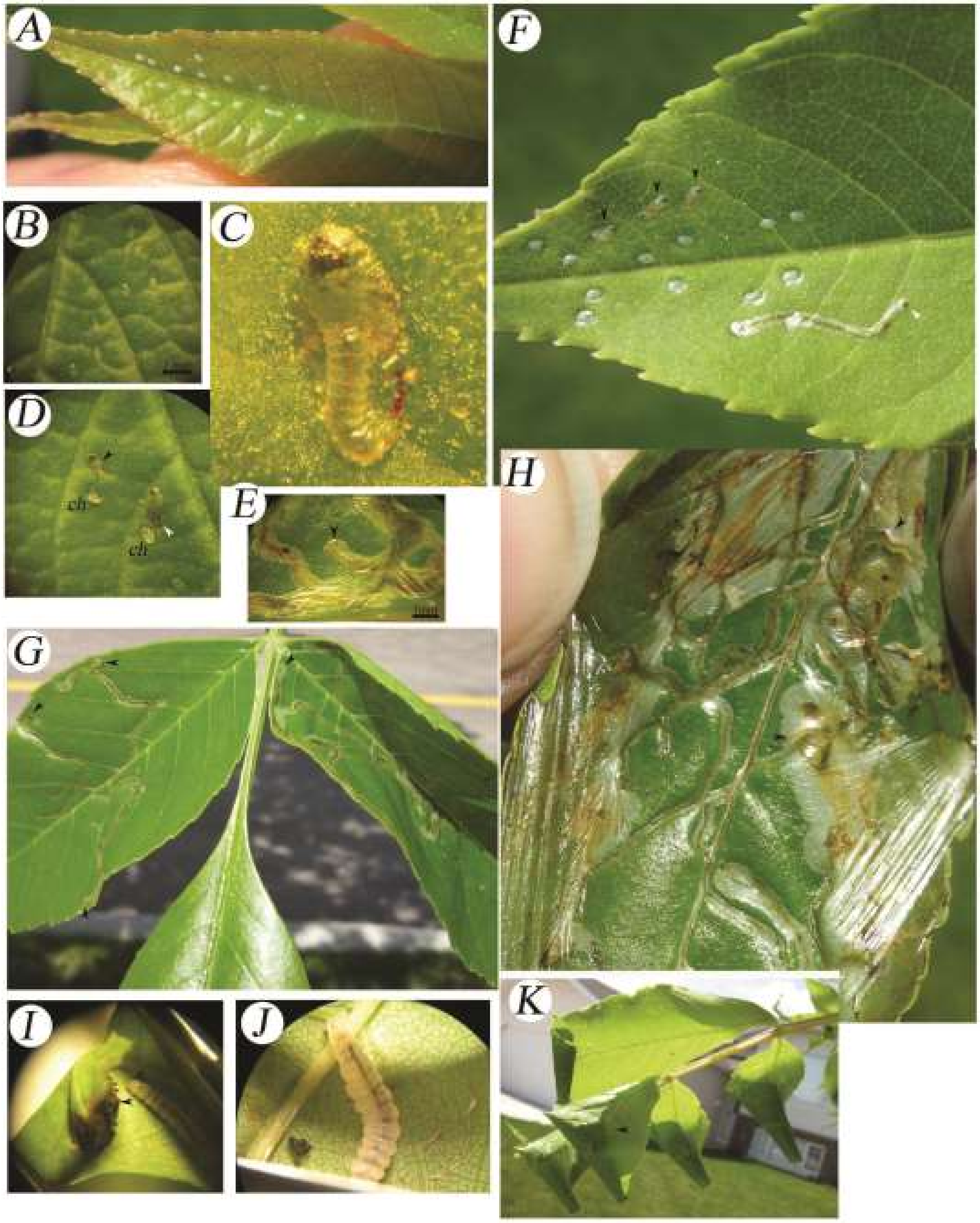
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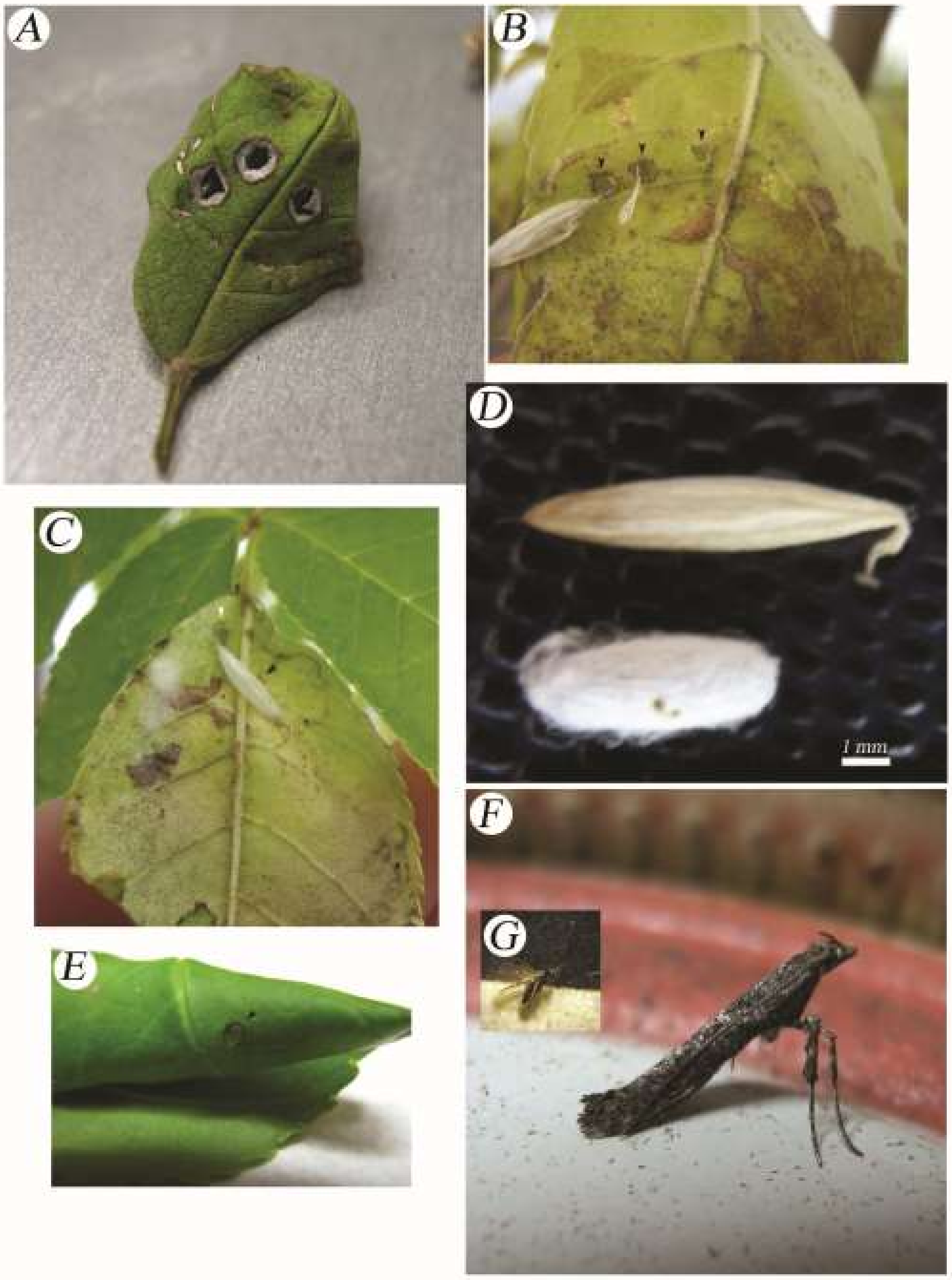
Figure 1-2. Pupal and adult stage of *Caloptilia fraxinella* (Lepidoptera: Gracillariidae) and the pupal and adult phase of its parasitoid *Apantles polychrosidis* (Hymenoptera: Braconidae). **A.** Triple exit windows in the pyramidal base of a *Fraxinus nigra* leaflet rolled by *C. fraxinella.* Note the cuticle ripped by the emerged moth. **B.** Interior of a *F. nigra* leaflet unrolled to show three open exit windows (black arrowheads) and one and a partial empty cocoon of *C. fraxinella*. **C.** *Fraxinus nigra* leaflet unrolled to show a single exit window (white arrowhead) and shed cocoon (black arrowhead) of *C. fraxinella*. "Cottony" material on the left of the cocoon indicates that this leaflet roll was inhabited by a cottony ash psyllid *Psyllopsis discrepans*, (Homoptera: Psyllidae) nymph. **D.** Comparison of the cocoons that contain pupae of *C. fraxinella* (top) and *Apanteles polychrosidis* (bottom). Scale= 1mm. **E.** Exit hole on the side of the pyramid made by *Apanteles polychrosidis* emergence from the leaflet roll. F. Newly emerged *C. fraxinella* moth. **G.** *Apanteles polychrosidis* parasitoid wasp.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Chapter 2: The volatile organic compound host location cues of *Caloptilia fraxinella*

Introduction

There are two competing hypotheses to explain the use of plant-produced volatile organic compounds (VOCs) (kairomones) for host location by herbivorous insects (Bruce et al. 2005). The "species-specific volatile" hypothesis was first proposed by Fraenkel (1959) to suggest that specialist insect herbivores detect and use taxon-specific VOCs to mediate host plant location. Support for the "species-specific volatile" hypothesis comes from host location behaviour of the diamondback moth, *Plutella xylostella* (*L.*) (Lepidoptera: Plutellidae), an oligophagous insect that specializes on brassicaceous plants. The diamondback moth uses a VOC specific to brassicaceous plants, allyl isothiocyanate*,* to find host plants (Han et al. 2001). The second hypothesis, the "ratio-specific volatile" hypothesis, is better supported with more empirical studies finding ratios to be important to host location and suggests that herbivorous insects respond to a specific ratio of common plant VOCs (Visser 1986) for host location (Bruce et al. 2005). Examples of insect herbivores that exploit specific ratios of common VOCs for host plant location include the tobacco budworm, *Manduca sexta* (*L.*) (Lepidoptera: Sphingidae) (Fraser et al. 2003), and the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomellidae) (Visser and Avé 1978). The generalist herbivore, the grapevine moth, *Lobesia botrana* (Dennis et Schiffermüller) (Lepidoptera: Tortricidae), is more attracted by a blend of VOCs specific to its preferred host, *Daphne gnidium* (Thymelaeaceae) than to VOCs released from its recently adopted grape host (Tasin et al. 2010). The grape specialist, the grape berry moth, *Paralobesia viteana* (Clemens) (Lepidoptera: Tortricidae) uses a ratio of common plant VOCs to locate its host and attraction is reduced to synthetic VOC lures that deviate from the attractive ratio (Cha et al. 2011). Many of the economically important microlepidopteran pests follow a mono or oligophagous strategy and are only capable of successful host location to and oviposition on a narrow range of host species (Hespenheide 1991).

There are more than seventy families of plants utilized as larval hosts by the microlepidopteran families Gelechiidae, Gracillariidae, and Tortricidae (Powell 1980); and many different host VOCs that could serve to mediate host plant location to adult moths. Host plant location is generally mediated by plant-produced VOCs in microlepidopteran leaf miners. Host location is particularly important for leaf-mining species as the offspring are constrained to develop within the plant host chosen by the adult female (Faeth 1985; Auerbach and Simberloff 1989). The specialist horse chestnut leaf miner, *Cameraria ohridella* Deschka and Dimić (Lepidoptera: Gracillariidae), can detect volatiles from damaged and undamaged leaves of horse chestnut, *Aesculus hippocastanum* (Hippocastanaceae) (Johne et al. 2006). This moth requires a specific blend of VOCs for host location and changes in single compounds can influence oviposition. The tomato leaf miner, *Tuta absoluta*, (Meyrick) (Lepidoptera: Gelechiidae) uses small variations in the identity and ratio of VOCs in host location to the most suitable host plant for larval development (Proffit et al. 2011).

Host plant VOCs may be harnessed for use in integrated pest management (IPM) as attractive lures used to monitor or manipulate the behaviour of pest populations (Harand and Schmolling 2006). Host plant VOCs are useful in semiochemical-based IPM of moths because they can attract gravid females and can increase the attractiveness of female-produced pheromone to males (Reddy and Guerrero 2004; Svatoš et al. 2009; von Arx et al. 2012). Traps baited with plant VOCs often catch both female and male moths (Light et al. 2001, Aurelian et al. 2012) as males are also physiologically capable of detection of plant VOCs (Ansebo et al. 2004).

Microscopic analysis of sensilla

Moths perceive host VOCs and pheromone through chemoreceptive sensillae on the antennae. Classes of chemoreceptive sensillae in lepidopterans have been identified through single sensillum recordings to perceive sex pheromones or host plant VOCs (Dickens 1989). Two sensillar types house olfactory receptor neurons sensitive to pheromones; sensilla auricillica (Ebbinghaus et al. 1998; Ansebo et. al. 2005) but sensilla trichodia on antennae of male moths house the main receptors for female-produced pheromone (Steinbrecht 1973; Zacharuk 1985; Hallberg et al. 1994; Hansson 1995). Other sensilla house olfactory receptor neurons sensitive to plant VOCs. Sensilla auricillica also receive plant VOCs (Anderson et al. 2000) in addition to pheromone. Sensilla coeloconica (Cuperus 1983, 1985; Pophof et al. 2005) and sensilla basiconica (Hansson 1995) only receive plant VOCs. These sensillae can be morphologically identified with a scanning electron microscope and compared to others of the same type with known chemoreceptive functions to infer the function of the sensillae. Female moths should have an abundance of receptors for host plant VOCs. Male moths are expected to have an abundance of sensillae that house pheromone receptors but host plant VOCs also influence male behavior in which VOCs synergize the sex pheromones of females (Dickens et al. 1993; Light et al. 1993; Reddy and Guerrero 2004).

Volatiles from Fraxinus spp.

Volatile profiles of green ash, *F. pennsylvanica* (Markovic et al. 1996; de Groot et al. 2008) and Manchurian ash, *F. mandshurica* (Rodriguez-Saona et al. 2006) have been identified. Several of the VOCs released from Manchurian ash seedlings attract ash specialists such as the emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Rodriguez-Saona et al. 2006). Four green leaf volatiles (GLVs) released by Manchurian and green ash; hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenol, and (*E*)-2-hexenol, are electrophysiologically active and attractive to emerald ash borer (Rodriguez-Saona et al. 2006; de Groot et al. 2008). Seven VOCs have been previously identified from the headspace of green ash leaves; *trans*-ocimene, linalool, methyl salicylate and *cis*-3 hexenyl acetate, (*E*) 4,8-dimethyl 1, 3, 7 nonatriene, α-farnesene, *trans*-nerolidol (Markovic et al. 1996) but the GLVs found in subsequent studies (Rodriguez-Saona et al. 2006; de Groot et al. 2008), were absent in this earlier study (Markovic et al. 1996). This could indicate that the emerald ash borer is extremely sensitive to low levels of GLVs released from its ash host.

Ash leaf cone-roller

The ash leaf-coneroller, *Caloptilia fraxinella* (Ely) (Lepidoptera: Gracillariidae) is an introduced leaf miner of horticultural ash (*Fraxinus* spp.) in Western Canada. Larvae mine and roll leaflets and under severe infestations reduce the aesthetic value and damage horticultural ash trees in that season. *Caloptilia fraxinella* females are specialists and selectively oviposit on newly flushed leaflets of ash in the spring (Pohl et al. 2004). Selectivity is further constrained by ash phenology as first instar larvae can only exploit newly flushed ash leaflets (Evenden 2009). The majority of *C. fraxinella* that eclose from pupae in July are in a state of reproductive diapause throughout a summer aestivation and overwintering period (Evenden et al. 2007) but a subset of the population is reproductively active and start a second generation if new ash growth is available (Wist, unpublished). *Caloptilia fraxinella* overwinter as adults (Evenden et al. 2007) away from their ash host, and females must find trees on which to oviposit in the spring. It is unknown how oviposition host location is accomplished but host VOCs are likely the most important cues. Newly flushed leaflets are targeted for oviposition (Evenden 2009) which suggests that the most attractive VOCs are released from new leaf flushes. Female response to host cues should be most acute when females are mated and physiologically able to produce eggs in the spring. Most females are mated and have developed eggs (Evenden et al. 2007) before leaf-flush and removal of these females during this “window of control” with a host-based VOC lure before they can oviposit on ash trees could reduce populations of this invasive leaf miner. *Caloptilia fraxinella* is a specialist on

Fraxinus species and as such, successful development of a lure based on host plant volatiles may be possible (Cha et al. 2008) and more effective than host-plant lures developed to attract generalist moths (Ansebo et al. 2004, Coracini et al. 2004, Mitchell et al. 2008).

Objectives

The overall objective of this study was to evaluate the role of VOCs in female host location behaviour of the ash specialist moth, *C. fraxinella* with the ultimate goal to create a host VOC-based lure to attract this invasive insect. The predictions of the species-specific hypothesis (Fraenkel 1959; Bruce et al. 2005) and ratio-specific hypothesis (Visser 1986; Bruce et al. 2005) are evaluated for this specialist moth. Within the framework of this goal, several host location hypotheses are explored: 1) VOCs of black and green ash are detected by *C. fraxinella* as tested using gas chromatographic-electroantennographic detection (GC-EAD); 2) VOCs mediate host location as tested by behavioural bioassays conducted in the field and wind tunnel; and 3) a blend of electrophysiologically active VOCs is attractive to *C. fraxinella*. Synthetic copies of the detected blend of VOCs are tested for attraction of female *C. fraxinella* in wind tunnel and field experiments.

Materials and Methods

Field sites

All field experiments were conducted in urban neighborhoods of Edmonton, AB (53°34′N, 113°31′W) with horticultural ash *Fraxinus* spp., planted outside of their natural range (Farrar 1998) as boulevard and park trees that housed high populations of *C. fraxinella*.

Insects

Insects used in laboratory tests were collected on the wing near overwintering sites in early spring (10-12 May 2010, 4-9 May 2011) in neighborhoods with horticultural ash plantings in Edmonton. Insects used in wind tunnel experiments were caught in the spring of 2010 and 2011 before natural ash leaf flush. Collected moths were separated by sex and sorted individually into 30 ml plastic cups and females were placed into a growth chamber on a reversed photo-regime (16L:8D) at 24°C and provided with a 10% sucrose solution through a cotton dental wick for 1-2 weeks to permit female egg development (Evenden et al. 2007) before moths were used in bioassays. Captured female *C. fraxinella* were assumed to be mated (Evenden *et al.* 2007) but a subset of females was dissected to test this assumption.

Plant material

One-year-old green and black ash seedlings (Jeffries Nurseries, Portage La Prairie Manitoba) and three-year-old black ash var. "Fallgold" (Advance Nurseries, Grand Forks, British Columbia) (approximately 1.5 m) were planted individually in pots containing Sunshine #4 potting mix (SunGro Horticulture Ltd., Agawam, MA, USA) placed into a growth chamber under summer conditions (16L:8D, 24.5 °C, 50 %RH) and watered as needed. One-year-old green and black ash seedlings were approximately the same size (~50-60 cm tall) and had similar amounts of foliage. Plants were forced to flush leaves in the spring with summer day lengths approximately one month early so that experimental trees were ready when moths were captured.

Microscopic analysis of sensilla

Scanning electron microscopy of the antennae of five *C. fraxinella* males and 17 females was conducted to assess the sensillar classes present on the antennae. Moths were reared from pupae collected in leaf rolls of green ash in neighbourhoods of Edmonton, AB, Canada (53°34′N, 113°31′W) in summer 2007 and frozen at -80 °C. Their heads, including intact antennae, were excised, placed into 70% ethanol and ultrasonicated (VWR Symphony, VWR International) for five seconds to remove the dense scales that cover the flagellomeres. Heads and antennae were dehydrated through a graded ethanol series (70%, 85%, and 95%), then a graded acetone series to 100%, critical point dried, and mounted on ridged SEM stubs. Insects were sputter-coated with gold (DC Sputter coater: Nanotech, Semprep) and observed at 5 kv in a Jeol JSM 630 IFXV Field Emission Scanning Electron Microscope. Sensillar types were classified on flagellomeres that had lost scales located along the flagellum from three males and four females. The putative terminal antennal segment of the antenna of one female was intact, and the general appearance of antennae was described from the scale-less and the scaled segments.

Detection of VOCs for host location

To test the hypothesis that female *C. fraxinella* respond to volatile cues emitted from host plant material, an initial field experiment (Experiment 1) was conducted using sentinel black ash trees that were positioned at field sites before the onset of leaf flush of trees in the field. Individually potted, three-year-old Fallgold black ash (Advance Nurseries, Grand Forks, British Columbia), the same cultivar as black ash planted in Edmonton, were forced to flush early with exposure to summer conditions in a growth chamber (16L:8D, 24.5 °C, 50 %RH). Three trees were positioned at each of three sites in Edmonton over eight nights. One tree at each site was left open with nothing obscuring the tree which provided both visual and volatile cues to foraging female *C. fraxinella*. The canopy of one tree at each site was covered with white organza mesh (mesh size 330 x 330 µm, IKEA Inc.) that obscured visual cues but allowed volatile cues. The canopy of the third tree was enclosed with a transparent plastic bag (65 x 100 cm, Home Depot Canada) that obscured volatile cues from the ash canopy but allowed visual cues. Two yellow sticky cards (7.26x12.7 cm) (Contech Enterprises Inc, Delta, BC) were positioned in the centre of the canopy on either side of each tree to capture *C. fraxinella* attracted to each tree. Trees were placed at field sites overnight, and checked the following day at which time the position of the trees was randomized and sticky cards were replaced if any moths were caught. The plastic and mesh bags were removed during the day to reduce stress on the trees and replaced to trap moths overnight. Moths were counted and separated by sex and a subset of females was dissected to determine mating status. The number of moths on the two sticky cards per tree was pooled to create one count per treatment at each site. Data were analyzed with three generalized linear mixed effects models (GLMM) that tested the effect of the tree treatment on the combined number of moths, the number of males or the number of females caught. The location of trees was nested within site and date with sites and date set as random factors and the error distribution specified as Poisson with a log link. The Poisson family of errors with a log link is suitable for count data (Crawley 2007) and the variance to mean ratio was close to one. Generalized linear models were created with R 2.13 (R Development Core team, 2012) using the Rcmdr library (Fox et al. 2011).

As the results of Experiment 1 provided evidence that female *C. fraxinella* use volatile cues for oviposition host location, a second experiment (Experiment 2) was conducted to identify what VOCs emitted from ash are detected by antennal receptors of *C. fraxinella*. Volatile emissions were collected from green and black ash seedlings with new leaf flush. Samples were collected by enclosing the crown of either one black ash “Fallgold” or three green ash seedlings (to sample a similar amount of foliage) at leaf flush with a large Look™ oven bag (35x43 cm, Terinex, Bedford, England) sealed around the trunk with a twist tie. Charcoal-filtered air was drawn through the bag with an aquarium pump system modified to pull air through Teflon® tubes at 1 L/min for 8 h onto an adsorbent tube filled with Porapak-Q beads in an SKC Sorbent Tube (OD 6 mm, length 110 mm; absorbent: front layer 150 mg, back layer 75 mg; separated by glass wool) (SKC Inc., PA, USA distributed by Supelco, Bellefonte, Pennsylvania, USA). Ten black ash var. “Fallgold” were sampled for 8h during the day and again for 8 h at night. Nine green ash seedlings (three groups of three) with newly flushing leaves were sampled for 8 h during daytime to maximize the amount of VOCs captured. Each VOC sampling session included a single control sample, collected simultaneously, of the air within the growth chamber. The back layer of Porapak-Q from each sample was also analyzed to ensure that no VOCs were pulled through the front layer of Porapak-Q in the tube. Captured VOCs were eluted with 1 ml pentane (Fisher, 99.6% purity) and analyzed using gas chromatography on a Hewlett Packard 5890A gas chromatograph equipped with a DB-5, column (each 30 m x 0.32 mm ID; JandW Scientific, Folsom, CA, USA) with a temperature program in which 50 ºC was held for one min, then increased by 20 ºC per min to 280 ºC, and held for five min. Black ash "Fallgold" daytime and night time treatments had the same VOCs, but the amount of VOCs captured at night was lower than during the day (Table 1). No VOCs "broke through" the front layer of Porapak-Q in the 8 hours of sampling and the control samples did not collect appreciable amounts of VOCs. All samples within an ash species with the highest peak areas were pooled for use in the GC-EAD analysis with an antenna of female *C. fraxinella* used as the EAD detector. Sixty mated female *C. fraxinella* were field collected in the spring (2009), fed 10% sugar water and shipped to Simon Fraser University where GC-EAD analyses were conducted by Dr. Regine Gries. An antenna was removed with fine forceps from the head of a female *C. fraxinella* and its base placed into the opening of a glass capillary electrode (1 x 0.58 x 70 mm) (A-M Systems, Carlsborg, WA, USA) filled with saline solution. The tip of the antenna was removed with fine spring microscissors (Fine Science Tools, North Vancouver, BC, Canada) and the cut antenna placed into the recording glass capillary electrode (Gries et al. 2002). Each black or green ash sample was tested against at least three individual antennae. Compounds in ash extracts that elicited a response from the antennae were identified using mass spectrometry (GC-MS) on a Varian Saturn Ion Trap with a column of the same type used for gas chromatography and GC-EAD analysis. The temperature regime was 50 ºC for five minutes, and then increased by 10 ºC per min to 280 ºC for five minutes with comparison of their retention indices (RI) (relative to alkane standards; Van den Dool and Kratz 1963) with those of authentic standards. The quantity of each identified compound in extracts from green and black ash seedlings was calculated from the peak areas of the GC traces (Table 1).

Wind-tunnel experiments

Synthetic chemicals used in experiments were (*E,E*) *α*-farnesene (67.2 % purity, with 23.1% (*Z,E*) *α*-farnesene), (*E*)-β-farnesene (98.25% purity), and (*E*)/(*Z*) mixture of β-Ocimene (70/30% E/Z) (Contech International Inc. Delta, B.C.), methyl salicylate (99% purity), linalool (97% purity) (Sigma-Aldrich, St. Louis, MO) and (*E*) 4,8-dimethyl 1, 3, 7 nonatriene (~99.8% purity) (Donated by Dr. Stefan Bartram of the Max-Planck Institute of Chemical Ecology in Jena, Germany); Experiments 3 and 4 tested blends of these electrophysiologically active VOCs in the concentrations released by green and black ash (Table 2) that were formulated into a biodegradable paste (Khaskin et al. 2006) to attract female *C. fraxinella* in wind tunnel bioassays. Treatments consisted of 50 ± 5 mg droplets of each formulation positioned on 1x1 cm foil tabs which were compared to inert droplets of paste without VOCs and ash seedlings as controls. Experiment 3 compared *C. fraxinella* female response to two 50 ± 5 mg droplets containing the synthetic formulation that mimicked green ash (Table 2) suspended from a plastic seedling at the upwind end of the wind tunnel with similarly placed blank control droplets and a living green ash seedling. Experiment 4 compared *C. fraxinella* female response to two 50 ± 5 mg droplets containing the synthetic formulation that mimicked black ash (Table 2) or blank control droplets suspended from a plastic seedling at the upwind end of the wind tunnel with a black ash seedling. In each experiment, the two foil tabs housing the droplets were positioned on the plastic seedling 30 cm apart. The source was placed 100 cm upwind of the release cage.

In the wind tunnel experiment, behavioural responses were recorded as "yes" or "no" for each moth so the resulting data were binomially distributed. Each recorded behaviour was analyzed separately with a generalized linear model (GLM) with a logit link function and binomial errors terms (lmer package in R 3.0.1, R Core Team 2013). The resulting analysis of deviance table was used to determine if female response was dependent on the semiochemical treatment, experimental day or their interaction. When the GLM was significant, multiple comparisons were made using a Tukey-contrasts test (R-package: multcomp), except where means of zero in the control group prevented Tukey's test. The Fisher's Exact test (R 3.0.1, R Core Team 2013) was employed to compare treatment groups with means of zero.

Field experiments with synthetic lures

The synthetic blends tested in the wind tunnel in experiments 3 and 4 were tested in the field in experiments 5 and 6. Both experiments were set up following a split plot design with each treatment (Table 2.2) placed twice in ten different neighbourhoods: once in alleys and backyards (alley) near overwintering sites and again in the canopy of green ash park trees (tree). Experiment 5 was conducted pre ash leaf flush in the field (6 May-20 May, 2011) when *C. fraxinella* would be expected to occur near overwintering sites. Experiment 6 was conducted for two weeks post ash leaf flush in the field (20 May-3 June, 2011) when *C. fraxinella* would be expected to be at ash trees for oviposition. At each site, a wing trap (Contech Enterprises Inc., Delta, BC) baited with a 50 ± 5 mg droplet of either the green or black ash lure or a control drop without volatile chemicals (Table 2) loaded on small foil tabs and suspended from the top of the trap were either hung individually from a 1 m wooden stake (alley) or in the canopy of a green ash (tree) with a minimum distance of 15 m between traps. Traps were checked weekly, sticky inserts were replaced and any captured moths were enumerated and separated by sex. In experiment 6, two additional treatments were added in trees. A 100 mg droplet of each of the synthetic volatile blends was hung in wing traps in two additional trees at each site to determine if a higher dose of volatiles (increased signal strength) might increase trap catch.

Experiments 5 and 6 were analyzed as split plot GLMM with the Negative Binomial family of errors (log link), suitable for overdispersed count data (Crawley 2007), with the lme4 package (Bates et al. 2011) in R 2.13. Treatment was nested within trap location (tree or alley) and nested within site. Site and date were set as random factors.

Results

Microscopic analysis of sensilla

Antennae of male and female ALCR are filiform, run the length of the body (Fig. 2-1A) and both dorsal and ventral surfaces are densely covered with scales (Figs 2-1A, 2-2 A). The scape and pedicel are densely covered with short scales (Fig. 2-1 A). Flagellar segments (flagellomeres) are entirely covered in scales with a sheath of long scales originating from the proximal end of the flagellomeres (Fig 2-1 C) and terminating beyond the proximal end (Figs 2-1E, J; 2C). Long scales are nested within a top scale (Fig. 2-1J, distal end; Fig. 2-2 G proximal end) and the nested properties can be inferred from associations of 5-7 antennal sockets at the proximal flagellar end that remain after long scales are lost (Figs 2-1D, E; 2-2 C,D, F). A sheath of short scales rings the distal end of each flagellomere. Short scales are born singly (Figs 2-1 E, J; 2-2 C, D, E, F, I, J) and this pattern is confirmed by the single ring of antennal sockets at the distal end of each flagellomere (Figs 2-1 D, E; 2-2 D, E, F). The cuticular surface of the antennae is wrinkled with wavy ridges that run longitudinally from the distal to proximal end of each flagellomere (Figs 2-1D, E, G; 2-2C, D, E, F) where they transition to horizontal ridges (Fig. 2-1 D, E, F). The flagellomeres closest to the head in both male and females are longer and have a sparse number of sensillae while the segments shorten as they get further from the head and the number and type of sensillae increase.

Three sensillar types protrude beyond the scales and several other sensillar types are concealed beneath the scales. Sensilla chaetica protrude perpendicularly beyond the scales at the proximal end of each flagellomere on both male (Fig. 2-2 B, C) and female antennae (Fig. 2-1 B, C) and are distinguished by their basal articulating socket (Figs 2-1E, L, 2-2 D, G) and helical ridges (Fig. 2-1 L). An apical pore seems to be present at the sensillar tip (Fig. 2-1 L). Sensilla trichodia and basiconica also protrude between and beyond long scales on the antennae of females (Fig. 2-1 B, C) and males (Fig. 2-2 B, D). A corrugated appearance caused by a repeated series of annular rings is evident on most of the sensilla trichodia on the male antenna (Fig. 2-2 L) and female antennae (Fig. 2-1 F, H). On scale-less sections, there were distinct differences in the number, appearance and distribution of sensilla trichodia on male (Fig. 2-2 D, E) versus female antennae (Fig. 2-1 D, E). Male moths have more sensilla trichodia than females and these are loosely organized into three wreaths around the entire segment; one that encircles the distal end and two closely associated wreaths that span the median to proximal ends of the flagellomeres (Fig. 2-2 D, E). The distal sheath of trichoid sensillae extends beyond the end of the flagellomeres and overlaps the following segment (Fig. 2-2 F, G). Sensilla trichodia on male antennae were approximately twice as long as on female antennae (Fig. 2-1 D, E *vs.* Fig. 2-2 D, E) and trichoid sensilla on females were the same length as and difficult to distinguish from sensilla basiconica except by their cuticular patterns (Fig. 2-1 G, F).

Sensilla basiconica (Fig. 2-1 G, F) with longitudinal striations composed of pores are scattered among the trichoid sensilla on the flagellar segments of female antennae. On female antennae the basiconic outnumber the trichoid sensilla and the opposite is true on male antennae. Another putative basiconic type sensillum was evident on one male segment (Fig. 2-2 G, H) with longitudinal striations but with a fuzzy surface.

One putative sensillum campaniformium was evident on a segment of a female antenna (Fig. 2-1 D, G) indicated by a cuticular depression with a possible central peg. At the current SEM angle, a basiconic sensillum is partially obscuring the sensillum campaniformium. One naked sensillum coeloconica (without cuticular spines) (Fig. 2-1 K) was observed on another female flagellomere. A sensilla styloconica sits at the distal end of some female flagellomeres (Fig. 2-1 D) and displays a small stylus on the end of an ovoid base (Fig. 2-1, I). This sensillar type was present on two female flagellomeres examined (Fig. 2-1 D, I).

At the distal end of the flagellomeres was at least one (Fig. 2-1 C, D, E, F, I, J) and potentially two (Fig. 2-1 J) sensilla auricillica on the female's putative terminal segment (Fig. 2-1 J). Sensilla auricillica appear to be of three types, rabbit-eared shoehorn smooth (Fig. 2-1 F, E) fuzzy, covered by granules (Fig. 2-1 J) and smooth regular shoehorn (Fig. 2-2 M). Rabbit eared and regular shoehorn types were evident at the distal end of both male (Fig. 2-2 C, D, E, F) and female flagellomeres. These sensillae lacked a socket (Fig. 2-1 F, J).

Detection of VOCs for host location

In Experiment 1, trees without bags allowed for perception of both visual and volatile cues by *C. fraxinella*, while trees with mesh bags blocked visual cues but allowed VOC cues and trees with plastic bags allowed visual cues but blocked VOC cues. The total number of *C. fraxinella* trapped differed among treatments with more moths trapped at unbagged trees than trees with plastic bags (Table 2-1) (Fig. 2-3) but not more than trees covered with mesh (Table 2-1). Although there was a trend for more capture of male and female moths on trees that emitted volatile cues, the number of males and females was not significantly different among the differently treated trees (Table 2-1, Fig. 2-3). However, low sample size reduced the power of the statistical test to detect a significant difference. All dissected female moths (n=12) were mated and had vitellogenic oocytes and were thus physiologically ready for oviposition.

In Experiment 2, the concentration of black ash VOC emmisions was lower at night compared to day (Table 2-2; Fig. 2-4). The GC EAD analyses of the volatile profiles of black and green ash revealed five VOCs each from black and green ash (Table 2-2; Fig. 2-4; Fig. 2-5) that consistently elicited responses from the antennae of female *C. fraxinella.* Four VOCs, *trans*-ocimene, linalool, methyl salicylate, and α-farnesene were common to each profile while one compound was specific to black ash, (*E*)-4,8-dimethyl 1,3,7 nonatriene, and one, β-farnesene, specific to green ash (Table 2-2; Fig. 2-4; Fig. 2-5). Two unidentified sesquiterpenes (at RI 11.68 and 12.12) in the volatile profile of black ash elicited small EAD responses (Table 2-2: Fig. 2-4). The ratio of electrophysiologically-active VOCs released by seedlings of each ash species was used to determine the percentage of each synthetic VOC used in volatile lures tested in bioassays (Table 2-3).

Wind-tunnel experiments

All dissected females had mated (n=10 per season) suggesting that all females captured in spring 2010 and 2011 had mated prior to use in the bioassays. In experiment 3, when moths were tested in response to natural and synthetic sources of green ash volatiles, take off by moths was not significantly different from the blank control (Table 2-4; Fig. 2-6). There were significant differences in moth responses among the synthetic green ash lure, control and green ash seedling in lock-on, oriented flight, and source contact behaviours (Table 2-4). Statistical differences were driven by the lack of host location behaviour to the control treatment (Fig. 2-6). There was no significant difference in the proportion of moths that locked-on and conducted oriented flight to the green ash seedling or to synthetic green ash lures suspended from a plastic seedling. More female moths contacted the green ash seedling than the plastic seedling with the synthetic VOC lure (Table 2-4).

In experiment 4, when moths were tested in response to natural and synthetic sources of black ash volatiles, take off was not significantly different among treatments (Table 2-5). There was no lock on, oriented flight or contact to the plastic seedling baited with blank droplets but this lack of response was not statistically different from the small female response to the plastic seedling baited with black ash droplets (Table 2-5, Fig. 2-7). There were no significant differences in lock on or oriented flight between black ash seedlings and synthetic black ash lures but there was significantly more source contact to black ash seedlings compared to unbaited plastic control seedlings (Table 2-5, Fig. 2-7).

Synthetic lure field experiments

In experiment 5 (pre-leaf flush) when the synthetic ash lures were tested in the field, the number of *C. fraxinella* moths caught on all traps was low (0 to 30 moths/trap) (Fig. 2-8). There was no difference in total number of moths, or number of males or females captured among the variously baited traps (Table 2-4). There were significantly more females caught at overwintering sites (location, Table 2-4) before ash flush than in traps positioned in trees (alley *vs.* tree, Fig. 2-8a). The difference in total moths between overwintering sites and trees before ash flush was driven by the number of female *C. fraxinella* captured, as the number of males was not different between alley and tree locations before ash flush (Table 2-4). The interaction between semiochemical treatment and trapping location was not significant in any of the three GLMM models (Table 2-4).

In experiment 6, conducted after ash leaf flush, there was also no significant difference in moth capture among the semiochemical treatments (Table 2-4, Fig. 2-8b) except that more males were trapped with the green lure than the control (Table 2-4, Fig. 2-8b). There was no significant difference among treatments when the signal strength was increased with 100 mg droplets (Table 2-4, Fig. 2-8c). There were significantly more moths; both male and female, captured in trees after leaf flush than at overwintering sites (Table 2-4; Fig. 2-8b). The interaction between treatment and trap location was never significant (Table 2-4).

Discussion

Microscopic analysis of sensilla

The entire surface of the antennae of male and female *C. fraxinella* is densely covered with five to seven layers of scales. Long scales on the antennae of the horse-chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić (Gracillariidae) are also clearly layered but with four nested scales (Kalinová et al. 2003; Fig. 1 B). Short scales on the antennae of *C. fraxinella* are arranged around the proximal end of each flagellomere beneath the long scales and may be another sensilla type, sensilla squamiformia (Schneider 1964, Sun et al. 2011) if they are innervated by a neuron. If these short scales are indeed sensilla, they are designed for mechanoreception (Schneider 1964; Faucheux 2011) and not chemoreception as they have non-porous walls (Sun et al. 2011). These scales cover the antennae and may help protect it from mechanical and thermal damage as this long-lived moth overwinters as an adult. Superficially, the antennae of male and female *C. fraxinella* are not sexually dimorphic with no molecular sieve arrangement of sensillae such as occurs in many male moths that use long-distance pheromones to locate females (Hansson 1995). *Caloptilia fraxinella* males do use long-distance pheromones to orient to females (Evenden and Gries 2008; Evenden et al. 2008) and there appears to be a greater number of sensilla trichodia on the scale-less segments observed in this study on male compared to female antennae. Sensilla trichodia house olfactory receptor neurons sensitive to pheromone signals in other moths (Hansson 1995). For example, sensilla trichodia of male European sunflower moths, *Homoeosoma nebulella* Denis and Schiffermüller (Pyralidae) (Faucheux, 1991) are speculated to contain pheromone receptor neurons (Steinbrecht 1973; Zacharuk 1985) as are sensilla trichodea of the silkworm, *Bombyx mori* L. (Bombycidae) Schneider 1957) and the European cornborer, *Ostrinia nubilalis* Hübner (Pyralidae) (Hallberg et al. 1994). Sensilla trichodia bear annular rings along their shaft between horizontal rows of pores that allow odour molecules into the lumen of the sensillum (Faucheux 1991). Antennae of male *C. fraxinella* respond physiologically to female-produced pheromone and male moths orient to pheromone in wind tunnel (Lemmen and Evenden 2009) and field experiments (Evenden et al. 2008).

Sensilla trichodia are also present in smaller numbers on the antennae of female *C. fraxinella*. Antennae of female *C. fraxinella* respond to female-produced pheromone (Dr. Joelle Lemmen, University of Alberta, personal communication) in contrast to sensilla trichodia on the antennae of some female moths (Hallberg et al. 1994; Pophof et al. 2005). The horse-chestnut leaf miner, has a sexually dimorphic sensilla trichodia with one type found only on male antennae and presumed to be used in pheromone perception (Kalinová et al. 2003; Fig 1.c). This type of sensilla trichodia closely resembles those found on antennae of male *C. fraxinella*. Sensilla campaniformium serve a proprioceptive function (Albert 1980) while sensilla styloconica are thermo and hygrosensitive (Schoonhoven 1967; Zimmermann 1992).

Several sensillar types are known chemoreceptors that house olfactory neurons that respond specifically to host plant volatiles. Sensilla chaetica are present on the distal end of each flagellomere of *C. fraxinella* antennae. They project out between the long scales and do not appear to be sexually dimorphic. Sensilla chaetica on other moths are chemo and mechanoreceptors (Maher and Thiery 2004) and are distinguished by their cuticular articulating socket or "collar" (Albert and Seabrook 1973) which indicates that their movement stimulates neuronal firing like a mechanoreceptor. However, these sensillae on male and female *C. fraxinella* resemble the porous sensilla chaetica type 1 "b" of the tortricid moth, *Lobesia botrana* (Maher and Thierry 2004) and so may also serve a chemoreceptive function. Apical pores were detected in sensilla chaetica of *L. botrana* and electrophysiological tip recordings showed that these sensilla play a chemo and mechanoreceptive role (Maher and Thiery 2004). Several other studies suggest a chemoreceptive function for sensilla chaetica (Chanda and Roome 1980; Marion-Poll et al. 1992) with the presence of an apical pore suggesting its use in contact chemoreception (Faucheux 2011).

Sensilla basiconica are characterized by longitudinal striations that are assemblages of pores (Sun et al. 2011; Cuperus 1985). Sensilla basiconica often house olfactory receptor neurons sensitive to plant volatiles (Hansson 1995). Female ermine moths, *Yponomeuta* spp. (Yponomeutidae), have approximately twice as many pores as those of males (Cuperus, 1985). Female *C. fraxinella* appear to have more sensilla basiconica than males which suggests that these are also involved in detection of VOCs for host location. Sensilla coeloconica also perceive plant VOCs (Cuperus 1983, 1985) especially acids and aldehydes (Pophof et al. 2005), and this sensilla type was rare and only present on female *C. fraxinella* antennae. One sensilla auricillica was found on the scale-less segments of most male and female *C. fraxinella* antennae and the terminal segment of the female antenna had two at the distal end of the segment. Males and females had both types of sensilla auricillica, rabbit eared shoehorn and regular shoehorn. Sensilla auricillica are often found on the distal end of flagellomeres (Callahan 1975; Ebbinghaus et al. 1998). With the lack of a socket, sensilla auricillica can only serve a chemoreceptive function for plant VOCs (Anderson et al. 2000) and pheromones in both male and female moths (Ebbinghaus et al. 1998; Ansebo et al. 2005). Sensilla basiconica and auricillica on the antennae of cactus moth *Cactoblastis cactorum* both respond to terpenes and green leaf volatiles (Pophof et al. 2005). Female and male *C. fraxinella* both have antennal sensillae to perceive host plant VOCs and males respond electrophysiologically to the same host plant VOCs as females (Dr. Joelle Lemmen, University of Alberta, personal communication) which is common in moths (Van der Pers 1981).

Detection of VOCs for host location

Gas chromatography of the headspace VOCs of black ash reveal that the night VOC profile contains the same VOCs as the day profile but the concentration of VOCs is much lower at night. The night profile of green ash was not analyzed. Manchurian ash also emits greater concentrations of VOCs during the day than at night (Rodriguez-Saona et al. 2006). Release of VOCs from apple trees at night is also lower (Bäckman et al. 2001) or the same as daytime levels (Casado et al. 2006). GC-EAD experiments identified six VOCs from the headspace profiles of green and black ash trees that are detected by the antennae of *C. fraxinella*. Four compounds are common to both ash species with (*E*) 4, 8-dimethyl 1, 3, 7 nonatriene only found in black ash and (*E*) *β*-farnesene only in green ash. With the exception of *β*-farnesene, all of these VOCs were previously reported from the headspace volatile profiles of green ash (Markovic et al. 1996) and Manchurian ash (Rodriguez-Saona et al. 2006). *Trans* ocimene, linalool, (*E*) 4, 8-dimethyl 1, 3, 7 nonatriene, methyl salicylate and *α* and *β*-farnesene were detected by the antennae of mated, female *C. fraxinella.* Detection of only six VOCs from green and black ash by *C. fraxinella* is low compared to the sixteen ash VOCs detected by the emerald ash borer (Rodriguez-Saona et al. 2006). However, only one VOC, (*Z*)-3-hexenol, is required to attract a significant number of emerald ash borers (de Groot et al. 2008) which supports the "species-specific" volatile hypothesis. These electrophysiologically active VOCs are leaf-derived compounds (Paré and Tumlinson 1999; Dudareva et al. 2006) and not bark-derived. Reports of *C. fraxinella* females on the trunk of ash trees prior to leaf flush (Pohl et al. 2004) suggest that bark volatiles may also be detected by female *C. fraxinella* but Manuka oil (Malure™) which shares four sesquiterpenes common to ash bark (Crook et al. 2008) were not attractive to female *C. fraxinella* (Fig. A-3, Table A-1).

Long-range host location by mated *C. fraxinella* females is mediated by VOCs from ash trees. Black ash trees placed outdoors before natural ash leaf flush in the area attracted more *C. fraxinella* when they were open and released VOCs than when they were enclosed in plastic. There were more females and males trapped on trees that released VOCs than those that were enclosed in plastic but the difference was not significant. However, the power of the statistical test to detect a difference was low due to the small sample size of black ash trees available for this experiment. Lack of a significant difference among treatments with and without VOC cues also suggests that visual cues function in host location. These trapped *C. fraxinella* females were mated and physiologically ready for oviposition with a full complement of eggs before the ash trees of the urban forest flushed leaves. Female *C. fraxinella* become active and mate in early to mid-April (Evenden et al. 2007; Evenden 2009) while ash trees do not typically flush leaves until the third week of May in Edmonton (personal observation). Females and males are capable of VOC-mediated host location to ash trees even before natural leaf flush. This result indicates that there is approximately a four to five week window prior to ash leaf flush to deploy VOC-based lures to monitor and potentially control females of this invasive leaf miner.

Wind tunnel experiments

Wind tunnel experiments indicate that VOC cues mediate host location of *C. fraxinella*. Ash seedlings and synthetic VOC lures presented on a plastic seedling elicit oriented upwind flight in female *C. fraxinella* towards the volatile source. The synthetic lures appear to replicate the VOC signal released by ash seedlings and received by female *C. fraxinella*. In contrast, *C. fraxinella* did not display similar behaviour to plastic seedlings alone. This result is expected as VOC cues are the main mechanism of oviposition host location in moths (Renwick and Chew 1994; Bruce et al. 2005).

These results demonstrate that synthetic volatiles can induce odour-mediated host location in female *C. fraxinella* moths and that the ratio of VOCs presented here elicits a response. Although response to the synthetic lures did not differ from that to ash seedlings, the overall level of response in the wind tunnel was low. Plant VOCs however, typically do not elicit behavioural responses from female moths that are as strong as those elicited in males by female pheromones (Hurtrel and Thiery 1999; Tasin et al. 2005; Tasin et al. 2006a,b) and even preferred oviposition substrates only elicit 10% upwind flight and source contact in grapevine moth females (Tasin et al. 2006b). However, 80% of female grape berry moths orient upwind to VOCs from a combination of host plant parts (Cha et al. 2008) which suggests that further optimization of *C. fraxinella* response to the VOCs released from ash trees may be warranted.

The six compounds from black and green ash that elicit an antennal response from *C. fraxinella*, with the exception of *trans*-ocimene, are electrophysiologically and behaviourally active in several other female moths (Hern and Dorn 2004; Tasin et al. 2006b, Cha et al. 2008, Knight et al. 2011). Two terpenoids, (*E*) 4,8-dimethyl-1,3,7-nonatriene and (*E*)-*β* farnesene and the ester methyl salicylate are electrophysiologically active and essential for grape berry moth host location while the terpenoid linalool was not essential for attraction even though it was detected (Cha et al. 2008). Further experiments with *C. fraxinella* may also reveal that one or more of these VOCs from ash are not essential to elicit attraction. The two sesquiterpenes, (*E*)-*β*-farnesene and (*E,E*) α-farnesene, attract codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Hern and Dorn 2004) as does (*E*) 4, 8-dimethyl 1, 3, 7 nonatriene with acetic acid (Knight et al. 2011). Methyl salicylate is a common attractant to over twenty species of moths (El-Sayed 2013), but attraction to *trans*-ocimene has not been reported. Antennae of *C. fraxinella* were not stimulated by common GLVs (C_6 alcohols, aldehydes and acetates) released by ash leaves that stimulate antennal receptors of the coleopteran ash folivore, the emerald ash borer (Rodriguez-Saona et al. 2006; de Groot et al. 2008). Common GLVs presented singly or as a blend also did not attract female *C. fraxinella* in field experiments (Fig. A-4, Table A-2).

These results, suggest that *C. fraxinella* uses a specific ratio of common VOCs to locate their ash hosts and lends support to the ratio-specific hypothesis of VOC-mediated host location to plants (Bruce et al. 2005). A subset of VOCs released from green ash presented singly in an initial field trial attracted a similar number of female *C. fraxinella* as controls (Fig A-4, Table A-2). *Caloptilia fraxinella* respond to four VOCs released by both green and black ash trees in a similar ratio but these are also commonly found in the headspace of other plants (Paré and Tumlinson 1999). Other moths can be attracted to two similar but distinctly different VOC blends, like *C. fraxinella* in the wind tunnel to the similar synthetic blends based on green and black ash. For example, the grapevine moth is most attracted by a blend of VOCs specific to its preferred host, *Daphne gnidium* (Thymelaeaceae), and is less attracted by a similar VOC blend released in a different ratio from its recently adopted grape host, *Vitis vinifera* (Vitaceae) (Tasin et al. 2010). Even a blend of only three essential VOCs elicits upwind flight in grapevine moth if the ratio of VOCs is maintained but not if the ratio is skewed towards that released by non-host apple trees (Tasin et al. 2006ba). Large deviations in the ratio of electrophysiologically active compounds however, did not alter attraction of *Cydia molesta* (Busck) (Lepidoptera: Tortricidae) females to a synthetic blend (Najar-Rodriguez et al. 2010). Thus ratio differences in the four VOCs common to black ash and green ash may not be significant enough to disqualify one ash species as a host plant. Although not directly compared in one test, response of female *C. fraxinella* to black ash seedlings was lower than that to green ash seedlings in wind tunnel experiments. The ratio of electrophysiologically active VOCs in the headspace of black ash may be less attractive than that of green ash in the wind tunnel (Chapter 3) or the two unique compounds, *β* farnesene and (*E*) 4,8-dimethyl 1, 3, 7 nonatriene might mediate the difference in female attraction between the two ash trees. In the field however, the difference in odour profile of the two ash species affects orientation as more *C. fraxinella* females were caught in black ash than green ash canopies at sites where both trees occur and more eggs are laid on black than green ash (Chapter 3). These results suggest that the ratio of these common plant VOCs identifies ash trees as hosts to mated, female *C. fraxinella* which is predicted by the ratio-specific hypothesis (Bruce et al. 2005).

While research on gracillariid pheromone-based lures is fruitful for attraction of male moths (Evenden et al. 2008; Svatoš et al. 2009), development of lures based on plant kairomones presents several challenges. These include low behavioural response by females to synthetic host VOCs (Tasin et al. 2005; Tasin et al. 1996) and the competing attractive VOCs released by the host plant. Female behaviour is not as easy to manipulate as males (Svatoš et al. 2009) as scramble competition motivates males to be the first to arrive at a calling female while females do not face such pressure to compete for oviposition sites amongst the thousands of available leaflets on an ash tree. Low trap catch of female moths in response to VOCs in the field is commonly reported (Coracini et al. 2004; Svatoš et al. 2009; Loeb et al. 2011). The small signal presented by a single 50 or 100 mg lure droplet in the field may not provide a distinct stimulus compared to an entire tree with newly flushed leaves or send a strong enough signal to attract females from their overwintering locations. Furthermore, female flight patterns coupled with trap design (Cha et al. 2013) may also play a role in the low capture of females in the field. *Caloptillia fraxinella* are observed to fly upwards when approaching the ash canopy (Chapter 3). A cylindrical trap, a yellow sticky card trap (Wist and Evenden 2013) or a panel trap (Loeb et al. 2011) with a vertical trap surface might be more appropriate in future studies to capture female moths as they fly upwards within the ash canopy. Grape berry moth has a similar tendency to fly upward with infrequent orientation to leaves and completely fails to enter delta shaped wing traps (Hurtrel and Thiéry 1999) unless traps are clear and marked with a grape shoot pattern (Cha et al. 2013). Upward flight from the lower to upper canopy might explain the tendency for female *C. fraxinella* to favour oviposition on leaflets in the lower canopy over the upper canopy (Evenden 2009; Wist and Evenden 2013) simply due to encounters with leaflets in the lower canopy first.

Summary

Sexual dimorphism is evident on the antennae of male and female *C. fraxinella* between the sensilla trichodia that putatively perceive pheromones and the sensilla basiconica that putatively perceive host plant VOCs. Volatile organic compound cues from ash trees mediate host location of *C. fraxinella* females. Female *C. fraxinella* are capable of host location before natural leaf flush in the field, thus a window of opportunity exists for deployment of a host VOC-based lures to monitor and control mated females before they oviposit. Electrophysiological experiments identify six VOCs in the volatile profiles of black and green ash that elicit antennal activity in mated, female *C. fraxinella*. A lure based on a blend of these VOCs presented in the ratio released by ash trees elicits as much upwind oriented flight as newly flushed seedlings but elicits very little source contact. The lure also does not increase trap catch of *C. fraxinella* male or female moths above unbaited control traps in the field. This study has laid the ground work for the development of a VOC lure to attract female *C. fraxinella* but additional research to optimize the VOC signal, delivery and trap design is required before plant-based VOCs can be incorporated into integrated pest management strategies for this invasive leaf miner.

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Table 2-1. ANOVA table of the main effects of the generalized linear mixed effects model on the total number of moths and numbers of male and female *Caloptilia fraxinella* caught on Fallgold black ash trees that display visual and volatile cues (open), volatile cues only (mesh) and visual cues only (closed) (2008). The variable treatment is nested within site which is nested within date and site and date are set as random. Tukey's *post hoc* tests separated significantly different treatments. Degrees of freedom are shown in subscripted brackets. Table corresponds to the mean number ± S.E. moths of Fig. 2-3.

	***Post hoc* comparisons**	**Total no. moths**		**No. males**		**No. females**	
Effect	***Post hoc* comparisons**	**Test statistic**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**
Treatments		$\chi^2_{(2)}$=6.87	**=0.032**	$\chi^2_{(2)}$=5.47	=0.065	$\chi^2_{(2)}$=2.42	=0.299
	open *vs.* closed	$z_{(1)}$=2.61	**=0.024**	$z_{(1)}$=2.15	=0.078	$z_{(1)}$=1.51	=0.282
	open *vs.* mesh	$z_{(1)}$=1.09	=0.520	$z_{(1)}$=1.46	=0.303	$z_{(1)}$=0.21	=0.975
	mesh *vs.* closed	$z_{(1)}$=1.69	=0.206	$z_{(1)}$=0.97	=0.589	$z_{(1)}$=1.33	=0.378

Table 2-2. Volatile organic compounds that elicit electrophysiological activity in the antennae of female *Caloptilia fraxinella* identified from the headspace volatile profile of black, *Fraxinus nigra* var. "Fallgold" (day and night time samples) and green (day samples), *F. pennsylvanica,* ash by gas chromatographic-electoantennographic detection and mass spectrometry.

Concentration of compounds in samples ng/µl/8 h

Tree species	*trans*-Ocimene	Linalool	Methyl-salicylate	(*E*)-β-Farnesene	(*E,E*) α-Farnesene	*E* 4,8-dimethyl 1, 3, 7 nonatriene	Unknown sesquiterpenes
Black ash (day)	15.2	5.5	4.2	0	12.6	23.8	0.46
Black ash (night)	0.38	0.52	0.33	0	1.28	0.42	0
Green ash (day)	12.0	5.7	7.4	1.2	7	0	0

Table 2-3. Chemical components of lures loaded into 50 ± 5 mg drops of biodegradable paste used in experiments 3-6. Identity and concentration of synthetic volatile organic compounds in lures is based on compounds that elicited antennal responses from mated, female *Caloptilia fraxinella* in the volatile profiles of black, *Fraxinus nigra* var. "Fallgold" and green ash, *F. pennsylvanica*. Blank control is a 50 ± 5 mg drop of biodegradable paste.

	Amount (µg) per 50 mg drop (% of total)					
Lure types	*trans*-Ocimene	Linalool	Methyl-salicylate	(*E*)-β-Farnesene	(*E,E*) α-Farnesene	*E* 4,8-dimethyl 1, 3, 7 nonatriene
Black ash lure	64 (24.8)	23 (8.97)	18 (6.85)	0 (0)	53 (20.55)	100 (38.83)
Green ash lure	100 (36.04)	48 (17.12)	62 (22.2)	10 (3.6)	58 (21.02)	0 (0)
Blank	0	0	0	0	0	0

Table 2-4. ANOVA table of the generalized linear models on the effect of green and black ash treatments: ash seedlings, synthetic lures, and control plastic seedling on the host location behaviours performed by female *Caloptilia fraxinella* in a wind tunnel. The day of flight and the interaction between treatments and day were not significant (α=0.05) in any model. Significant treatments are separated by *post hoc* Tukey's tests. "*"indicates where p values were calculated with Fisher's Exact test when means of zero prevented Tukey's *post hoc* analysis. Degrees of freedom are shown in subscripted brackets. Table corresponds to Figs. 2-6, 2-7.

Experiment 3: Green ash lure and seedling in wind tunnel

		Take Off		**Lock On**		**Oriented Upwind Flight**		**Contact**	
Effect	***Post hoc* comparison**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**
Treatments		$\chi^2_{(2)}=19.56$	0.761	$\chi^2_{(2)}=9.69$	**0.002**	$\chi^2_{(2)}=11.93$	**<0.001**	$\chi^2_{(2)}=10.34$	**<0.001**
	lure *vs.* control	$z_{(1)}=0.53$	0.859	-	**0.034***	-	0.239	-	1.0*
	seedling *vs.* control	$z_{(1)}=0.45$	0.897	-	**<0.001***	-	**0.008***	-	**0.008***
	seedling *vs.* lure	$z_{(1)}=0.11$	0.994	$z_{(1)}=1.56$	0.223	$z_{(1)}=1.29$	0.353	$z_{(1)}=2.10$	0.071

Experiment 4: Black ash lure and seedling in wind tunnel

		Take Off		**Lock On**		**Oriented Upwind Flight**		**Source contact**	
Effect	***Post hoc* comparison**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**
Treatments		$\chi^2_{(2)}=7.86$	0.385	$\chi^2_{(2)}=3.75$	**0.01**	$\chi^2_{(2)}=3.75$	**0.01**	$\chi^2_{(2)}=2.01$	**0.003**
	lure *vs.* control	$z_{(1)}=1.43$	0.323	-	0.239*	-	0.239*	-	1.0*
	seedling *vs.* control	$z_{(1)}=0.18$	0.982	-	**0.024**	-	**0.024***	-	**0.050***
	seedling *vs.* lure	$z_{(1)}=1.27$	0.410	$z_{(1)}=1.13$	0.452	$z_{(1)}=1.13$	0.452	$z_{(1)}=2.10$	**0.022***

Table 2-5. Split plot ANOVA table of the main effects and interactions of the generalized linear mixed effects model of the total number of *Caloptilia fraxinella* and the total number of males and females, caught in wing traps baited with synthetic ash volatile organic compound lures (see Table 2-3) in Edmonton (2008). The variable site is random. Degrees of freedom are shown in subscripted brackets. Table corresponds to Fig. 2-8.

Experiment 5: Pre flush

	Total no. moths		No. males		No. females	
Effect	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**
Treatments	$\chi^2_{(2)}=2.67$	0.263	$\chi^2_{(2)}=2.72$	0.257	$\chi^2_{(2)}=2.06$	0.358
Location (tree vs. alley)	$\chi^2_{(1)}=10.83$	**<0.001**	$\chi^2_{(1)}=1.82$	0.178	$\chi^2_{(1)}=16.93$	**<0.001**
Treatment * Location	$\chi^2_{(2)}=0.30$	0.861	$\chi^2_{(2)}=0.07$	0.967	$\chi^2_{(2)}=1.16$	0.559

Experiment 6: Post flush

Treatments	$\chi^2_{(2)}=3.88$	0.144	$\chi^2_{(2)}=6.46$	**0.040**	$\chi^2_{(2)}=0.25$	0.413
Green lure vs. Control	-	-	$z_{(1)}=2.47$	**0.036**	-	-
Location (tree vs. alley)	$\chi^2_{(1)}=35.91$	**<0.001**	$\chi^2_{(1)}=33.27$	**<0.001**	$\chi^2_{(1)}=10.85$	**<0.001**
Treatment * Location	$\chi^2_{(2)}=0.20$	0.905	$\chi^2_{(2)}=2.09$	0.351	$\chi^2_{(2)}=0.56$	0.757

Experiment 6: Post flush with 100 mg lures

Treatments	$\chi^2_{(4)}=8.38$	0.079	$\chi^2_{(4)}=8.17$	0.086	$\chi^2_{(4)}=4.30$	0.367

Figure 2-1. Scanning electron microscopic images of the antennae of female *Caloptilia fraxinella* (Lepidoptera: Gracillaridae).

A. Head and left antenna of a female. pd=pedicel, sp=scape, flg=flagellum B. Sensilla protruding beyond the scaled covering of a flagella segment (arrowheads) ch=sensilla chaeticum, tr=sensilla trichodia. C. Segments along the flagellum of a female antenna. Sensilla chaetica (ch) extend perpendicularly beyond the covering of long scales (l sc) with other sensillar types running longitudinally between the long scales. D. Flagellar segment with scales removed to show sensilla pointing towards the distal end of the antennae. Assemblages of scale sockets (ss l) from long scales (l sc) are visible at the proximal end of the segment with single sockets of the short scales (ss) at the distal end. Ca= sensilla campaniformium, st= sensillum styloconica, ch=sensillum chaeticum E. Junction of two segments showing overlap of short scales (s sc), covering of long scales (l sc), single scale sockets (ss) and multiple sensillar types including sensilla chaetica. F. Sensillum basiconicum (ba) with longitudinal striations in close association with a sensillum trichodium (tr) with corrugated helical ridges and a blunt tip. G. Magnification of segment from image D with sensilla campaniformium (ca), trichodia (tr) and basiconica (ba). Scale socket (ss), long scale (l sc). H. Sensillum trichodium with a sharp tip. I. Sensillum styloconica, with a short stylus (st). J. Putative terminal flagellar segment with two sensilla auricillica (au), long scale (l sc) overlapping short scales (s sc) and several other sensilla (not labelled). K. Sensillum coeloconica. L. Sensillum chaeticum with basal articulating socket and apical pore.

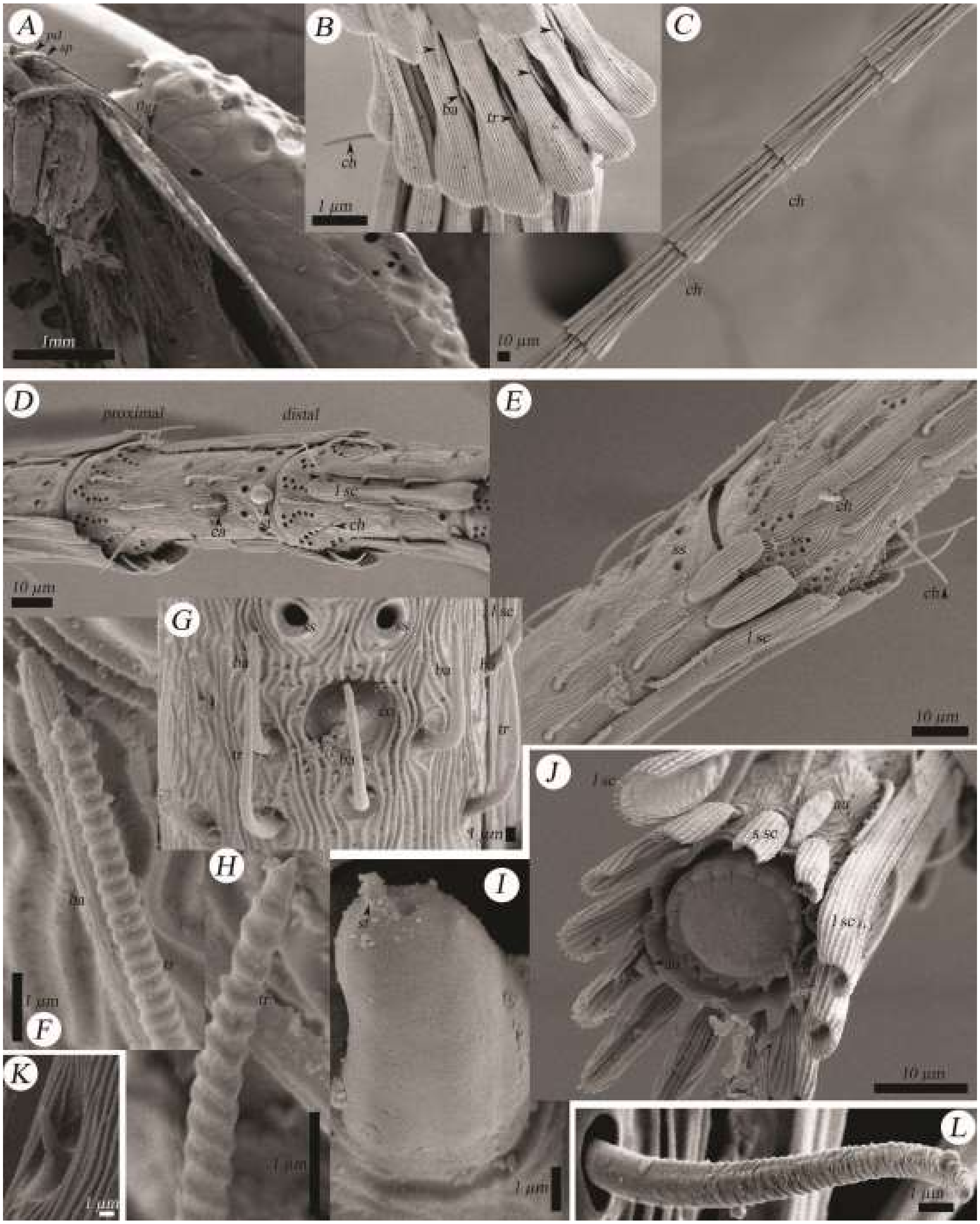
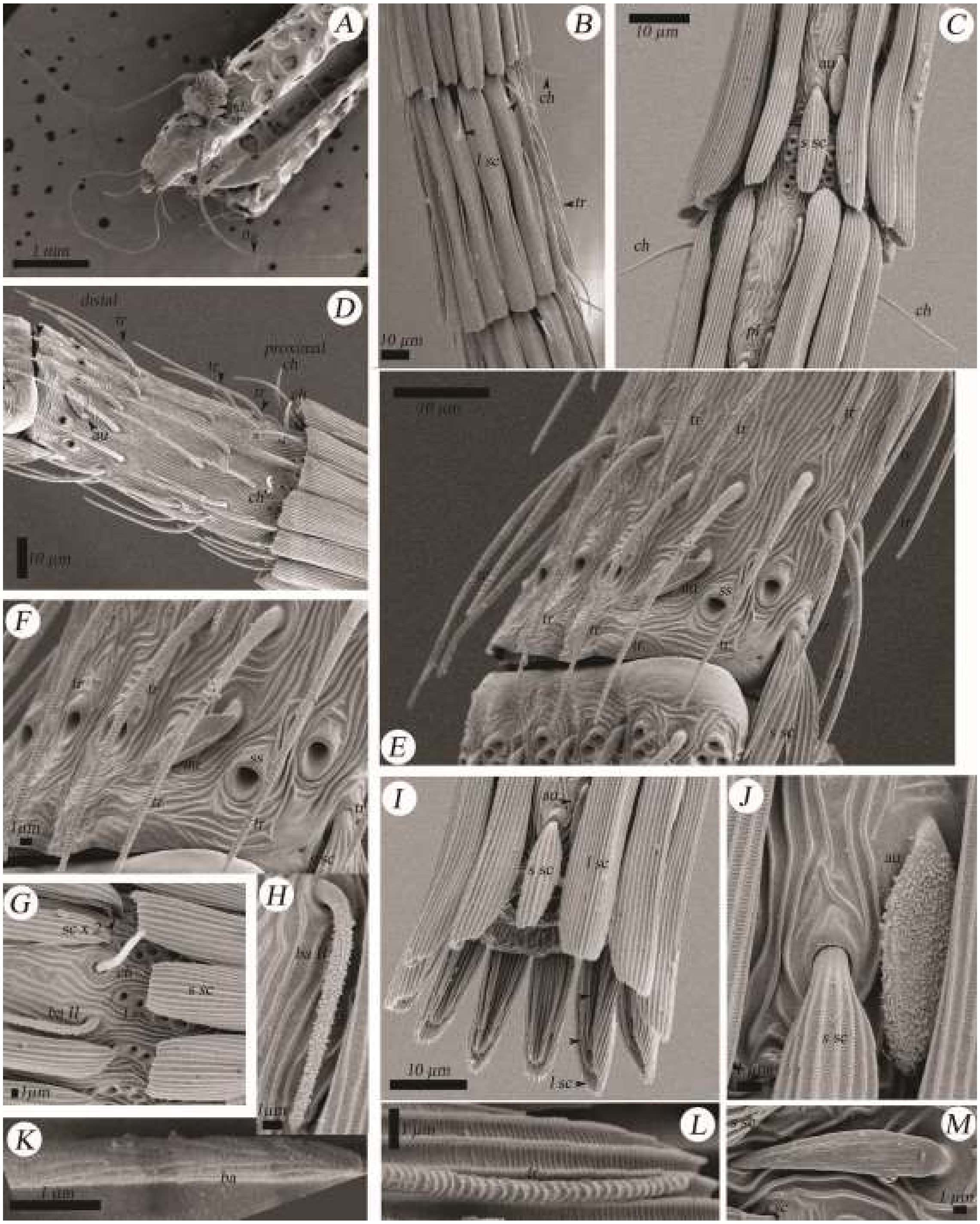

A
pd
sp
ba
1mm
B
ba
tr
ch
1 µm
C
ch
ch
10 µm
D
proximal
distal
l sc
co
st
ch
10 µm
E
ch
ss
l sc
ch
10 µm
G
ss
ss
l sc
ba
ba
co
tr
ba
tr
1 µm
F
ba
tr
1 µm
H
tr
1 µm
I
st
1 µm
J
l sc
aa
s sc
l sc
aa
10 µm
K
1 µm
L
1 µm

Figure 2-2. Scanning electron microscopic images of the antennae of male *Caloptilia fraxinella* (Lepidoptera: Gracillaridae).

A. Head and both antennae of a male. pd=pedicel, sp=scape, flg=flagellum B. Sensilla protruding beyond the scaled covering of a flagella segment (arrowheads) ch=sensilla chaeticum, tr=sensilla trichodia, long scales (l sc). C. Partially de-scaled flagellar segments with sensilla placodium (pl) at the distal end of each. Two sensilla chaetica (ch) protrude from the proximal end of the lower segment. S sc=short scale. D. Scaleless flagellar segment densely covered with distally pointing sensilla trichodia (tr) loosely arranged into three wreaths that encircle the segment (arrowheads). Three sensilla chaetica (ch) are evident at the proximal end below the scale sockets of long scales. au=sensilla auricillica E. Magnification of segment in D to show a sensillum auricillica (au) and the distal sheath of sensilla trichodia (tr; below) and median sheath (tr; above). Note the two remaining short scales (s sc) and their ring of single scale sockets (ss). F. Magnification of segment in D, E to show helical ridges on sensilla trichodia (tr). G. Proximal end of a segment with two overlapping scales (sc x 2), long scale socket assemblage (l sc), and short scales overlapping from the previous segment (s sc), sensillum chaeticum (ch) and a fuzzy sensillum basiconica (ba II). H. Magnification of fuzzy sensillum basiconica (ba II). I. Sensillum auricillica (au) on the distal end of a segment beside a short scale (s sc) and the underside of several long scales (l sc) where two scales can be seen nested within the longer, top scale (arrowheads). J. Fuzzy sensillum auricillica (au) beside a short scale (s sc). K. Sensillum basiconica (ba) with longitudinal striations. L. Sensillum trichodia (tr) with characteristic helical ridges and a blunt tip. M. Regular shoehorn sensillum auricillica on distal end of flagellomere. S sc= short scales.


A
1 mm
B
ch
l sc
tr
10 µm
C
10 µm
au
s sc
ch
ch
pi
D
distal
tr
proximal
ch
tr
tr
ch
au
ch
10 µm
E
10 µm
tr
tr
tr
au
ss
tr
tr
tr
tr
s sc
F
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tr
au
ss
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1 µm
G
sc x 2
ch
s sc
ba II
1 µm
H
ba II
1 µm
I
au
s sc
l sc
10 µm
l sc
J
au
s sc
1 µm
K
ba
1 µm
L
1 µm
M
s sc
sc
1 µm

Figure 2-3. Mean (± S.E.) total number of adult *Caloptilia fraxinella* moths caught per treatment on sentinel black ash trees (Open treatment allowed both visual and volatile cues, mesh allowed volatile cues only, closed allowed visual cues only). Statistically significant differences (GLMM) among treatments in the mean number of moths are represented by different lower case letters.

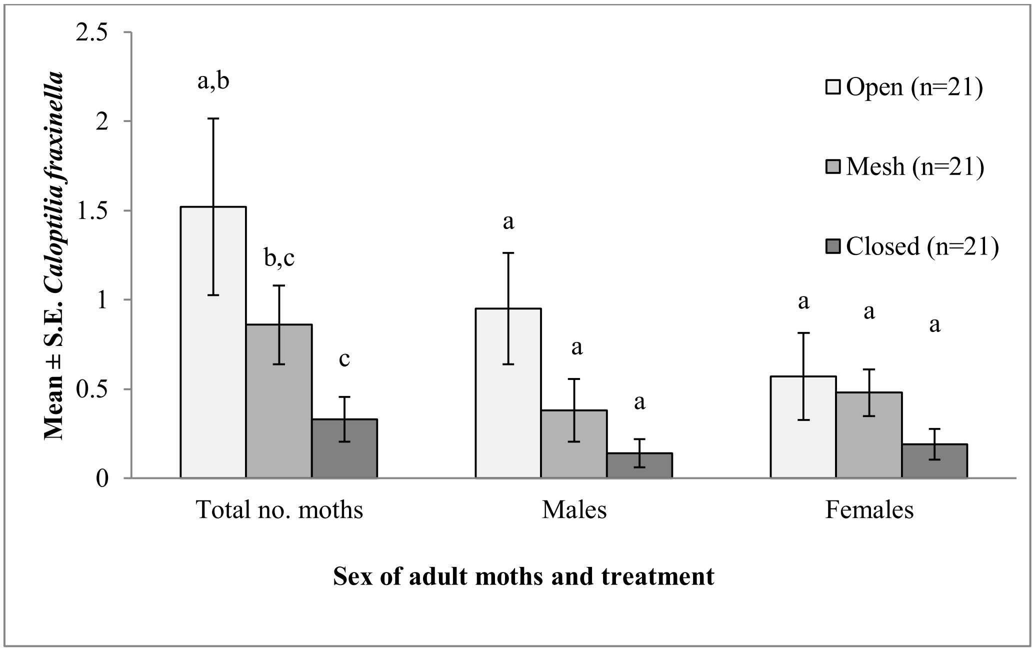


Figure 2-4. Simultaneously recorded GC–EAD analysis of headspace volatile organic compounds collected during the day and night from newly flushing black ash, *Fraxinus nigra*. The upper trace represents the Flame Ionization Detector (FID) response and the lower trace the antennal responses (EAD) of mated female *Caloptilia fraxinella*. Antennal activity was found to the following compounds. Retention indices are bracketed: 1) *trans* ocimene (7.84) 2) Linalool (8.41) 3) (*E)* 4,8-dimethyl 1,3,7 nonatriene (8.55), 4) Methyl salicylate (9.40) 5) Unknown sesquiterpene (11.68) 6) (*E,E*)-α-farnesene (11.79) 7) Unknown sesquiterpene (12.12).

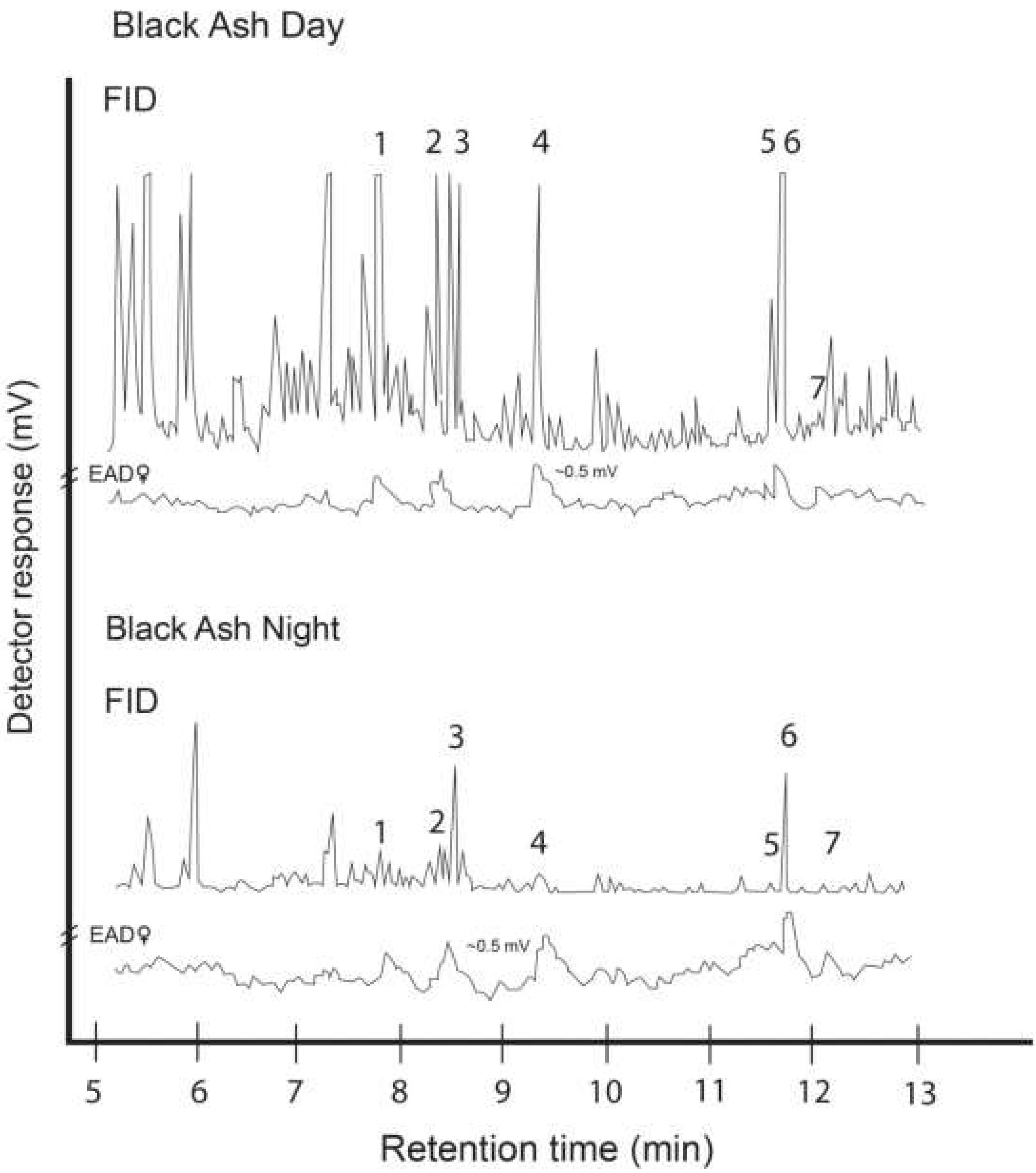

Black Ash Day
FID
1
2 3
4
5 6
7
EAD♀
~0.5 mV
Black Ash Night
FID
3
6
1
2
4
5
7
EAD♀
~0.5 mV
Detector response (mV)
5
6
7
8
9
10
11
12
13
Retention time (min)

Figure 2-5. Simultaneously recorded GC–EAD analysis of headspace volatile organic compounds collected during the day from newly flushing green ash, *Fraxinus pennsylvanica*. The upper trace represents the Flame Ionization Detector (FID) response and the lower trace the antennal responses (EAD) of female *Caloptilia fraxinella*. Antennal activity was found to the following compounds. Retention indices are bracketed: 1) *trans* ocimene (4.68) 2) Linalool (5.17) 3) Methyl salicylate (6.02) 4) (*E*)-β-farnesene (8.27) 5) (*E,E*)-α-farnesene (8.33).

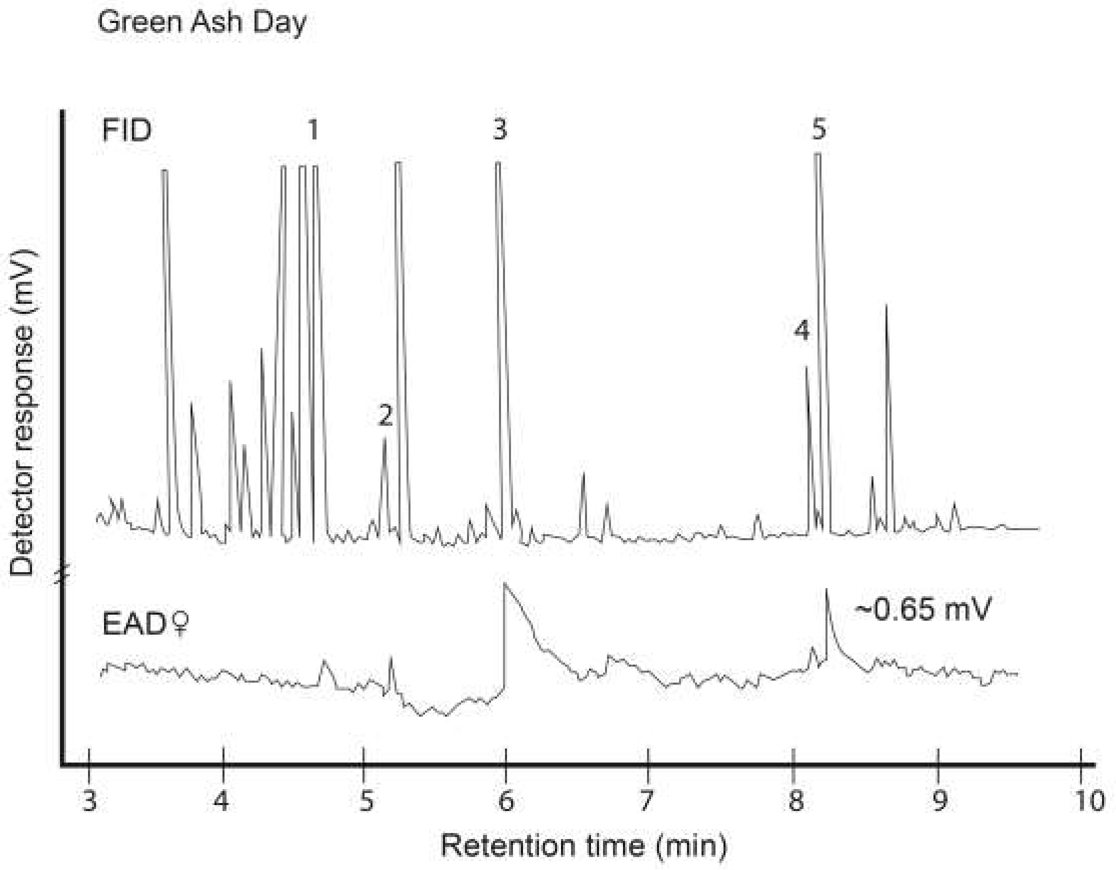

Green Ash Day

Figure 2-6. Host location behaviour of spring-caught, mated female *Caloptilia fraxinella* to green ash seedlings, synthetic green ash lures (volatile organic compounds in 50 mg drops of biodegradable paste) and a control plastic plant (2011) with inert 50 mg drops of paste. Statistically significant differences (GLMM and Fisher's Exact Test) among treatments are represented by different lower case letters.

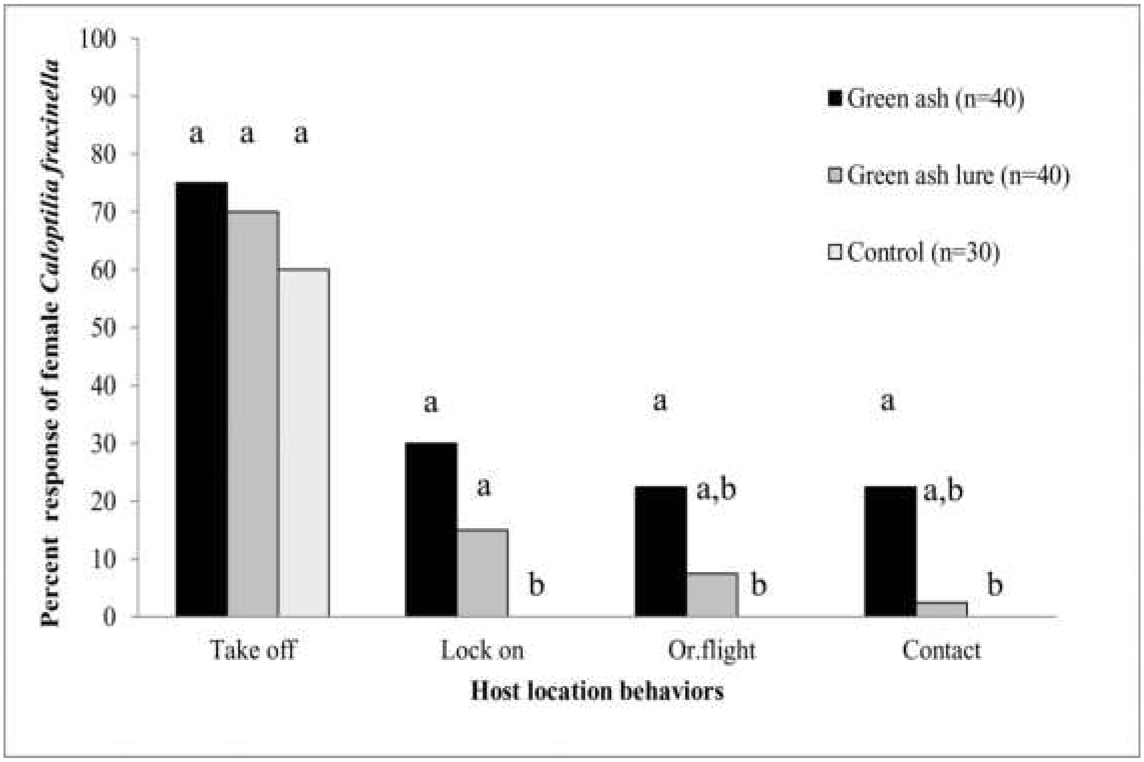


Figure 2-7. Host location behaviour of spring-caught, mated female *Caloptilia fraxinella* to black ash seedlings, synthetic black ash lures (volatile organic compounds in drops of biodegradable paste) and a control plastic plant (2011) with inert drops of paste. Statistically significant differences (GLMM and Fisher's Exact Test) among treatments are represented by different lower case letters.

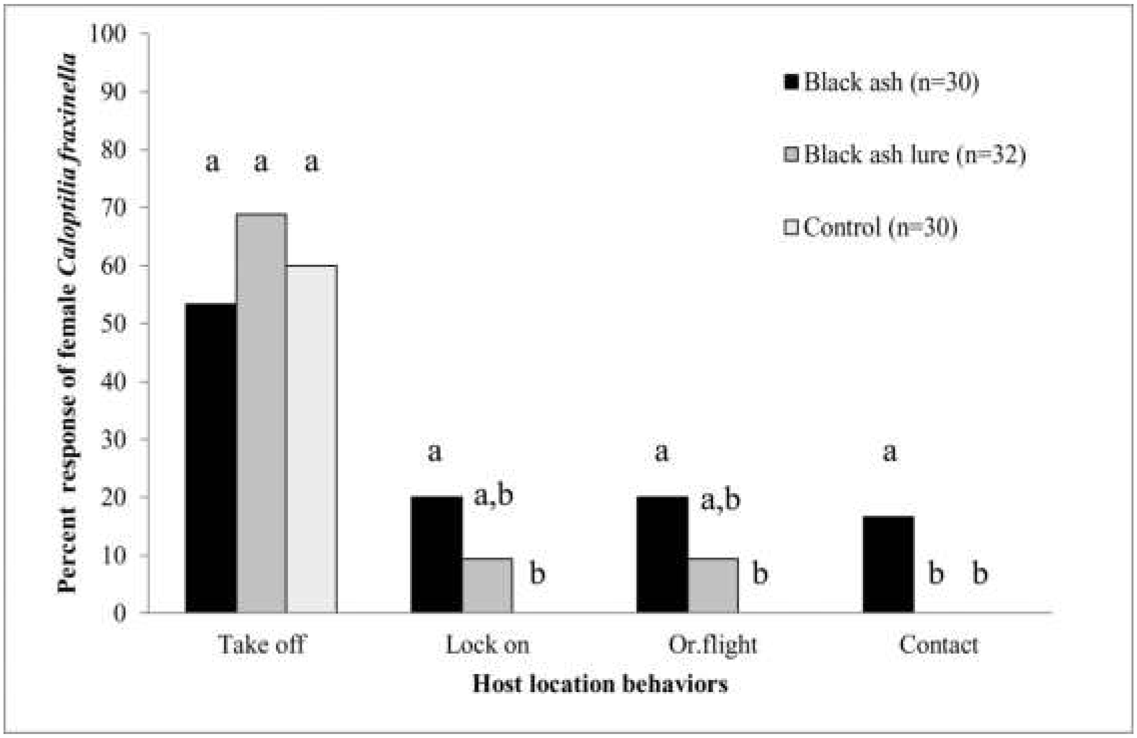
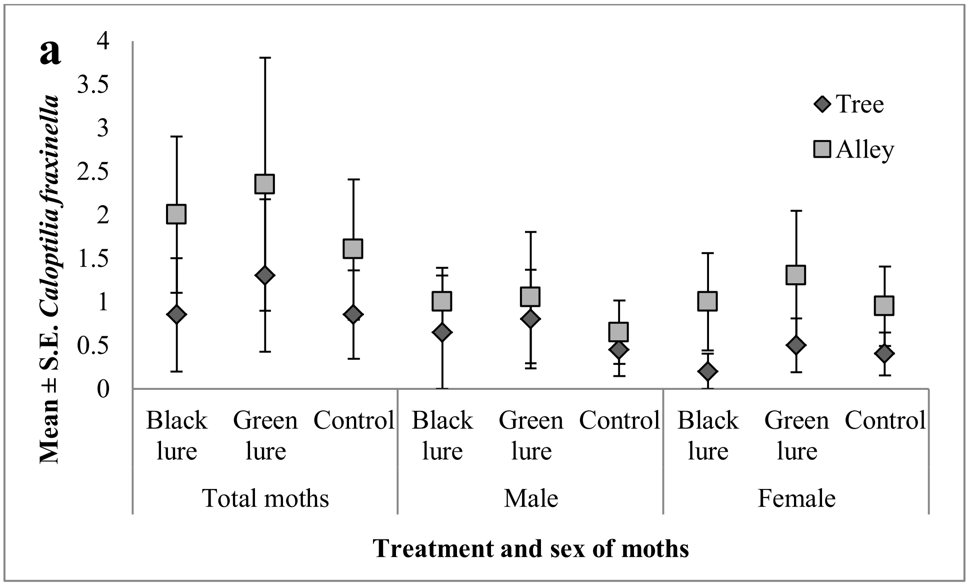
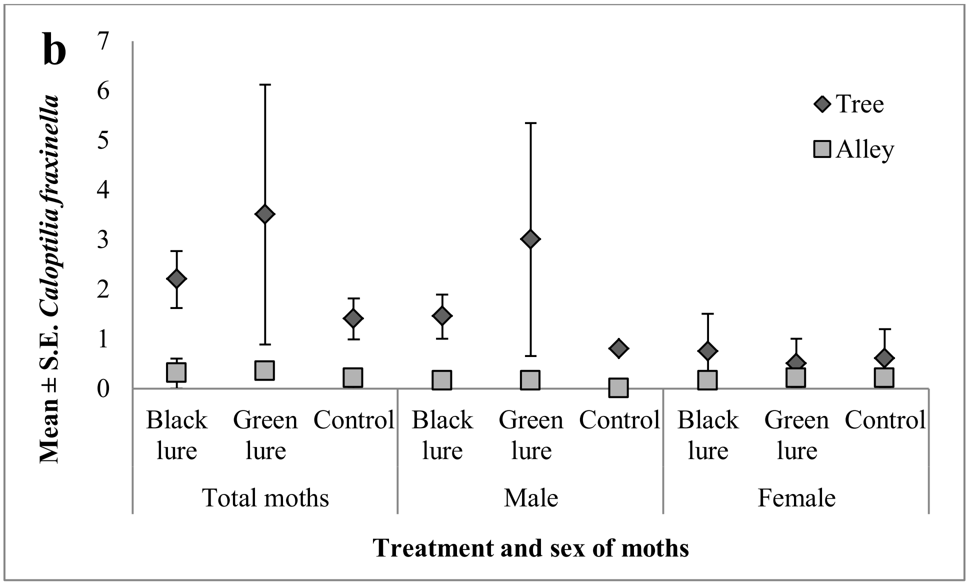
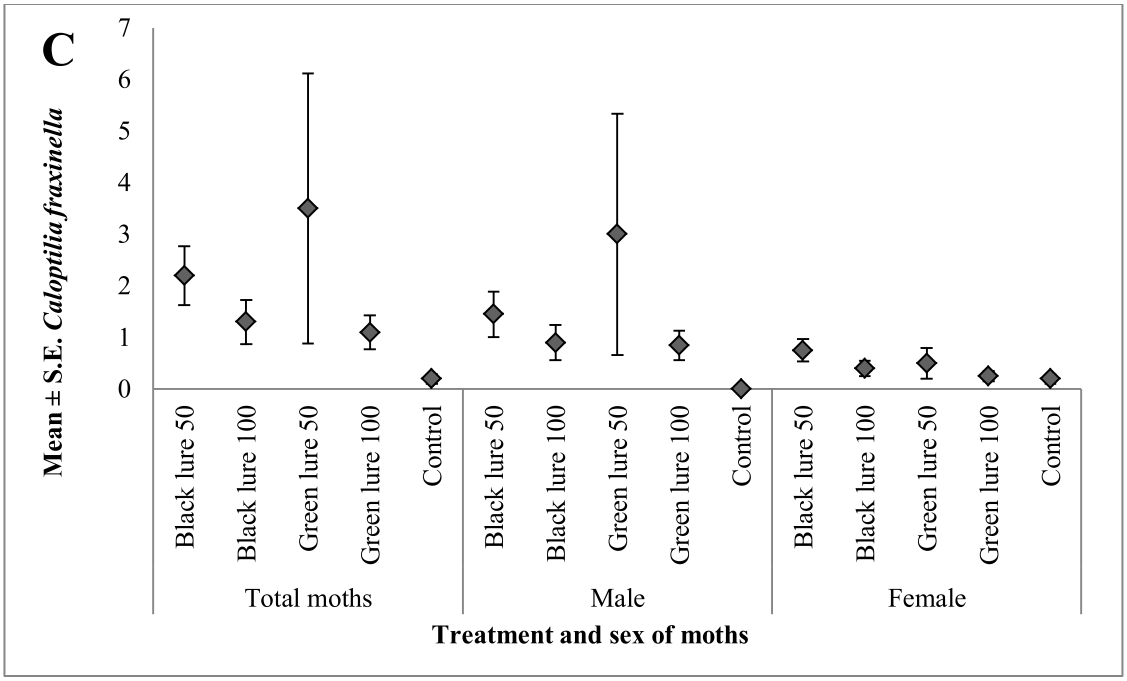


Figure 2-8. Field trapping results, experiments 5 (a) and 6 (b,c): Mean ± S.E. number of female *Caloptilia fraxinella* by treatment and trap location, (a) before leaf flush, (b) after leaf flush and (c) tree only results with 100 mg black and green ash lures after leaf flush.






C
Mean ± S.E. *Caloptilia fraxinella*
0
1
2
3
4
5
6
7
Black lure 50
Black lure 100
Green lure 50
Green lure 100
Control
Total moths
Male
Female
Treatment and sex of moths

Chapter 3: Preference and performance of a leaf-mining moth on two *Fraxinus* species

Introduction

Evolutionary theory predicts that insects should prefer to oviposit on host plants that maximize the fitness of their offspring (Jaenike, 1978; Thompson, 1988), thus, host plant preference of the female should be positively linked to offspring performance, as suggested in the "Mother-knows-best" (Gripenberg et al., 2010) or preference-performance hypothesis (Jaenike, 1978), also called "the optimal-oviposition" hypothesis (Thompson, 1988). A positive preference-performance linkage is especially important for leaf-mining insects with non-motile larvae in which host location is entirely dependent upon the oviposition choice of the female (Faeth 1985; Auerbach & Simberloff, 1989). The choice of a suboptimal host plant by the female can significantly reduce her fitness, even to the point of complete failure (Kagata & Ohgushi, 2001). Positive linkages between preference and performance are expected and frequently occur; however negative linkages are also reported (see references in Thompson, 1988; Thompson & Pellmyr, 1991; Mayhew, 1997; Gripenberg et al., 2010) and sometimes no preference or difference in performance occurs among hosts (Goane et al., 2008).

Stimulation of oviposition behaviour can involve the integration of cues via several sensory modalities (Ramaswamy, 1988) such as perception of volatile organic compounds (VOCs), visual and contact cues and can involve perception of both stimulatory and inhibitory signals. To achieve host plant location, sensory information is integrated in the central nervous system of the adult female insect. If the experimental design eliminates some host cues or interrupts the sequence of host selection (Kostal & Finch, 1994), then the true host plant preference may be obscured under artificial experimental conditions.

Host location for oviposition by moths (Lepidoptera) can be divided into a sequence of several search steps that are often mediated by chemical cues (Ramaswamy 1988; Liu et al., 2006). Host-habitat location, host location and host acceptance are the three main steps in the host finding sequence (Ramaswamy 1988). Host habitat location is a long range endeavour in which a female moth follows a stimulus or stimuli in order to find the host environment or host plant. Host location to single plants occurs once the habitat of the host plant has been found. Host acceptance occurs after host location to a plant and involves stimuli on the surface of leaves that indicates if a plant is suitable for oviposition. At each step, female moths must make a correct choice based on the cues that she has available, or risk oviposition on an inferior host plant. Long and short range orientation of female moths to host plants is often mediated by VOCs released from plants (Renwick and Chew, 1994) and contact with surface chemicals often guides host acceptance (Ramaswamy, 1988; Honda, 1995).

Host plant VOCs are implicated as the main host location cue for leaf-mining micro lepidopterans (Proffit et al. 2011). Vision is likely not as important in host location as VOC cues for moths that seek oviposition sites at night but can contribute somewhat to host location (Ramaswamy, 1988 and references therein). The tomato leaf miner, *Tuta absoluta*, (Meyrick) (Lepidoptera: Gelechiidae) uses small variations in the identity and ratio of plant VOCs in host location (Proffit et al. 2011). Four single compounds in the VOC profile of horse chestnut, *Aesculus hippocastanum* (Hippocastanaceae) are short range attractants that elicit oviposition by the horse chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić (Lepidoptera: Gracillariidae), and two compounds unique to damaged leaves deter oviposition (Johne et al. 2006). Female *Stilbosis juvantis* (Cosmopterigidae), an oak (*Quercus* spp.) leaf miner preferentially oviposit on undamaged rather than damaged leaves (Faeth 1985), suggesting an inhibitory chemical cue from damaged oak leaves deters oviposition. Contact with leaf surface chemicals and the physical structure of leaf surfaces also mediates host acceptance for oviposition in leaf-mining microlepidoptera (Fenemore, 1980; Liu et al., 2006). An *Agnippe* sp. Chambers (Lepidoptera: Gelechiidae) leaf miner prefers to oviposit on young *vs*. old leaves due to the lower tannin content and higher nitrogen and water levels in younger leaflets (Ishino et al., 2011).

This study examines the relationship between female preference (oviposition and host location) and larval performance of a leaf-mining moth, *Caloptilia fraxinella* (Ely) (Lepidoptera: Gracillariidae), on two host plant species in the genus *Fraxinus*. *Caloptilia fraxinella* is an oligophagous herbivore that feeds exclusively on ash trees in the genus *Fraxinus* (Oleaceae) (Pohl et al., 2004) and uses green ash (*F. pennsylvanica*), black ash (*F. nigra*), white ash (*F. americana*) and Manchurian ash, *F. manshurica* Rupr as hosts (Pohl et al., 2004, and specimens in the Great Lakes Forestry Centre, Canadian Forestry Service ON, and the Laurentian Forestry Research Collection, Canadian Forest Service, QC). In western Canadian cities, the two main hosts of *C. fraxinella* are green ash and black ash (Pohl et al., 2004) both planted outside of their natural range (Farrar, 1998).

Caloptilia fraxinella larvae serpentine mine leaflets during the first two instars, transition from second to third instar in a blotch mine on the leaflet edge, then emerge to travel to new leaflets that are rolled into pyramidal-shaped cones where they pupate and emerge as adults in mid-summer (Pohl et al., 2004; Evenden, 2009; Wist & Evenden, 2013). As movement of leaf-miner larvae is restricted within the leaflet, host preference is dictated by the oviposition of the female (Valladares & Lawton, 1991; Auerbach & Alberts, 1992; Stein & Price, 1995) that occurs in the spring (Evenden, 2009). *Caloptilia fraxinella* females and males arrive at ash trees as leaves flush (Wist & Evenden, 2013) and females are constrained to lay eggs on freshly flushed ash leaflets in the spring (Evenden, 2009). Leaf miner host preference can be affected by the phenological stage of the host plant that can further dictate host-plant suitability (Denno & McClure, 1983; Mattson et al., 1988). *Caloptilia fraxinella* oviposits on new leaflets of ash (Evenden, 2009) but in the spring when females arrive at host trees, all available leaflets are new and this preference for new leaflets may not constitute a true choice. It is not clear if females prefer new leaflets over older leaflets or if they are constrained to new leaflets by tree phenology.

Metrics used to measure offspring performance in Lepidoptera include larval survival and development and pupal mass (Kagata & Ohgushi, 2001; Yamasaki & Fujisaki, 2010; Videla et al., 2012; Zhang et al., 2012). Development time of offspring is a good indicator of the performance of larvae on a given host plant (Kagata & Ohgushi, 2001) and slower development can indicate the presence of host defensive compounds that slow growth and increase the susceptibility of larvae to predation and parasitism (Benrey & Denno, 1997). Pupal weight correlates with adult body size and reproductive capacity in many moths and can reflect the nutrient value of the host plant (Lill & Marquis, 2001). This study is designed to test the following hypotheses: (1) That female *C. fraxinella* prefer on ash species over another for host location and oviposition; (2) that performance of larvae differs between ash species; (3) that there is a positive link between the host plant preference of female *C. fraxinella* and the performance of larvae on host plants. Does "Mother know best" in the *C. fraxinella-Fraxinus* system?

Materials and methods

Insects

Insects used in oviposition experiments (collected 28 April to 6 May, 2008, 16-22 April 2009), laboratory larval development experiments (collected 4-6 May, 2011) and wind tunnel experiments (collected 20 April to 3 May, 2010) were collected as adult moths on the wing after emergence from overwintering sites each spring in several ash-dense neighbourhoods in Edmonton, AB (53º32′N/-133º30′W) prior to leaf flush of the ash trees. *Caloptilia fraxinella* mates in early spring (Evenden et al., 2007) and collected females were assumed to be mated. A subset of ten females from each collected cohort was dissected and were all mated. Moths were separated by sex and sorted individually into 30 ml plastic cups with clear lids (SOLO cup company, Lake Forest, Illinois, U.S.A.) and placed into an environmental chamber maintained at 24 °C on a reversed photo-regime (16L:8D). Moths were fed sugar water (10%

sucrose, *ad libitum*) through a cotton dental wick for 7-14 days after collection to promote egg maturation (Evenden et al., 2007) prior to use in bioassays.

Plant material

The plant material used in wind tunnel experiments and oviposition and larval development experiments in the laboratory was primarily derived from ash seedlings except where noted. One-year-old green ash and black ash seedlings (Jeffries Nurseries, Portage La Prairie, Manitoba) were planted individually in pots filled with Sunshine #4 potting mix (SunGro Horticulture Ltd., Agawam, MA, U.S.A.), in a greenhouse under summer conditions (16L:8D), 24.5 °C, 50 %RH) and were watered as required. Seedlings were approximately ~50-60 cm tall and had similar amounts of foliage at the start of trials, although green ash continued to produce leaves (indeterminate growth) while growth of black ash was determinate.

Oviposition preference

Oviposition preference experiments were conducted as choice and non-choice experiments at three scales using: 1) excised leaflets (Fig. 3-1A,B); 2) excised whole leaves (Fig. 3-1C,D); and 3) intact whole seedlings (Table 3-1).

Excised leaflet assays

Most oviposition experiments were carried out in an environmental chamber (24 °C, 70% R.H), under 16L:8D except where noted. To determine oviposition preference and the potential cues used by ovipositing females, adult females caught in the spring were placed singly into oviposition arenas in several experimental configurations. Oviposition arenas were constructed from an individual 90 mm Petri dish with one, two (Fig 3-1A) or three (Fig. 3-1B) centrally located 20 mm diam. holes that were equidistantly positioned. The Petri dish bottom with holes was inverted over the experimental leaflets that sat on a moist disc of Whatman no. 1 filter paper inserted into the lid of the Petri dish. This arrangement permitted a similar leaflet area of each treatment to be available to the moth. After the female moth was placed in the assay, the arena was covered with another Petri dish lid and sealed with Parafilm™. Eggs were counted after each scotophase period until the moth died. The number of distinct oviposition events was recorded throughout the bioassay. Non-responsive moths were removed from the final analysis except in experiments in which only the wax paper control substrate was provided to females.

Leaflets presented to moths in the excised leaflet experiments were field collected from the south side of three separate green ash and black ash var. "Fallgold" trees from two sites, and leaflets of roughly the same size and age from the fourth position on each leaf were chosen and used immediately in oviposition arenas. Five excised leaflet experiments were conducted in the laboratory in 2008 and 2009 (Table 1). Experiment 1(n=15) was a no-choice experiment with a control that tested oviposition on wax paper through a single 20 mm hole. Wax paper provided an oviposition substrate similar to the waxy cuticle of a leaflet but without any host-associated cues. This wax paper control was run at the same time as a no-choice experiment in which a single leaflet of green ash was offered to each female positioned under a single 20 mm hole (n=28). Experiment 2 (n=23) tested oviposition choice between a green ash leaflet and a wax paper disc each positioned under a 20 mm hole. Experiment 3 (n=30 total) provided a choice between a black ash leaflet and a wax paper disc each positioned under a 20 mm hole (Fig 3-1A). This experiment was conducted over two field seasons in 2008 and 2009 because of premature moth mortality in 2008. Experiment 4a was conducted in 2009 and was nearly identical to and run concurrently with one rep of experiment 3. In experiment 4a however, arenas were positioned on the lab bench next to a window so that moths could experience natural photoperiod conditions (n=10). In experiment 4b, ten replicates of arenas with only wax paper was conducted concurrently with experiment 4a in arenas also positioned on the lab bench under natural photoperiod conditions. In these arenas the wax paper was cut into the shape of leaflets with "leaflet veins" traced on the surface with a dissection needle to provide visual cues to females. Experiment 5 was conducted in consecutive years 2008 (n=15) and

2009 (n=11) and offered three oviposition choices; one similarly sized black and green ash leaflet and wax paper all offered through 20 mm holes in the Petri dish (Fig. 3-1B), as described above.

Oviposition data were not normally distributed (Kolmogorov-Smirnov tests). Leaflet oviposition experiments (1-5) were analyzed with the Kruskal-Wallace test followed by non-parametric *post hoc* Tukey's HSD tests to separate mean differences among groups when multiple comparisons occurred (SigmaPlot 12, Systat Software, San Jose, CA) with the exception of experiments 1 and 5. In these two experiments, the number of eggs per treatment was analyzed with a generalized linear model (GLM) with a log link function and a negative binomial error term (R 3.0.1, R Core Team 2013) due to overdispersion of the response variable. In experiment 1, the number of eggs oviposited in each no choice treatment (wax paper or green ash leaflet) was compared in addition to the placement of the eggs (surface or onto the Petrie plate). In experiment 5, eggs laid on leaflets or wax paper, above leaflets and on the plate near leaflets were pooled according to the three treatments; black ash leaflet, green ash leaflet and wax paper control. The resulting analysis of deviance table was used to determine if the number of eggs laid by female *C. fraxinella* was dependent on the treatment (black ash, green ash, wax paper control) or year of the experiment. The two-way interaction was included in the model. Significant effects of treatments on the number of eggs laid were separated with *post hoc* Tukey's tests ("multcomp" package, in R 3.0.1, R Core Team 2013).

Excised whole leaf assays

Leaf assays conducted in caged arenas with entire leaves allowed for more in-depth questions to be tested and allowed some flight behaviour within the arena by female *C. fraxinella*. Whole leaf arenas used in Experiment 6 (n=15) (Table 3-1) each contained one entire leaf of green ash and one of black ash (Fig 3-1C) of the same approximate size and age placed in separate small vials of water covered by Parafilm™ except where petioles penetrated. Leaves were placed into a plastic container (15 cm height, 7.5 cm diameter) with a transparent lid. One female *C. fraxinella* was placed into each arena and oviposition was scored as in leaflet assays until moths died except that multiple leaflets per leaf were examined. Leaf assay arenas were placed into an environmental chamber as above. At the completion of the bioassay, moths were dissected to determine mating status and presence of vitellogenic eggs.

The number of eggs on the leaves of the two ash species was compared with a generalized linear mixed effects model (GLMM) with a log link function and Poisson errors terms (lme4 package, R 3.0.1, R Core Team 2013). The resulting analysis of deviance table was used to determine if number of eggs laid by female *C. fraxinella* was dependent on the species of ash leaf (black or green) with the individual moth and leaflet number within the leaf specified as random factors. Significant treatment effects in the GLMM were separated by Tukey's HSD *post hoc* tests (R package multcomp).

Intact whole seedling assays

Experiments 7 and 8 tested the hypothesis that female oviposition behaviour was influenced by host tree species and leaflet age (Table 3-1). Green and black ash seedlings, planted as described above, were positioned at leaf flush in cages (60 x 60 x 60 cm) (BugDorm 2120, MegaView Science, Taichung, Taiwan) kept at ambient room temperature (~24.5 ºC) under a natural photoperiod. Plants were watered as required, sugar water was provided *ad libitum* to moths and the experiment ran 11-23 May 2009 (Table 3-1). Moth oviposition was recorded in two caged seedling experiments: 7) species choice where one black ash and one green ash seedling per cage (n=7 cages); and 8) no species choice where moths were either caged with two black ash seedlings (n=7) or two green ash seedlings (n=7). Two female moths were introduced per cage when approximately half the leaflets were older than four days. Leaflets were designated as "new" if they were younger than four days and old if they were older than four days. Green ash continued to produce new leaves and leaflets while black ash stopped at the four leaf stage. The black ash seedlings exhibited determinate growth and produced one single flush of leaves (four leaves total) while the green ash seedlings exhibited indeterminate growth and continued to elongate their twigs and produce new buds over the course of the experiment. On green ash, new leaflets were continuously available for oviposition while after the first few days of the experiment the black ash leaflets were all considered old. Oviposition was scored after each scotophase until both moths died. Moths recovered from cages were dissected to determine mating status and to count the vitellogenic eggs that remained. The number of eggs laid per leaflet in each experiment was analyzed with a GLMM with a log link function and negative binomial errors terms to correct for overdispersion (R 3.0.1, R Core Team 2013). The resulting two analyse of deviance tables were used to determine if the number of eggs laid was dependent on the ash species (black or green) (Experiment 8) and age of the leaflets (new or old) with the replicate nested within the experimental day and set as a random factor. In the analyses of experiments 7 and 8, the two-way interaction between ash species and leaflet age was included in the model.

Host location preference

Wind tunnel assay

A wind tunnel experiment was conducted (4-7 May, 2010) to assess the long distance host location behaviour of female *C. fraxinella*. The experiment tested the hypothesis that the host location response of mated female *C. fraxinella* would be similar to green ash and black ash seedlings. Groups of ten moths were flown individually to each ash species and a control plastic seedling over four consecutive days for a total of forty moths in each treatment.

Green ash and black ash seedlings were planted as described above for oviposition experiments. Insects used in the wind tunnel experiment were caught in the spring of 2010 and were separated by sex and placed individually in 30 ml plastic cups and provided with sugar water (10% v/v) through a dental wick for at least seven days to develop eggs (Evenden et al.

2007) before use in the bioassay. Cups were distributed evenly among three environmental chambers each programmed for a reversed photo-regime (16L:8D) with staggered initiation of the scotophase period so that continuous experimentation could occur during daylight hours.

The wind tunnel was 1.7 m long, 0.85 m high and 0.9 m wide. A pattern of randomly distributed red dots on a white background was on the chamber floor (diameter 5-10 cm) to provide visual stimuli for optomotor anemotaxis. The wind tunnel experiment was conducted under red light (0.11 lux), with a wind speed of 0.3 m/s and a temperature of 24 °C. Wind tunnel assays occurred between one hour prior to and three hours after the initiation of scotophase. Moths were transferred to the wind tunnel room 0.5 hour prior to initiation of the wind tunnel assay to acclimatize to the experimental conditions. Females were placed individually into cylindrical release cages (5cm d x 6cm h). Release cages were placed 65 cm from the tunnel floor and 100 cm from source plants. Each moth was given 30 s in the release cage once placed in the tunnel before release, five minutes to leave the cage before being declared a non-responder and a total of ten minutes in the wind tunnel including the time in the release cage. Moth behaviour was recorded as: wing fanning, take-off from the release device, lock on to the odour plume, oriented upwind flight (or.flight) and contact with the seedling. Moths were used only once and were dissected after flight to determine mating status, presence of vitellogenic eggs and egg load.

In the wind tunnel experiment, behavioural responses were recorded as “yes” or “no” for each moth so the resulting data were binomially distributed. Each recorded behaviour was analyzed separately with a GLMM with a logit link function and binomial errors terms (lmer package in R 3.0.1). The resulting analysis of deviance table was used to determine if female response was dependent on the ash treatment with experimental day as the random factor. When the GLMM was significant, multiple comparisons were made using a Tukey-contrasts test (R-package: multcomp), except if means of zero in the control group prevented Tukey’s test. The Fisher’s exact test was employed to compare treatment groups with means of zero.

Field assay

A field experiment to test if female *C. fraxinella* prefers to fly to black or green ash was conducted at ash leaf flush when oviposition of *C. fraxinella* is known to occur in the field (Pohl et al. 2004; Evenden 2009). In addition, the daily flight phenology of *C. fraxinella* was followed to determine the circadian flight activity of male and female moths. Fight activity of *C. fraxinella* was observed on three green ash and three black ash trees at each of four sites over three 24-h periods in Edmonton, on 18-19, 20-21 May and 5-6 June 2010. At each date, experimental trees were at leaf flush and *C. fraxinella* eggs were visible on young leaflets. Six yellow sticky cards (10 x 15.5 cm; Contech, Delta, British Columbia, Canada) were positioned in the canopy of each tree, one at each of the cardinal directions within the mid-canopy and two opposite each other on the lowest branches. Traps were checked five times within each 24-h period and moths caught in each time period were marked with a coloured dot that corresponded to that time period. After each 24-h period, traps were removed and *C. fraxinella* moths were enumerated by sex under a microscope. Sunset occurred at approximately 9:55 PM and a drop in light intensity to zero at this point was confirmed by HOBO pendant data loggers (Onset Computer Corporation, Pocasset, MA) (1/site) that recorded light intensity and temperature. The number of female and male *C. fraxinella* (dependent variables) per time period and ash species (independent variables) were analysed in two separate split plot GLMM with "site" as a random factor in R 3.0.1. Ash species (green or black) was nested within site and the Poisson (log link) family of errors was used. *Post hoc* comparisons on time of day were performed with Tukey's contrasts (R package "multcomp").

Larval performance

Laboratory study

Experimental seedlings were housed in a greenhouse under a natural photoperiod at 23ºC (50% RH) and placed into insect cages (60cm x 60cm x 60cm, BugDorm 2120, Megaview Science, Taichung, Taiwan) from 16 May-25 June, 2011. Two cages each held five seedlings of either green or black ash with newly flushed leaves. Seedlings were experimentally infested with *C. fraxinella* through introduction of ten female and five male field-collected (5-9 May 2010) adult moths into each of the four insect cages. Ten percent sugar water (v/v) was provided *ad libitum.* Larval development time and survival were followed daily until larvae either died or pupated. Pupae, along with their silken cocoons, were weighed as soon as the presence of emergence windows appeared in rolled leaflets.

Two GLMMs compared the proportion of *C. fraxinella* that survived from egg to pupae, and the development time from egg to third instar per tree species with individual tree specified as a random factor using the Poisson family of errors in both models (lme4 library, Bates et al., 2011) in R (R 3.0.1). GLMM was also used to compare the mass of *C. fraxinella* pupae on each ash species with the individual tree specified as a random factor (R 3.0.1) with the Gaussian family of errors (identity link) after examination of the response variable on a quantile comparison plot (Crawley, 2007). The number of eggs per leaflet between black ash and green ash species was analyzed in R (R 3.0.1, R Core team 2013) with a GLMM with Poisson errors with tree number specified as the random factor.

Field study

The larval performance field experiment was conducted on horticultural plantings of ash, *Fraxinus* spp., when trees were approximately 8-10 years old. Study trees were confined to boulevard and park trees in which the majority of the canopy could be sampled from the ground or with 1.8 and 2.4 m ladders. At the time of the study, *C. fraxinella* had been present in horticultural plantings of ash in Edmonton, AB for approximately twelve years (Pohl et al., 2004). Larval survival and development time were followed on each of three trees of each ash species at two sites and two trees of each species at one additional site in Edmonton from 18-19 May to 23-June 2010. Ten leaflets per tree with C. *fraxinella* eggs were marked with flagging tape. The initial number of eggs was recorded and the larval instar was determined

(Evenden, 2009) at three day intervals until the presence of emergence windows in rolled leaflets near marked leaflets signalled pupation. *Caloptilia fraxinella* within these rolled leaflets had likely dispersed from marked leaflets. Pupae positioned in leaf cones near each experimental leaflet were removed and weighed along with their silken cocoons. Development time in days from first instar to third instar when larvae exited natal leaflets and travelled to new leaflets was used for larval development because time of neonate hatch was better known than time of oviposition and dispersing 4th instar larvae could not be tracked reliably. Survival was the number of *C. fraxinella* that survived from the egg stage to the third instar.

A GLMM compared the effect of ash species on the number of eggs per leaflet with the individual tree nested within site and both set as random factors with the error distribution specified as Poisson with the log link. Two GLMMs compared the proportion of *C. fraxinella* that survived to pupae, and the development time from first to third instar per species with tree and site specified as random factors using the Poisson family of errors. Another GLMM assessed the effect of ash species on the mass of pupae with site nested within date specified as random factors. A quantile comparison plot of the mass of pupae on green and black ash revealed that most of the pupal weights fell along the normal line so a Gaussian distribution with the identity link was used to model the error structure (Crawley, 2007). All GLMMs used the lme4 library (Bates et al., 2011) in R 3.0.1.

Results

Oviposition preference

Experiment 1: No choice, green leaflet or wax paper control. When females were presented with a wax paper oviposition substrate, four eggs total were laid randomly by two females within the Petri dish arena in two separate oviposition events (mean number of eggs = 0.33 ± 0.21 S.E., n=15, range 0-3 eggs, with no significant differences between the surface of the wax paper and the plate (Table 3-1; Fig 3.2). Significantly more eggs were laid in green ash alone arenas but most eggs were laid on the surface of the Petri dish and not on the green ash leaflet

(Table 3-1; Fig 3.2). When green ash leaflets were tested alone (Fig. 3-2), fifty percent of females oviposited (Total n=28) with an average of 16.54 ± 5.40 S.E. eggs per responder (range 3-66) in a total of 34 separate oviposition events. Most eggs were deposited haphazardly on the Petri dish, significantly more (Table 3-1) than those oviposited directly onto the green ash leaflet (Table 3-1, Fig 3.2). Twelve eggs were oviposited in four separate oviposition events onto the Petri dish in a pattern that clearly followed the outline of the leaflet below (Fig. 3-1b).

Experiment 2: Choice between green ash and wax substrate. When green ash and wax substrates were directly compared in the same Petri dish arenas, the results were similar to experiment 1 (Fig. 3-3). Fifty percent of females (Total n=23) laid an average of 20.5 ± 5.16 (range 1-51) in 46 oviposition events. There were no significant differences in the number of eggs laid among the positions within the arena (Table 3-1). Some females oviposited in the pattern of the leaflet veins on the Petri dish (Above leaflet, Fig 3-3).

Experiment 3: Choice between black ash and wax substrate (environmental chamber). Thirty three percent of females oviposited (total n=30) in experiment 3 with an average number of 12.4 ± 4.7 (range 1-42, n=9) per female moth. There was a significant difference in number of eggs among areas within the arena (Fig. 3-4a; Table 3-1). Significantly more eggs were deposited on the leaflet than the wax paper disc (Table 2-1), and the plate on the control side (Table 3-1; Fig. 3-4a). Egg deposition above the leaflet occurred (Fig. 3-4a) and eggs were also laid on the leaflet side of the Petri dish.

Experiment 4: a) Choice between black ash and wax substrate (natural photoperiod). Under a natural photoperiod 50% of females responded (n=10) and oviposited directly onto the black ash leaflet (Fig. 3-4b) (Table 3-1) with an average number of 33.4 ± 13.8 (range 2-85, n=5) eggs per responding female. With the experiment outside of an environmental chamber, the oviposition behaviour of females could be observed. Females often dragged their ovipositor across leaflet tissue and this behaviour sometimes preceded oviposition. **b)** In the ten control arenas run concurrently under a natural photoperiod, females did not oviposit on the wax paper that visually simulated a leaflet. Two of ten females (20%) oviposited one and 18 eggs, respectively on the Petri dish with no significant differences in oviposition location within the arena (Table 3-1).

Experiment 5: Choice among black ash, green ash and wax substrate. The Petri dish arena was divided into three areas that corresponded to the position of each leaflet (black ash or green ash) or wax paper disc. In 2008, fifty nine percent of moths laid eggs (n= 17) with an average number of eggs per moth of 33.78 ± 12.04 (range 1 to 107). In 2009, seventy three percent of females laid eggs (n=11) with an average of 20.38 ± 7.97 (range 2 to 69). The mean number of eggs laid (Fig. 3-5) was significantly different among areas (Table 3-1). The mean number of eggs was not different between the wax paper disc and black ash leaflets (Table 3-1; Fig. 3-5). There was no effect of experimental year on the number of eggs laid (Table 3-1). The interaction between treatment and year was significant with more eggs laid on the control than green ash in 2008 and the opposite occurred in 2009 (Fig. 3-5). A clear preference for oviposition on black ash over green ash was evident (Table 3-1; Fig. 3-5) in both years.

Experiment 6: Choice between whole leaves. In the cage arenas, female *C. fraxinella* had some ability to fly and could choose from all of the leaflets on a leaf of green ash and black ash. Moths lived an average of 4.2 ± 0.55 days during the experiment (range 1-8 days). Nine of 15 *C. fraxinella* females (60%) oviposited in the caged arena. All but one of the experimental moths had mated and one of the non-responders had not developed vitellogenic eggs. Female *C. fraxinella* chose black ash five times more for oviposition than green ash (χ^2_1=50.7, P<0.001) (Fig. 3-6). Females deposited more eggs (342 total) per leaf on black ash compared to green ash (63 total) (Table 3-1; Fig. 3-6). Five of the nine moths oviposited during the first night period. Two moths began ovipositing in the second day, one started in the fifth night and one waited until the seventh night to oviposit 11 eggs (Fig. 3-7). A host switch occurred twice where a female oviposited for two nights on green ash then switched to black ash and another oviposited on black ash for three nights then green ash for the fourth. *Caloptilia fraxinella* females oviposited multiple times during one scotophase on several leaflets (3.16 ± 0.38, range 3-7). Six females (67%) targeted leaflets upon which they had previously oviposited (mean number of times 2.5 ± 0.5, range 1-4). The average number of leaflets used per female was 4.67 ±0.85 (range 1-9). Females laid an average of 42.5 ± 11.1 eggs in the experiment (range 5 to 94) and oviposited an average of 6.76 ± 0.91 eggs per leaflet (range 1-33, n=11 responders). Most moths began to oviposit within 24 h of exposure and continued over 3-4 days with the majority of eggs oviposited within 48 h (Fig. 3-7).

Experiment 7: Choice between green and black ash seedlings and new and old leaflets. Ninety-five percent of dissected females (n=21) had mated and 71% still had vitellogenic eggs (mean=19 ± 4.24, n=13) after the choice (experiment 7) and no choice (experiment 8) experiments. Females laid more eggs per leaflet on green ash (4.51 ± 0.43, range 1-26, n=114) than on black ash (1.47 ± 0.19, range 1-4, n=17) ($t_{(2), \mathrm{df}=129}$=2.72, *P*=0.007) in cages that contained both ash species. Female *C. fraxinella* preferred to oviposit on green ash over black ash (Table 3-1) and new leaflets over old leaflets (Table 3-1) (Fig. 3-7a). A significant interaction effect between ash species and leaflet age affected the number of eggs laid (Table 3-1).

Experiment 8: No choice between green and black ash seedlings and new and old leaflets. In no choice cages, leaflet age and species of ash tree significantly affected the number of eggs oviposited per leaflet (Table 3-1, Fig. 3-7b). More eggs were laid on new leaflets than old leaflets in which new leaflets of black ash (n=67) had a higher number of eggs per leaflet than new leaflets of green ash (n=806) (Table 3-1, Fig 3-7b). Old leaflets of black ash (n=507) and green ash (n=885) had lower numbers of eggs than the new leaflets of those species (Table 3-1, Fig. 3.7b). The interaction between leaflet age and ash species was significant (Table 3-1) and indicates that more eggs were laid on old green ash leaflets than old black ash leaflets while the converse was true for new leaflets (Fig. 3-7b).

Host location preference

Wind tunnel assay

There was a significant difference in all host location behaviours among treatments, caused in part by a lack of host location behaviour to the control treatment except for a small amount of undirected take-off flight. The experimental day only significantly affected the incidence of contact with seedlings (Table 3-2). Female *C. fraxinella* responded more to green ash than black ash (Fig. 3-8) with significant differences in lock on, oriented flight and source contact but not in take off (Table 3-2). All dissected females (n=72, 61% of total) had mated and developed vitellogenic eggs (per female 46.04 ± 2.62, range 2-110).

Field assay

Significantly more *C. fraxinella* (females and males) were captured on black ash than green ash (Fig. 3-9, Ash species, Table 3-3). Fight activity of both male and female moths, was low until 2200 h (10:00 PM) (Fig. 3-9), which corresponded to sunset, when both males and females were active. Flight activity decreased significantly (Table 3-3) after 0100 h (1:00 AM) and for male *C. fraxinella,* increased again between 0300 and 1200 (3:00 AM-12:00 PM) (Fig. 3-9, Table 3-3). Male flight activity mirrored female flight activity except in the 0300 to 1200 h time period when male activity was as high as the 2200-0100 h (Table 3-3).

Larval performance

Laboratory experiment- Oviposition occurred on eleven leaflets of four black ash seedlings and thirty four leaflets of seven green ash seedlings. Female *C. fraxinella* laid more eggs on green ash (188) than on black ash (63) but the mean number of eggs per leaflet was not significantly different between ash species (Table 3-4). Survival from egg to pupa on green ash was tenfold higher than on black ash (Table 3-4) with most mortality on both ash species occurring at the egg stage. Mortality (of the 20% of insects that died) on green ash occurred most often at the egg stage (67%), followed by second instar (17%), first instar (8%), and fourth instar (8%). Mortality (of the 92% of insects that died) on black ash also occurred most often at the egg stage (91%), with 9% at the first instar. There was no difference between mass of pupae, or developmental time from egg to third instar (Table 3-4) on green ash vs. black ash seedlings but the number of *C. fraxinella* that survived on black ash to be weighed at pupation was small (Table 3-4).

Field experiment- An equal number of leaflets with eggs was followed on both species of ash but more eggs were deposited on black ash (625) than green ash (449). The mean number of eggs per leaflet was higher on black ash (Table 3-4). Larvae of *C. fraxinella* developed faster from first to third instar on green ash than on black ash and had higher survival from first instar to pupae on green ash (Table 3-4). Mortality on black ash occurred at the egg stage (20%) and first (60%) and second (20%) instar larvae within mines. Other eggs on black ash leaflets outside of the marked study eggs also failed to hatch. The mass of pupae was higher in *C. fraxinella* reared on black ash than green ash (Table 3-4).

Discussion

Oviposition cues

Oviposition experiments with *C. fraxinella* suggest that several ovipositional cues are used in host plant acceptance by female moths. *Caloptilia fraxinella* will only oviposit in the presence of ash leaflets which suggests that a chemical cue from the host plant is essential for oviposition. In the absence of leaflets, female *C. fraxinella* failed to lay eggs and in most of the control arenas, females died without laying eggs. In no choice experiments with single ash leaflets, most oviposition was haphazard on the surface of the Petri dish arena and not localized on the leaflet, which indicates that a volatile organic compounds (VOC) may be the only host plant cue required to trigger oviposition in *C. fraxinella*. This is different than other moths such as *Choristoneura* sp. (Lepidoptera: Tortricidae) leaf roller and leaf tier moths, that do not require host plant material to stimulate oviposition and oviposit on filter paper (Grant & Langevin, 1994) or a wax paper substrate (Wins-Purdy et al. 2009). The potato moth, *Phthorimaea operculella* Zell. (Lepidoptera: Gelechidae) also does not require host plant VOCs to stimulate oviposition (Fenemore, 1978) but fecundity increases in the presence of host VOCs (Fenemore, 1979). Host plant VOCs increased egg deposition in another leaf miner, *Paraleucoptera sinuella* Reutii (Lepidoptera: Lyonetiidae) (Kagata & Ohgushi, 2002). The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), locates tomato plants with VOCs and also requires VOCs to stimulate oviposition (Proffit et al. 2011). The number of eggs laid however, increases when females can contact the leaf surface (Proffit et al. 2011).

Host plant acceptance by this leaf miner probably integrates chemical cues from close range VOCs, contact chemoreception with mechanoreception and visual signals as occurs in other lepidopterans (Reed et al., 1988; Smallegange et al., 2006; Teles Pontes et al., 2010). Visual cues also seem to be important for females to properly target leaflets. Eggs were sometimes deposited on Petri dishes above leaflets and on the plastic surface above leaflet veins, which could only be accomplished through vision. When confined to the environmental chamber, these females must have oviposited during the light cycle. Under a natural photoperiod which allows for a slow transition to darkness, *C. fraxinella* deposits all of its eggs directly onto the surface of the leaflet, which is further evidence of the importance of vision to oviposition. This importance of vision is unexpected in a crepuscular moth with the peak flight activity of *C. fraxinella* at sunset when very low light levels are present. However, almost no oviposition occurs in the absence of VOC cues from leaflet tissue, even when the size, shape and leaflet vein pattern is duplicated with wax paper cut-outs so host VOC cues are more important than visual cues. Visual cues alone do not induce oviposition in grapevine moth, *L. botrana* but impact host-plant recognition when combined with plant VOCs (Tasin et al., 2011). In the field, oviposition by *C. fraxinella* occurs along veins on the adaxial (upper) surface of the leaflet. Where the smooth surface of a Petri dish prevented oviposition directly onto the leaflet, females still chose to oviposit along the pattern of veins, which suggests that mechano or contact-chemo sensory cues from the leaflet are not as important as the visual pattern of leaflet veins. However, ovipositor drag by females on the leaflet tissue or the Petri dish surface suggests that mechano or chemoreceptors on the ovipositor are involved with host plant acceptance. In instances where eggs were laid on the Petri dish above the leaflet, eggs were often also placed on the leaflet surface, so oviposition probably started with contact cues on the leaflet surface. Horticultural oil on the surface of lemon leaves disrupts oviposition by the citrus leaf miner, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) by masking contact host acceptance cues, but does not disrupt host location flight to leaves (Liu et al., 2006). Ovipositor drag can disrupt the cuticular wax layer of a leaf if the ovipositor is sclerotized and brings chemoreceptors on the ovipositor into contact with chemical cues in the underlying cuticular layer (Städler, 2002). However, the ovipositor of *C. fraxinella* is not sclerotized so surface chemicals most likely mediate host acceptance.

Several ovipositional patterns emerge in cage arenas with whole leaves, when females can fly and choose from all leaflets on leaves of both ash species. *Caloptilia fraxinella* oviposits on the adaxial surface of the leaflet over multiple nights (often successive). Oviposition on the upper leaflet surface also occurs in the field (Pohl et al. 2004), and this oviposition site is less common in the Gracillariidae than oviposition on the abaxial (lower) leaf surface (Reavey & Gaston, 1991). The reproductive effort is spread across several leaflets and females will oviposit onto a leaflet they have previously used, which suggests that no epideictic pheromone cues are deposited at oviposition (Anderson, 2002; Renwick, 1989; McNeil & Quiring, 1983). Host plant preference of females is plastic with several instances of host switches between ash species. Females are capable of oviposition and storage of over one hundred eggs, but forty to fifty eggs per female is more common. Another similarly sized gracillariid leaf miner, *Lithocolletis blancardella* Fabr., produces a similar number of eggs per female (Pottinger & LeRoux, 1971).

Oviposition preference

Choice tests are the standard for determination of a host plant preference (Singer, 1986; Thompson, 1988) and in these tests *C. fraxinella* females prefer black ash for oviposition when offered the choice between leaves and leaflets of black ash and green ash. However, on whole seedlings, green ash was the preferred host when both species were presented together, but the preference for new leaflets confounded the observed host plant preference. The preference for new leaflets was also evident in no choice caged experiments in which new leaflets received more eggs than old leaflets and new leaflets of black ash received more eggs per leaflet than green ash. In the field at leaf flush, which is when females search for oviposition sites, more mated female and male *C. fraxinella* were trapped in the canopy of black ash than green ash trees at the same sites which suggests a greater host location preference for black ash under natural conditions. In contrast, green ash elicits more host location behaviour by mated, female *C. fraxinella* in the wind tunnel than black ash. This suggests that the VOCs emitted from the green ash seedlings are more attractive to female moths than VOCs emitted from the black ash seedlings when presented one meter upwind. Long distance oriented flight to host plants is duplicated in wind tunnel experiments and is the second step in the host selection process (Ramaswamy, 1988; Renwick, 1989). The first step is host habitat location (Ramaswamy, 1988; Renwick, 1989) and greater attraction to black ash trees in the field may indicate that *C. fraxinella* prefers this ash at this step. Host acceptance is the final step (Ramaswamy, 1988; Renwick, 1989) and *C. fraxinella* prefers to oviposit on black ash. Host recognition cues perceived post-alightment such as mechano and contact chemoreception and vision (Singer & Ng, 1988) are involved. Experiments in Petri dish arenas only tested host acceptance. At close range, black ash is the preferred host, over medium distances, green ash evokes more host location behaviour and over the longest range tested in the field study, black ash is preferred, which may indicate that the host preference of this leaf miner is only fully realized when all of its host location signals are present.

Larval performance

Of the three methods used to assess the performance of *C. fraxinella* larvae, survival, followed by development time, proved most useful. Pupal mass was not a good predictor of larval performance with no difference between the mass of pupae reared on each ash species. In the other two measures of larval performance, *C. fraxinella* performs significantly better on green ash than on black ash in field and greenhouse experiments. Larvae develop faster and most survive to pupation on green ash. Most *C. fraxinella* larvae hatch, mine and successfully pupate on green ash. In contrast, larvae on black ash develop more slowly than on green ash and most do not survive to pupation. Eggs often fail to hatch on black ash, especially in the greenhouse experiment, and if hatching occurs, most fail at the mining stage. For leaflets on which development was followed, most of the hatched larvae on black ash died in mines during the first and second instars. These results suggest that leaf cuticular “toughness”, as occurs when leaves toughen with age (Müller & Riederer, 2005), is not a major source of mortality, at least when eggs are laid on new leaflets. The presence of domatia-like trichomes on the underside of black ash leaflets and no such structures on green ash may indicate that black ash encourages colonization of predatory mites, thrips or bugs (Grostal & O’Dowd, 1994; Agrawal et al., 2000; Romero & Benson, 2004), which could explain the high mortality of egg and first instar larvae on black ash in the field. Death of eggs and neonates on the leaflet surface could indicate an antibiosis response by black ash in which secondary metabolites present in the epicuticular leaf layer (Carter et al., 1989; Fatouros et al., 2012; Tamiru et al., 2012). Egg deposition can induce production of ovicidal secondary metabolites in leaflet tissue either through egg contact or surface damage from ovipositor drags (Hilker & Meiners, 2006). The failure of many first and second instar miners within the leaflets of black ash suggests toxic secondary metabolites (Woods, 2010) as the cause of death. Black ash produces significantly higher amounts of VOCs (Pureswaran & Poland, 2009) than green ash, which could indicate that black ash has higher levels of secondary plant compounds that are precursors to VOCs. However, green ash has higher levels of defensive compounds such as phenolics and trypsin inhibitors, but lower levels of chymotrypsin inhibitors than black ash (Chen & Poland, 2010). Failure of early instars in mines on black ash plants may result from an innate plant defense but a difference in nutritional content between black ash and green ash (Chen & Poland, 2010) may also contribute to the difference in larval performance. Black ash and green ash leaves have a similar macro and micronutrient content, except for a lower starch content in black ash that creates an unbalanced nitrogen-carbohydrate ratio (Chen & Poland, 2010). However, the mass of pupae is the same regardless of ash species, which might indicate that the difference in nutritional value of the foliage of both ash species does not impact growth of *C. fraxinella* larvae (Slansky, 1993).

The slower development of *C. fraxinella* on black than green ash can expose larvae to an increased risk of mortality from predation and parasitism (Benrey & Denno, 1997). As expected, parasitism rates of *C. fraxinella* in Edmonton by *Apanteles polychrosidis* Vierek (Hymenoptera: Braconidae) are significantly higher on black ash than green ash (Chapter 5). The dominant parasitoid of *C. fraxinella*, *A. polychrosidis*, utilizes *C. fraxinella* larvae mainly at the larval migration phase (Wist & Evenden, 2013). Slower growth of larvae on black ash may result in better synchrony of parasitoids with the vulnerable migratory larval phase. A shift of host preference (Agosta 2006) from black to green ash could support the enemy-free space hypothesis (Bernays & Graham, 1988) in this system. *Caloptilia fraxinella* females may be shifting their host plant preference to green ash, as suggested in wind tunnel flights and seedling experiments, to escape the higher parasitism and death of eggs and early instars on black ash.

Preference for new leaflets over old

Caloptilia fraxinella females prefer new leaflets for oviposition. The seedling experiment indicates that female *C. fraxinella* choose new leaflets not because they are the only ones available when females are ready to oviposit but because they are preferred over older leaflets. Utilization of new foliage thus is a choice made by females and not a constraint of tree phenology. The strong preference of *C. fraxinella* for new foliage may obscure any host plant choice in the whole seedling experiment in the current study. Similar confounding effects of a preference for young leaves on host choice are found in another leaf miner (Kursar et al., 2006). The optical profile (scattering and reflectance of light) of leaf surfaces changes as leaves senesce (Major et al., 1993), and *C. fraxinella* may be able to judge the age of leaflets visually. Vision could account for the ovipositional preference for new leaflets over old in the seedling experiment. Previous wind tunnel experiments (unpublished) suggest that new leaflets release a distinct volatile cue that is more attractive to female *C. fraxinella* than that released from older leaflets. The preference of *C. fraxinella* females to oviposit on new leaflets could be driven by several factors. First, leaves increase in toughness as they age (Müller & Riederer, 2005) and the cuticle hardens. Newly hatched *C. fraxinella* larvae may not be able to penetrate the thicker wax cuticle of an older leaflet or penetrate the increasingly sclerotized cross veins of leaflets (Neilsen, 1968). Second, new leaflets may confer a nutritional advantage to developing larvae over older leaflets. Lepidopteran larvae often have increased survival on newer leaves than older leaves (Smyth et al., 2003). Female citrus leaf miners, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) also selectively oviposit on new leaflets which are of higher nutritional value than older leaves (Kharrat & Jarraya, 2005). Another leafminer, *Agnippe* spp., prefers new leaves of *Erythroxylum tortuosum*

(Erythroxylaceae) for oviposition which have lower tannin content and higher nitrogen and water levels than older leaves (Ishino et al., 2011). However, concentrations of plant-defensive compounds are often higher in younger leaves and may cause increased mortality of herbivores (Stein & Price, 1995). The nutritional quality of new ash leaflets may not be a factor in oviposition selection by *C. fraxinella* as mature green ash leaflets contain a higher amino acid and protein content than new leaflets (Chen & Poland, 2009). Third, new green ash leaflets exhibit higher levels of anti-herbivore secondary metabolites such as trypsin and chymotrypsin inhibitors and phenolics than older leaflets (Chen & Poland, 2009); compounds which reduce the nutrional quality of plant tissue. Development in the newest leaves might be adaptive to ensure that leaflets used later for construction of leaflet rolls for pupation are as young and soft as possible to minimize exposure to predation and parasitism while rolling leaflets. Older, less pliable leaflets greatly increase the time required to complete a leaflet roll (Müeller & Dearing, 1994) and *A. polychrosidis* attacks exposed larvae as they transition from leaf mines to leaf rolls (Wist & Evenden, 2013).

Summary

The preference-performance hypothesis is partially supported for *C. fraxinella* as females show an oviposition preference for black ash in most bioassays but offspring performance is lower on black than green ash. *Caloptilia fraxinella* females are unable to consistently select the host plant that increases the performance of their offspring. Wind tunnel flights perhaps incorporate an additional essential element of the host selection process omitted in the oviposition choice host acceptance experiments (Hoffman, 1985). Petri dish experiments may not permit natural host location behaviours by female *C. fraxinella* (Singer, 1986) if flight behaviour to trees is a necessary step before host acceptance of leaflets. Caged arenas with whole leaves offer the opportunity for limited flight and should increase the realism of the experiment, yet black ash is still preferred for oviposition. More females were caught in black ash at leaf flush on trees in the field and this comparison is the most "natural" of the experiments. Several other leaf miners show a negative link between oviposition preference and larval performance (Gratton & Welter, 1998; Marini-Filho et al., 1997). Negative preference-performance linkages though, often occur in outbreak populations of herbivorous insects (Leyva et al., 2003) and common infestation levels of 40-80% of available leaflets in the field suggest that the population pressure may be contributing to oviposition behaviour in the field (Ellis, 2008).

The host location preference for green ash in the wind tunnel, taken together with significantly greater larval performance on green ash, show that the *C. fraxinella* in Edmonton are more adapted to green than black ash. Most of the horticultural black ash have been lost in Edmonton due to drought stress and cottony ash psyllid, *Psyllopsis discrepans* (Flor) (Homoptera: Psyllidae), infestation (Saunders et al., 2004) and moths in these experiments have used green ash almost exclusively for 10-12 generations. The few black ash that remain attract more *C. fraxinella* at leaf flush than green ash at the same sites over the same time period. This result may support the Hopkins host selection theory (Hopkins, 1916) in which experimental *C. fraxinella* prefer to fly to green ash in wind tunnel bioassays because they fed upon its foliage as larvae and were conditioned to use it as a host plant when they emerged as an adult moth from a rolled green ash leaflet. *Caloptilia fraxinella* does retain their ovipositional preference for black ash which may present a more attractive contact chemosensory cue (Renwick & Chew, 1994) or visual cue than green ash. After generations of development on green ash, this leaf miner may now be adapted to green ash and have lost their ability to defend against potentially different secondary plant compounds (Scribner et al., 2008) encountered in black ash foliage, hence their reduced larval performance on black ash leaflets. Future studies on this herbivore-host plant interaction should investigate the source of the resistance of black ash to *C. fraxinella* infestation and look further at breeding a black ash cultivar for increased resistance to *C. fraxinella*.

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Table 3-1. Results of non-parametric ANOVA (Petri dish arenas), generalized linear model (GLM) and generalized linear mixed effect model (GLMM) statistical tests in oviposition experiments. Response variable is the number of eggs laid by female *Caloptilia fraxinella*. The Kruskal-Wallace test returns *H* values, *post hoc* non-parametric Tukey's returns *q* values and the Mann-Whiney *U* test returns *U* values. The GLM and GLMM returns χ^2 values with any *post hoc* Tukey's comparisons returning *z* values. Degrees of freedom are shown in subscripted brackets and sample size shown (n) is with non-responders removed except in Experiment 1 and the control treatment of experiment 4b.

Petri dish arena Leaflet experiment	**Year**	**Location**	**Treatment**	**Test statistic (df)**	***P* value**
1. No choice: Green ash and wax paper control (n=28 GA,15 C)	2008	Env. Chamber	Leaflet arena *vs.* wax paper arena	$\chi2_{(1)}$ =14.05	**<0.001**
			Placement within arena	$\chi2_{(1)}$ =10.47	**=0.001**
			Arena * placement	$\chi2_{(1)}$ =0.86	=0.353
2. Choice: Green ash *vs.* wax (n=14)	2008	Env. chamber	Within arena	$H_{(4)}$=2.98	=0.560
3. Choice: Black ash *vs.* wax (n=10)	2008/9	Env. chamber	Within arena	$H_{(4)}$=17.78	**=0.001**
			Leaflet *vs.* wax	$q_{(1)}$ =4.01	**<0.050**
			Leaflet *vs.* plate	$q_{(1)}$ =4.01	**<0.050**
4. a. Choice: Black ash *vs.* wax (n=5)	2009	Natural photoperiod	Within arena	$H_{(4)}$= 15.37	**=0.004**
			Leaflet *vs.* Control	$q_{(1)}$= 3.16	**<0.050**
			Leaflet *vs.* leaflet plate	$q_{(1)}$= 3.16	**<0.050**
			Leaflet *vs.* above leaflet	$q_{(1)}$= 3.16	**<0.050**

			Leaflet *vs.* control plate	$q_{(1)} = 2.52$	**<0.050**
4.b No choice: Control (n=10)	2009	Natural photoperiod, wax paper like leaflet	Within arena	$H_{(2)} = 6.30$	=0.050
5. Choice: Black ash *vs.* green ash *vs.* wax (n=18)	2008, 2009	Env. chamber	Among treatments	$\chi^2_{(2)} = 18.12$	**<0.001**
			Year	$\chi^2_{(1)} = 1.37$	=0.242
			Treatment * year	$\chi^2_{(2)} = 16.95$	**<0.001**
			Black ash *vs.* green ash	$z_{(1)} = 3.70$	**<0.001**
			Black ash vs. wax	$z_{(1)} = 1.41$	=0.336
			Green ash vs. wax	$z_{(1)} = 2.25$	=0.064
Caged arenas					
6. Choice: Leaves of black ash and green ash (n=9), Random (Moth, leaflet)	2008	Env. chamber	Ash species	$\chi^2_{(1)} = 51.96$	**<0.001**
7. Choice: Seedlings of black and green ash (n=7)	2009	Natural photoperiod	Ash species	$\chi^2_{(1)} = 9.12$	**=0.003**
			Leaflet age	$\chi^2_{(1)} = 99.45$	**<0.001**

	Ash species * leaflet age	$\chi^2_{(1)}$ =13.11	**<0.001**
8. No choice: Seedlings of black and green ash (n=7)	Ash species	$\chi^2_{(1)}$ =74.66	**=0.005**
	Leaflet age	$\chi^2_{(1)}$ =218.34	**<0.001**
	Ash species * leaflet age	$\chi^2_{(1)}$ =0.14	=0.710

Table 3-2. ANOVA table of the generalized linear models on the effect of green and black ash seedlings and control plastic seedlings on the host location behaviours performed by female *Caloptilia fraxinella* in a wind tunnel. Significant treatments are separated by *post hoc* Tukey's tests. "*"indicates where p values were calculated with Fisher's exact test when means of zero prevented Tukey's *post hoc* analysis. Degrees of freedom are shown in subscripted brackets.

		Take Off		**Lock On**		**Oriented Upwind Flight**		**Source contact**	
Effect	***Post hoc* comparisons**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**
Treatments		$\chi^2_{(2)}=18.64$	**<0.001**	$\chi^2_{(2)}=8.94$	**0.011**	$\chi^2_{(2)}= 8.94$	**0.011**	$\chi^2_{(2)}=8.06$	**0.018**
Experimental day (random)									
	Green ash *vs.* control	$z_{(1)}=4.00$	**<0.001**	-	**<0.001***	-	**<0.001**	-	**<0.001***
	Black ash *vs.* control	$z_{(1)}=3.15$	**=0.004**	-	**=0.002***	-	**=0.002***	-	**=0.012***
	Green ash *vs.* Black ash	$z_{(1)}=0.118$	=0.502	$z_{(1)}=2.99$	**=0.006**	$z_{(1)}=2.99$	**=0.006**	$z_{(1)}=2.84$	**=0.009**

Table 3-3. ANOVA table from two generalized linear mixed effect models (GLMMs) of the number of male and female *Caloptilia fraxinella* captured in field experiments over three 24 hour periods. GLMMs show the influence of time of day, and ash species (black or green ash) nested within site on the number of male and female moths captured. The factor "site" is random. Degrees of freedom are shown in subscripted brackets.

		No. males		**No. females**	
Effect	***Post hoc* contrast**	**Test stat**	***P* value**	**Test stat**	***P* value**
Time of day		$\chi^2_{(5)}=209.91$	**<0.001**	$\chi^2_{(5)}=538.51$	**<0.001**
Ash species		$\chi^2_{(1)}=1.42$	0.233	$\chi^2_{(1)}=6.69$	**0.009**
Time * Ash species		$\chi^2_{(5)}=8.27$	0.142	$\chi^2_{(5)}=20.92$	**0.001**
	22:00-00:59 *vs.* all other times	$z_{(1)}=6.76\text{-}7.74$	**<0.001**	$z_{(1)}=9.41\text{-}11.12$	**<0.001**
	19:00-21:59 *vs.* 1:00-2:59	$z_{(1)}=1.98$	0.315	$z_{(1)}=3.56$	**0.005**
	3:00-11:59 *vs.* all other times	$z_{(1)}=6.14\text{-}6.57$	**<0.001**	$z_{(1)}=0.00\text{-}2.52$	0.106-1.0
	3:00-11:59 *vs.* 22:00-00:59	$z_{(1)}=2.17$	0.218	$z_{(1)}=11.03$	**<0.001**

Table 3-4. The number of eggs per leaflet, development time (days), survival (mean number *Caloptilia fraxinella* per leaflet ± SE, percentage in brackets) and pupal mass (mg) of *C. fraxinella* on black ash and green ash in laboratory (top) and field experiment (bottom). Degrees of freedom are shown in subscripted brackets.

Response variable		**Black ash**		**Green ash**	**Test statistics**
	n	*Laboratory* (2011)	n		
Mean no. eggs/leaflet	11	5.73 ± 0.95	34	5.53 ± 1.27	$\chi^2_{(1)}=0.058$, P=0.81
Mean no. surviving to pupation per leaflet	11	0.45 ± 0.21(8%)	34	4.41 ± 1.29 (80%)	$\chi^2_{(1)}=10.04$, **P=0.0015**
Development time (egg-third instar) (days)	4	9.75 ± 0.63	28	8.23 ± 0.27	$\chi^2_{(1)}=0.92$, P=0.34
Pupal mass (mg)	8	4.86 ± 0.28	79	5.22 ± 0.079	$\chi^2_{(1)}=3.01$, P=0.083
	n	*Field* (2010)	n		
Mean no. eggs/leaflet	69	9.06 ± 0.72	60	7.48 ± 0.55	$\chi^2_{(1)}=6.06$, **P=0.014**
Mean no. surviving to pupation per leaflet	69	3.2 ± 0.62 (35%)	60	7.23 ± 0.28 (97%)	$\chi^2_{(1)}=19.05$, **P<0.001**
Development time (first-third instar) (days)	23	13.92 ±1.69	53	11.75 ± 0.73	$\chi^2_{(1)}=46.20$, **P<0.001**
Pupal mass (mg)	138	5.10 ±0.065	352	4.97 ± 0.039	$\chi^2_{(1)}=3.37$, P=0.066

Figure 3-1. Oviposition arena experimental setup with Petri dish (A and B) and caged arenas (C and D). A. Wax paper control and black ash leaflet. B. Wax paper control, black ash leaflet (left: note eggs on leaflet and above leaflet), green ash leaflet (right). C. Caged arena with green ash leaf (bottom) and black ash leaf (top). D. Black ash leaf with eggs on 2nd and 3rd leaflets.

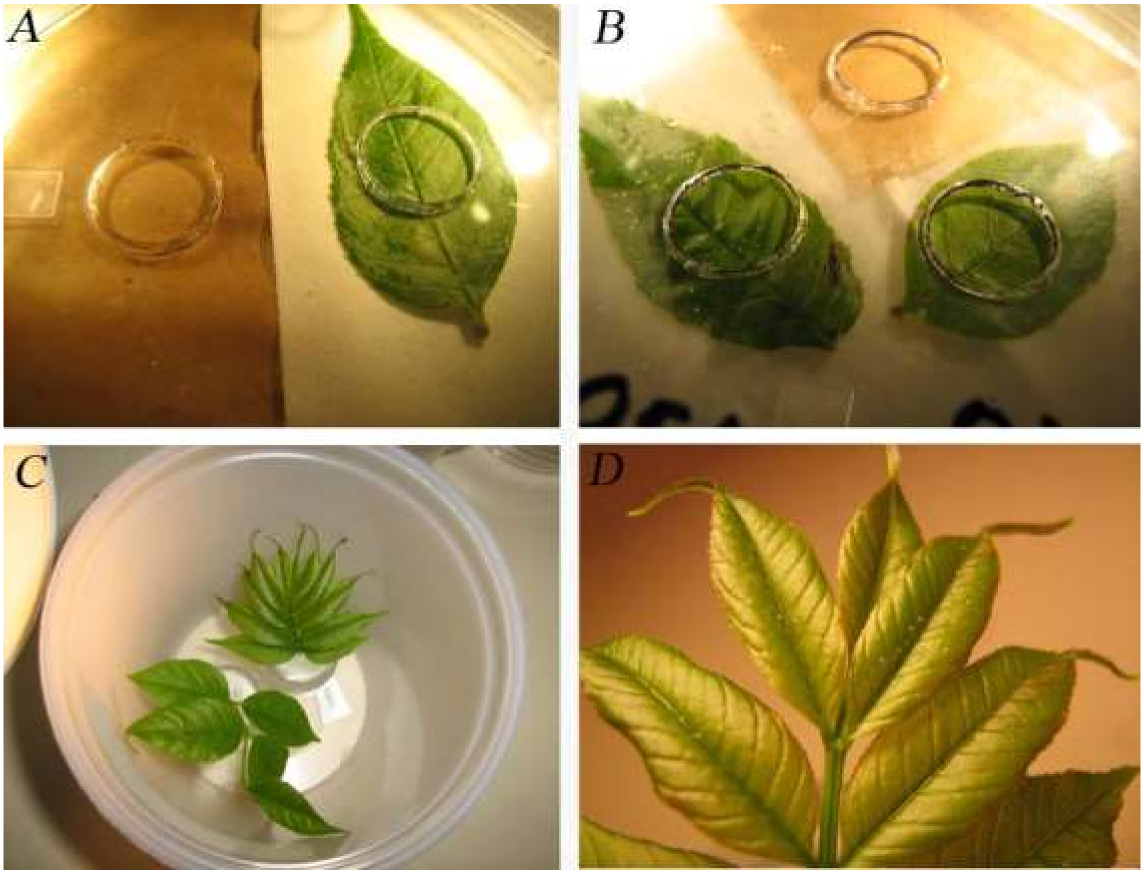


Figure 3-2. Mean ± S.E. number of eggs oviposited by female *Caloptilia fraxinella* and the location of egg deposition in no choice leaflet assay experiments to green ash leaflets alone and control arenas with wax paper (Experiment 1 2008). Significant differences at α=0.05 occur between the mean number of eggs in each arena "$" and between the placement of eggs within the arenas "*".

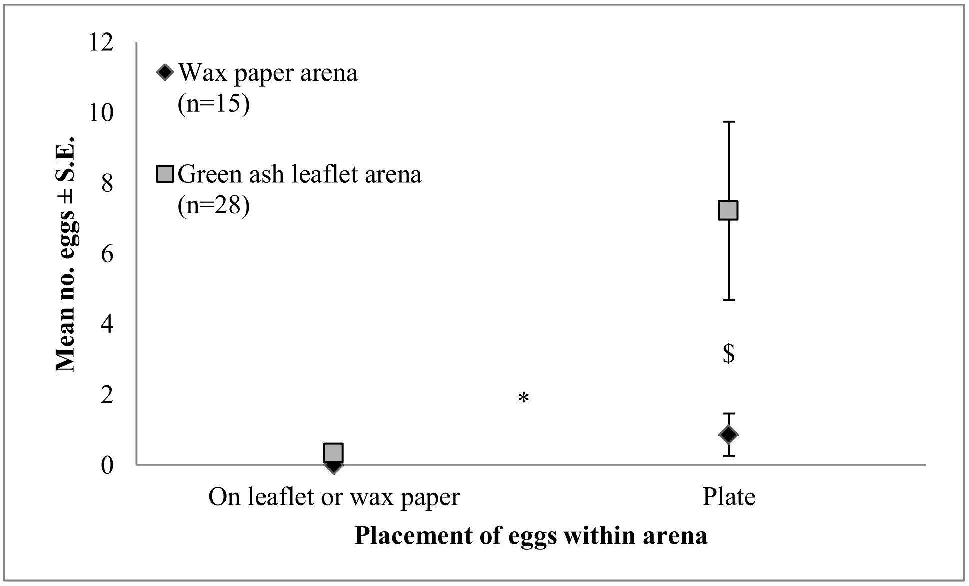


Figure 3-3. Mean ± S.E. number of eggs oviposited by female *Caloptilia fraxinella* and the location of egg deposition in a choice leaflet assay experiments to green ash leaflets and a wax paper disc (Experiment 2 2008). “Green plate” refers to eggs that were laid on the Petri dish plate on the leaflet half of the dish or on the control side of the dish (Wax plate) but did not follow the outline of the leaflet or wax disc. No significant differences in egg location were found.

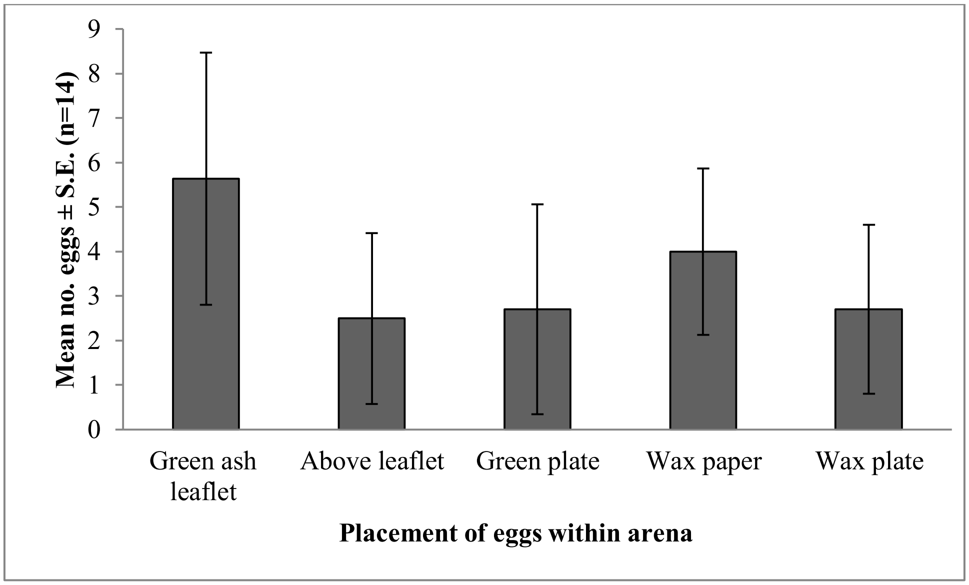


Figure 3-4. Mean ± S.E. number of eggs oviposited by female *Caloptilia fraxinella* and the location of egg deposition in leaf let assay choice experiments to black ash leaflets and a wax paper disc in an a) environmental chamber (Experiment 4 2008-2009) and b) under a natural photoperiod. Plate (ba) represents eggs that were laid on the leaflet side of the plate but did not follow the outline of the leaflet (Above leaflet). Statistically significant differences (α=0.05) among treatments are represented by different lowercase letters.

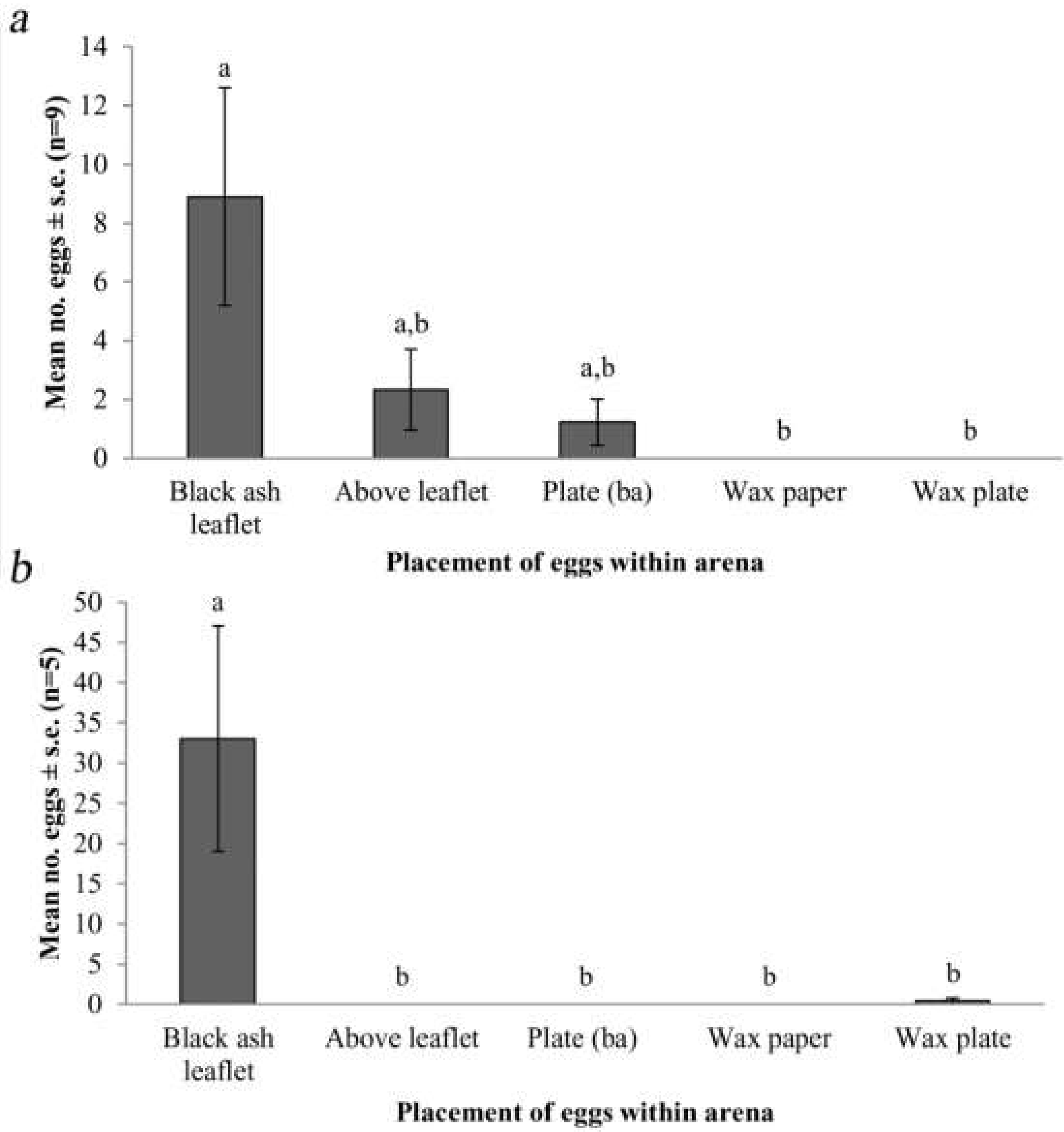


Figure 3-5. Mean ± S.E. number of eggs oviposited by female *Caloptilia fraxinella* and the location of egg deposition in excised leafletlet choice experiments to green ash and black ash leaflets and a wax paper disc in an environmental chamber (Experiment 5 2008,2009). Statistically significant differences (α=0.05) among treatments are represented by different lowercase letters.

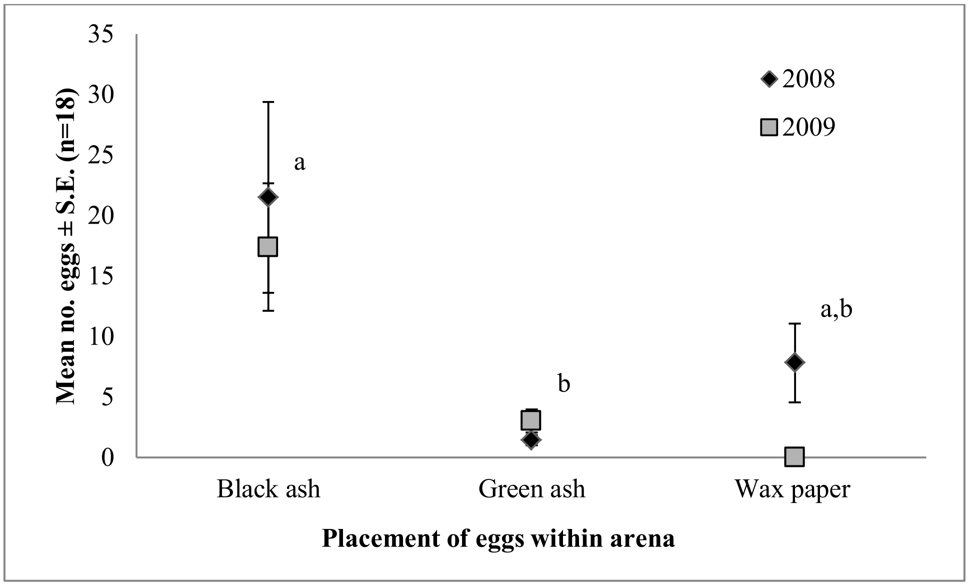


Figure 3-6. Mean ± S.E. number of eggs oviposited by female *Caloptilia fraxinella* and the number of oviposition choices on green ash and black ash leaves in a 1 L arena in an environmental chamber (Experiment 6, 2008). Statistically significant differences (α=0.05) between mean number of eggs laid on black and green ash are represented by different uppercase letters and differences between oviposition events are represented by lowercase letters.

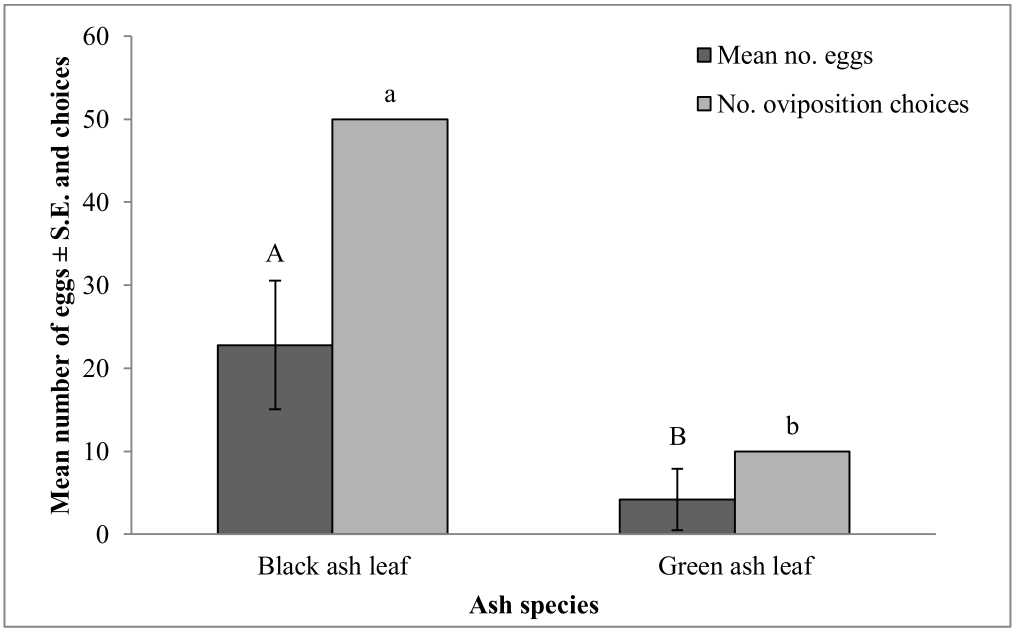


Figure 3-7. a.Mean ± S.E. number of eggs per leaflet oviposited by female *Caloptilia fraxinella* on new and old leaflets of black and green ash seedlings in experiment 7 with a choice of species. b.Mean ± S.E. number of eggs per leaflet oviposited by female *Caloptilia fraxinella* on new and old leaflets of black and green ash seedlings in experiment 8 with no choice of species. Significant differences at α=0.05 occur between the mean number of eggs on leaflets of black and green ash "$" and between new and old leaflets.

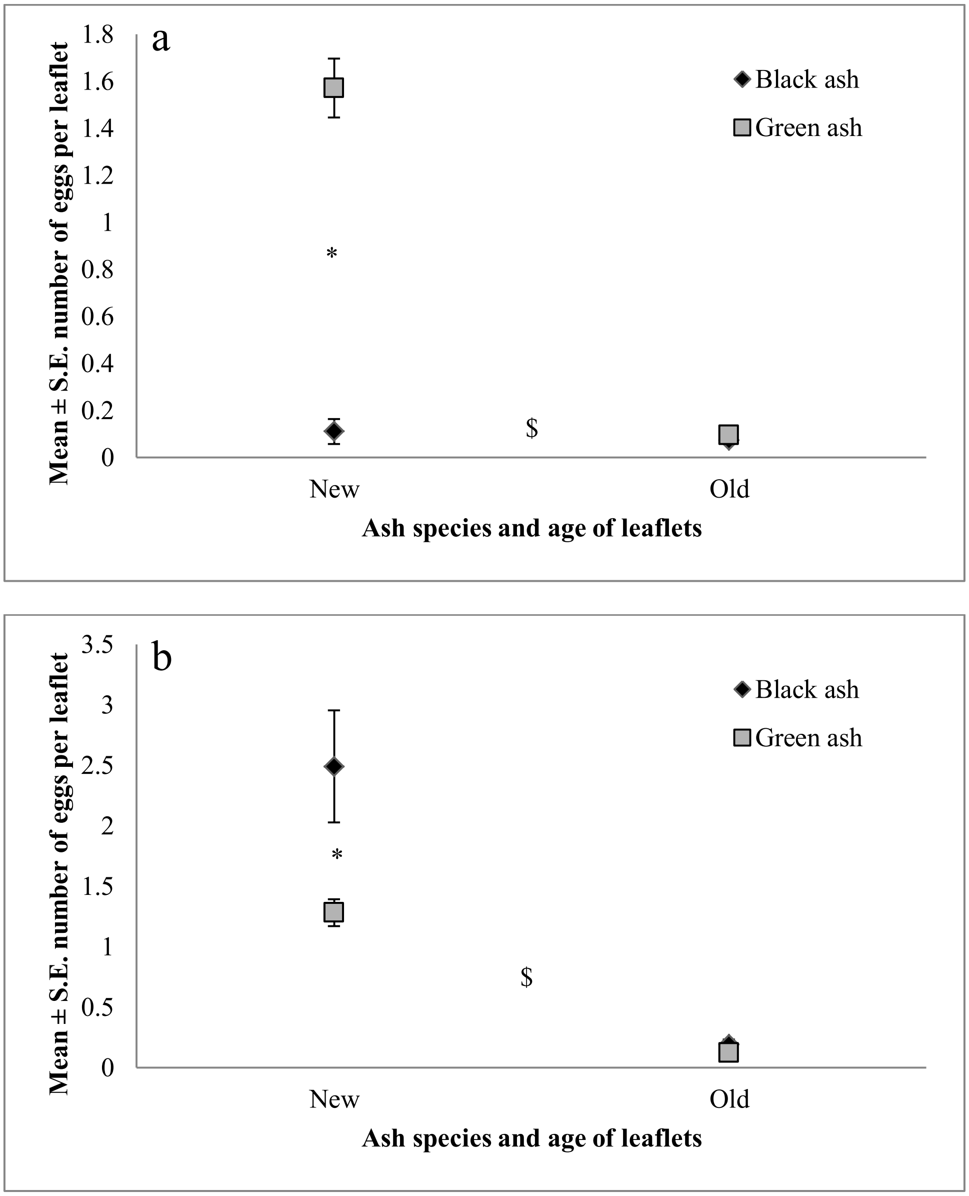


Figure 3-8. Percentage of mated, female *Caloptilia fraxinella* that performed host location behaviours to black ash, green ash and a plastic control seedling in a wind tunnel (2010). Significant differences in behaviour to each treatment are represented by different lowercase letters (Tukey's tests and Fisher's exact tests, α=0.05).

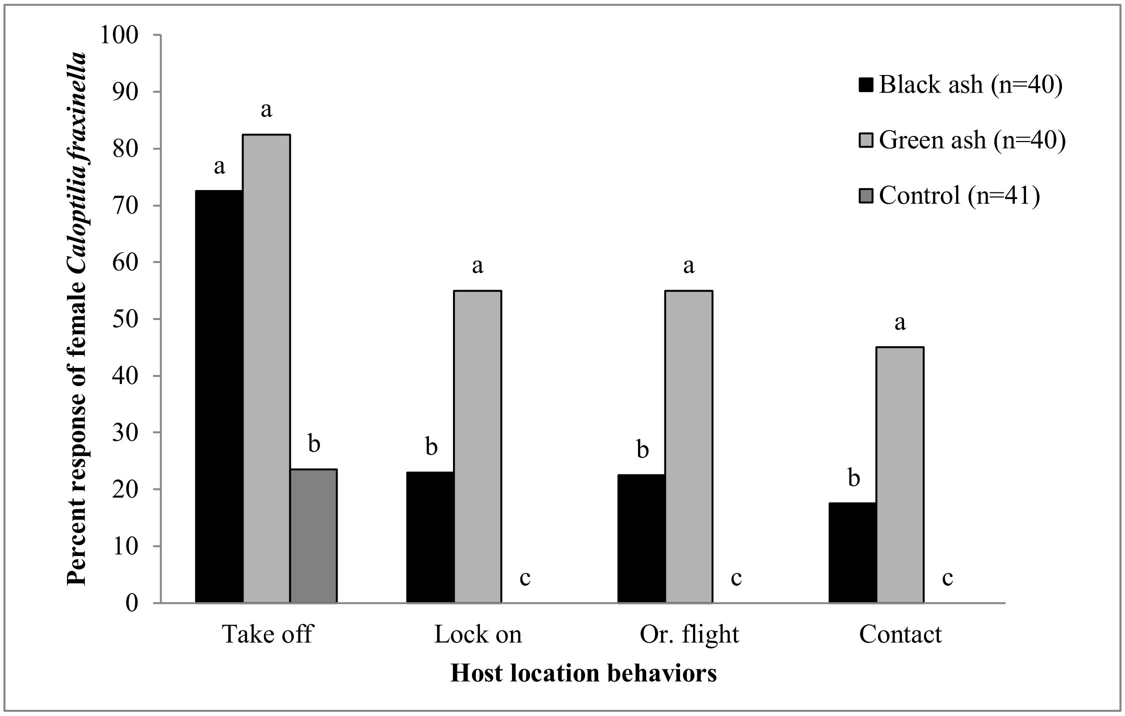
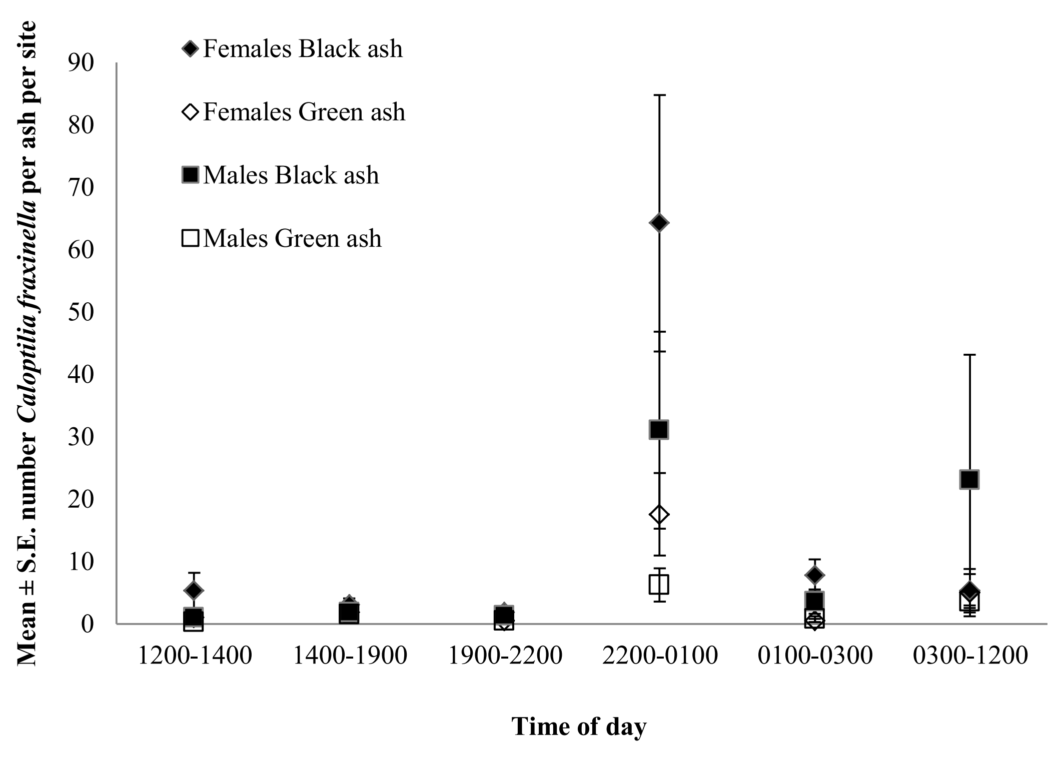


Figure 3-9. Daily flight phenology of *Caloptilia fraxinella* males and females on each ash species. Flights over three 24h periods are represented as the mean number of male and female moths at each time period per species of ash tree (n=3/site).



Chapter 4: Parasitoid complex and bionomics of *Apanteles polychrosidis* on the ash leaf cone-roller

(A version of this chapter has been published as Wist T.J. and Evenden M.E. (2013) Parasitoid complex and bionomics of *Apanteles polychrosidis* on the ash leaf cone-roller. The Canadian Entomologist 145: 416-429.)

Introduction

The ash leaf-cone roller, *Caloptilia fraxinella*, (Ely) (Lepidoptera: Gracillariidae) is an invasive, cosmetic pest of horticultural ash trees, *Fraxinus* spp. (Oleaceae) in several municipalities in the Western Prairie provinces. Infestations have occurred in Edmonton, AB since 1999 (Pohl *et al.* 2004), Saskatoon, SK since 1998 (personal observation) and have been reported in Calgary, AB (City of Calgary Factsheet, Dr. Joelle Lemmen, University of Alberta, personal communication) and Regina SK (personal observation). *Caloptilia fraxinella* mines leaflets of green (*F. pennsylvanica*), black (*F. nigra*) and white (*F. americana*) ash and collection records prior to 1998 place the native range of *C. fraxinella* in Southern Ontario (specimens deposited in the Great Lakes Forestry Centre, Canadian Forestry Service 1219 Queen Street East, Sault Ste. Marie, ON, (Kathryn Nystrom, curator)) and Québec (specimens deposited in the Laurentian Forestry Research Collection, Canadian Forest Service, Natural Resources Canada, 1055 du P.E.P.S., P.O. Box 10380, Stn. Sainte-Foy, Quebec, QC, (Georges Pelletier, curator) Pohl *et. al* 2004), and into the eastern United States (Forbes 1923). Black ash occurs as far west as southeastern Manitoba and green ash is native as far west as southeastern Alberta (Farrar 1998). Since the appearance of *C. fraxinella* in the urban forests of Western Prairie cities its main hosts are green and black ash.

Caloptilia fraxinella overwinter as adults and exhibit a prolonged reproductive diapause over fall and winter after eclosion from cone-rolled leaflets in the summer (Evenden *et al.* 2007). Adults mate in early spring and eggs are laid preferentially in the lower canopy as soon as host ash trees flush new leaflets in late May (Evenden 2009). Eggs hatch within several days and larvae develop through five instars (Pohl *et al.* 2004; Evenden 2009). First and second instars serpentine mine the mesophyll layer of leaflets. Second and third instars create blotch-mines on the outer edges of leaflets. Fourth instar larvae disperse on silken lines to new leaves which they roll into characteristic pyramidal cones where development to the fifth instar occurs followed by pupation and adult eclosion in mid-July (Evenden 2009). *Caloptilia fraxinella* does not significantly damage its host tree (Pohl *et al.* 2004), but a severely infested ash may present with every available leaflet rolled which reduces the cosmetic value of boulevard and park trees. Insecticide sprays are undesirable in an urban setting and are unlikely to be effective on internally feeding larvae but natural biological control is possible from the parasitoid complex. In this study, we identify the parasitoid complex of *C. fraxinella* in its expanded range and assess the parasitism rate and other parasitoid characteristics to determine the potential for biological control of this invasive leafminer.

Parasitoids previously documented to attack *C. fraxinella* in its expanded range include *Apanteles* sp. (Hymenoptera: Braconidae), *Pteromalus phycidus* (Ashmead) (Hymenoptera: Pteromalidae), *Zagrammosoma americanum* Girault and *Elachertus argissa* (Walker) (Hymenoptera: Eulophidae) (Pohl *et al.* 2004). The microgastrine parasitoid, *Apanteles* sp. was the dominant parasitoid reared from *C. fraxinella* in the initial seasons after its appearance in Edmonton (Pohl *et al.* 2004) and in Saskatoon (personal observation). Other *Apanteles* parasitize early instars (first-third) of internally-feeding lepidopteran larvae such as *Plutella xylostella* (Linneaus) (Lepidoptera: Plutellidae) (Cardona and Oatman 1975) and *Phthorimae operculella* (Zeller) (Lepidoptera: Gelechiidae) (Salazar and Rivera 1995). Little is known about the life history of the *Apanteles* sp. associated with *C. fraxinella* and in the current study it is identified to species as *Apanteles polychrosidis* Vierek (Hymenoptera: Braconidae).

Apanteles polychrosidis is a generalist parasitoid reported mainly from lepidopteran hosts in the family Tortricidae with leaf-rolling life strategies on various angiosperms (Biddinger *et al.* 1994; Cossentine *et al.* 2004, 2005; LaGasa *et al.* 2000; Li *et al.* 1999;

Seaman *et al.* 1990) or defoliators of conifers (Huber *et al.* 1996). *Apanteles polychrosidis* parasitizes first and second instar obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) and emerges from third and fourth instar hosts before pupation (Cossentine *et al.* 2005). Many solitary *Apanteles* spp. alternate hosts within a season and overwinter either as a prepupae or as a larva within an overwintering host (Shaw and Huddleston 1991). In British Columbia, *A. polychrosidis* parasitizes both generations of *C. rosaceana* and overwinters within larvae of the second generation (Cossentine *et al.* 2004). *Apanteles polychrosidis* has never previously been documented from a non-tortricid or from leaf-mining larvae.

The objectives of this study were: 1) to identify the parasitoid community of *C. fraxinella* in its expanded range in Western Canada; 2) describe the life histories of these parasitoids in association with *C. fraxinella* in the ash urban forest; and 3) determine the phenology and distribution of host use, adult eclosion patterns, and reproductive biology, of the dominant parasitoid in this system, *Apanteles polychrosidis.* Rearings of field collected *C. fraxinella* infesting green ash and traps positioned in the ash canopy were used to identify and determine the phenology of species within the parasitoid complex. The life history of *A. polychrosidis* after eclosion from *C. fraxinella* was followed with laboratory observations of its mating, courtship and oviposition behaviour into an alternate host, *C. rosaceana*.

Materials and Methods

Parasitoid complex

To determine the parasitoid complex of *C. fraxinella* in its expanded range, rolled leaflets with emergence windows made by *C. fraxinella* (to avoid collection of *Apanteles polychrosidis* individuals) were collected haphazardly from 3-5 green ash trees at 5-7 sites in Edmonton, AB, Canada (53º34 N 113º31 W) in June 2009, 2010, 2011 (range 200-2000 rolled leaflets/tree) except in 2010 when leaflets without emergence windows (which signified *A. polychrosidis* parasitism) were collected. Collection sites were parks with green ash dominant or boulevards with green ash planted as infill trees among a taller canopy of American elm, *Ulmus americana* L., (Ulmacea) and older green ash. Leaflet rolls were kept in individual 30 ml transparent plastic cups covered with clear plastic lids (SOLO cup company, Lake Forest, Illinois, U.S.A.) (10,067 in 2009, 23,863 in 2010 and 17,159 in 2011) and groups of 100 cups were placed in transparent plastic bags with a damp paper towel to maintain humidity. Bags were held under summer conditions in a walk in growth chamber at 24°C with a 16L:8D cycle. Eclosed parasitoids were collected and stored in 95% ethanol prior to identification. Twenty putative braconid parasitoids, four from each of five sites, were collected in 2008 and identified by Daryl Williams at the Northern Forestry Centre, Canadian Forest Service (Edmonton, AB, Canada) to *Apanteles* sp. The *Apanteles* sp. specimens were further identified to species as *Apanteles polychrosidis* by José Fernandez and Henri Goulet of Agriculture and Agri-Food Canada (AAFC) and the National Reference Collection (NRC) in Ottawa, Canada. Other hymenopteran parasitoid specimens were identified by Gary Gibson (Pteromalidae), John Huber (Chalcidae) and Andrew Bennett (Ichneumonidae) also of AAFC and NRC. Parasitoid specimens and adult samples of their host are vouchered in the Canadian National Collection (CNC).

Percentage parasitism caused by each identified parasitoid species in 2009 to 2011 was calculated as the number of parasitoids that eclosed from *C. fraxinella* pupae divided by the total number of pupae collected per year. In 2010, *A. polychrosidis* parasitism was similarly scored from emergence from leaflet rolls (23,863) collected from three trees at each of five sites in Edmonton. In 2008 and 2009, the percentage of parasitism by *A. polychrosidis* was determined through *in situ* counts in the field from six green ash trees at each of nine sites. To estimate percent parasitism, one hundred leaflet rolls per tree were haphazardly selected throughout the canopy and scored as un-parasitized (with a *C. fraxinella* cocoon) or parasitized (with an *A. polychrosidis* cocoon).

Phenology of host use

Larval dissections- Several hundred eggs, and first, second and third instar *C. fraxinella* larvae were field collected (27, 28 May and 3, 5 June, 2008) from two trees at each of eight sites to determine levels of endoparasitism at the egg, serpentine and blotch-mine stages. Leaflets with eggs and larvae were placed on Whatman no. 1 filter paper moistened with distilled water in a sealed Petri dish and kept in a growth chamber at 24 °C with a 16L:8D cycle until emergence of fourth instars from blotch mines. A total of 363 recovered 4th instar larvae were dissected under a compound microscope at 60 X to assess the presence of endoparasitic larvae within *C. fraxinella* collected at the different life stages in the field (egg=22, first instar = 155, second instar = 39, third instar = 147).

Host and parasitoid phenology - Development of the host, *C. fraxinella*, was sampled twice-weekly on three green ash trees at each of five sites in Edmonton, AB from 13 May to 15 July, 2008 to encompass the entire development period of *C. fraxinella*. The terminal 30 cm of four branches per tree were sampled from each of four positions (1) upper 1/3 from the North side of the canopy (2) lower 1/3 from the North side of the canopy (3) upper 1/3 from the South canopy and (4) lower 1/3 from the South canopy. Branches were individually bagged, and frozen overnight (-20 ºC) before assessment of the developmental stage of *C. fraxinella*. Concurrent counts of adult parasitoids in the sampled ash trees were obtained from four yellow sticky cards (10 x 15.5 cm, Contech, Delta, BC) similarly positioned in the canopy of each tree. Numbers of each identified parasitoid species captured on the four yellow cards in each tree at each developmental stage of the host were enumerated until host larvae were in the fourth and fifth instar inside leaflet rolls. Data loggers (HOBO UA-002-64, Onset Computer Corporation, Cape Cod, Massachusetts) tracked temperature (ºC) and light intensity (lx) at the same four positions within the canopy of one tree at each site. All statistical analyses were conducted with generalized linear mixed effect models (GLMM) in R (R 2.13, R development team 2011) using the Poisson family of errors with a log link recommended for count data

(Crawley 2007). One GLMM was used to determine the within tree preference for oviposition by *C. fraxinella*. *Caloptilia fraxinella* counts of non-motile stages (egg through third instar) were specified as the dependent variable, location within the canopy was the independent fixed variable and tree nested within site specified as random factor with leaflets without *C. fraxinella* removed from the analysis. Generalized mixed effect models were also used to assess variation of the number of each parasitoid species with sample date and location within the tree. Number of each parasitoid was specified as the dependent variable in each model and sample date and location within the tree were independent fixed variables with individual trees nested within site and specified as a random factor. Two models were constructed for female parasitoids in which sex could be determined (one each for *A. polychrosidis* and *Diadegma* sp.) and three for combined males and females in which parasitoid sex could not be determined (*Sympiesis* sp. 1, and *Sympiesis* spp. 2 and 3 combined and *Pteromalus phycidis*). If models were significant, a *post hoc* Tukey's HSD test (MASS library on CRAN) was used to identify differences in parasitoid abundance among sample dates and location within trees. Sample date was a categorical factor. Temperature and light intensity within the canopy were analyzed individually as dependent variables in two GLMMs with location within the canopy as the independent fixed variable, and site and date specified as random factors. *Post hoc* Tukey's HSD tests were used to identify significant differences in temperature and light intensity among the four locations within the canopy.

Phenology of parasitoid emergence

Emergence sleeves, made from white nylon organza (30 cm diameter x 70 cm length, mesh size 330 x 330 µm), were placed over branches with visible leaflet rolls in the mid-canopy of green ash trees and were sealed at both ends with flagging tape. Five trees at each of five sites were sampled every 2 days from 4-12 July, 2008 with one sleeve per tree. At each sample date, bags with branches were removed and one new branch in each tree was bagged. Branch samples were frozen at (-20ºC) overnight and parasitoids captured in the bags were enumerated, identified and separated by sex when possible. To determine if lack of an emergence window, normally created on leaflet rolls by fifth instar *C. fraxinella* (Pohl *et al.* 2004), is a reliable diagnostic feature of *A. polychrosidis* parasitism, all collected leaflet rolls that harboured *A. polychrosidis* (103) were scored for the presence or absence of an emergence window and analyzed with a binomial exact test (R.2.13, R development team 2011). To determine if there was a temporal difference in eclosion between the sexes of each parasitoid species recovered, two generalized linear mixed effects models were used with error terms specified as the Poisson family with a log link (R 2.13, R development team 2011). The number of *A. polychrosidis*, or *Diadegma* sp. was specified as the dependent variable with sex and sample date as independent variables and tree nested within site was designated as a random variable. An additional experiment was conducted in 2009 to further assess the emergence phenology of male and female *A. polychrosidis*. Leaflet rolls that contained *A. polychrosidis* cocoons were collected from five trees at each of seven sites in Edmonton, AB, on 2 July 2009, placed in clear plastic emergence containers in groups of 50 leaflets and held indoors under natural light next to a window. Emergence containers were checked every two days from 3 to 31 July 2009 and the number and sex of emerged wasps were recorded. The number of *A. polychrosidis* was the dependent variable and sex and sample date were independent fixed variables with site designated as a random variable in a GLMM with the Poisson family of errors and log link specified (R 2.13, R development team 2011).

Courtship and mating behaviour of Apanteles polychrosidis

Mating behaviour of *A. polychrosidis* was observed using individuals from the emergence phenology experiment in 2009 (above). Wasps were separated by sex as they eclosed and were kept under natural light conditions next to a window in the laboratory in clear plastic containers and fed sterile 10% honey water *ad libitum*. Twenty-five females (1-4 days old) were subsequently placed individually into clear glass vials. To determine if males would mate with multiple females, five males were introduced in succession to each of five virgin females and allowed five minutes to mate and perform courtship behaviours. Prior mating observations showed that mating occurs quickly once males and females are paired.

Acceptance of another host by Apanteles polychrosidis reared from C. fraxinella

To test the hypothesis that *A. polychrosidis* reared from *C. fraxinella* can oviposit in another known host, *C. rosaceana* (Cossentine *et al.* 2004, 2005), several hundred male and female *A. polychrosidis* were held together in dome-shaped cages (60 x 60 x 60 cm, BugDorm, Megaview Science, Taichung, Taiwan) under natural light conditions in the laboratory and fed sterile 10% honey water *ad libitum* for a minimum of two days to ensure they were mated before use in the bioassay. First-third instar *C. rosaceana* larvae were obtained from a laboratory colony held at 25ºC 16L:8D and fed a modified pinto-bean based diet (Shorey and Hale 1965). Larvae were introduced individually in 30 ml cups that contained the artificial diet on which they were reared to ten male and five female *A. polychrosidis* within clear, plastic 500 ml containers. *C. rosaceana* larvae were exposed to the wasps for 4 hours between 1200 and 1600 and then were returned to rearing conditions. *Choristoneura rosaceana* larvae exposed to *A. polychrosidis* were checked for parasitoid emergence daily.

Results

Parasitoid complex

All braconid parasitoid specimens reared from *C. fraxinella* in this study were the same species previously found to parasitize *C. fraxinella* in Edmonton (Pohl *et al.* 2004). This species was identified here as *Apanteles polychrosidis* Veirek (Hymenoptera: Braconidae) and was the most common parasitoid of *C. fraxinella* in this study (Table 4-1). Leaflet rolls are created (Fig. 4-1C) by fourth instar *C. fraxinella* before endoparasitoids emerge. *Apanteles polychrosidis* emerge from and kill fifth instar *C. fraxinella* before hosts can construct the characteristic emergence window in the rolled leaflet (Pohl *et al.* 2004) thus, absence of the moth emergence window is diagnostic for *A. polychrosidis* parasitism ($Z_{1,103}$= 8.52, *P*<0.0001). Solitary, endoparasitic *A. polychrosidis* larvae emerge from *C. fraxinella* within the leaflet roll created by its host and spin a hammock-like cocoon (Fig. 4-1A) in which pupation occurs. After eclosion, the adult wasp chews an exit hole (Fig. 4-1A) in the side of the cone-rolled leaflet that is morphologically different from the emergence window created by *C. fraxinella* larvae (Fig. 4-1B). *Diadegma* sp. (near *fenestrale*) (Hymenoptera: Ichneumonidae: Campopleginae) is a larval endoparasitoid that spins its cocoon within the cocoon of *C. fraxinella* (Fig. 4-1D) and was the second most common adult (Fig. 4-1E) parasitoid reared (Table 4-1). Three ectoparasitic *Sympiesis* species (Hymenoptera: Eulophidae) emerged from *C. fraxinella* pupae. Two of the *Sympiesis* species (*Sympiesis* spp. 1 and 2) did not match specimens in the CNC and were not identified below the level of genus but sp. 2 keyed out closely to *Sympiesis* near *viridula* (Thompson). The third species was *Sympiesis sericeicornis* (Nees). *Sympiesis* sp. 2 (near *viridula*) and *Sympiesis* sp. 1 (Fig. 4-1H) were also recorded in the present study as facultative hyperparasitoids of *A. polychrosidis* pupae. From one to five *Sympiesis* utilized one *A. polychrosidis* host (Fig. 4-1F). Larvae fed externally on *C. fraxinella* or *A. polychrosidis* and chewed a hole through the cocoon to access the host pupa inside (Fig. 4-1G). None of the *Sympiesis* species spun cocoons; instead they all pupated as naked pupae (Fig. 4-1F) within the abdominal cavity of *C. fraxinella* (Fig. 4-1I) or *A. polychrosidis* pupae (Fig. 4-1F). *Pteromalus phycidis* (Ashmead) (Hymenoptera: Pteromalidae) was the least common parasitoid reared from *C. fraxinella* (Fig. 4-1J) in this study (Table 4-1).

Phenology of host use

Larval dissections - No parasitoids were recovered from *C. fraxinella* collected as eggs or first and second instars. Four parasitoid larvae were recovered from *C. fraxinella* collected as third instar larvae (over-all parasitism rate = 0.01%). These parasitoids could have been either *A. polychrosidis* or *Diadegma* sp. based on their endoparasitic habit.

Host and parasitoid phenology -Capture of *A. polychrosidis* females on yellow sticky cards positioned in the ash tree canopy varied throughout the developmental period of its host (χ^2_4=160.68, P<0.001) with none caught before hosts were present (Fig. 4-2). Significantly more *A. polychrosidis* females were captured during the migration of fourth instar *C. fraxinella* than at the egg (z_1=7.32, P<0.001), serpentine miner (z_1=8.83, P<0.001), and blotch miner (z_1= 6.99, P<0.001) stages (Fig. 4-2). The number of female *A. polychrosidis* did not vary by location within the tree (χ^2_3= 2.24, P=0.524). The number of female *Diadegma* sp. captured also varied during host development (χ^2_4= 41.95, P<0.001) and *Diadegma* sp. females were most commonly found during fourth instar larval migration than at any other stage of host development (Fig. 4-2). The number of female *Diadegma* sp. did not vary by location within the tree (χ^2_3= 2.37, P=0.499). The number of *Sympiesis* sp.1 captured on yellow sticky traps remained low and constant throughout the larval development of *C. fraxinella* (χ^2_4= 3.17, P=0.530) (Fig. 4-2) and did not vary within the tree canopy (χ^2_3=3.81, P=0.283). Capture of the other *Sympiesis* species (*Sympiesis* sp. 2 and *S. sericeicornis*) varied with host development (χ^2_4= 58.71, P<0.001) and peaked during the egg and migration host stages (Fig. 4-2). The location of *Sympiesis* spp. varied within the canopy of the tree (χ^2_3=13.79, P=0.0032) with fewer trapped low in the canopy on the south side than at either high (z_1=3.44, P=0.0033) or low (z_1=2.82, P=0.025) positions on the north side. Numbers of *P. phycidis* captured on yellow sticky cards varied with host developmental stage (χ^2_4= 147.06, P<0.001). More *P. phycidis* adults were captured at the fourth instar dispersal stage than the blotch-mine (z_1= 2.62, P=0.044) and serpentine-mine stages of the host (z_1=10.42, p<0.001), and more at the blotch than serpentine-mine stage (z_1=7.98, P<0.001). More *P. phycidis* were captured in traps located high on the south side of the tree compared to low on the north side (z_1=2.67, P=0.038).

There was a significant effect of position within the canopy on the number of early stage *C. fraxinella* (χ^2_3= 41.37, P<0.001). There were more *C. fraxinella* in the lower canopy than the upper with significantly more *C. fraxinella* in the low south than high south (z_1=5.10, P<0.001) and low north canopy than high north canopy (z_1=3.82, P<0.001). There was no difference between north and south in either the lower (z_1=0.07, P=0.999) or upper (z_1=1.42, P=0.487) canopy.

Temperature varied within the canopy (χ^2_3= 144.02, P<0.001) with no difference between the high southern and northern canopies (z_1=1.76, P=0.294) or at low and high positions on the south side of trees (z_1=2.37, P=0.0834). The low southern canopy was cooler than the high northern canopy (z_1=3.99, P<0.001). The low northern canopy was cooler than the high northern canopy (z_1=11.19, P<0.001), and both the low southern (z_1=6.36, P<0.001) and high southern (z_1=9.43, P<0.001) canopies. The low northern canopy was the coolest at 21.197 ± 0.065 ºC and the high northern canopy the warmest at 21.81 ± 0.064 ºC. Light intensity was significantly different in all four positions within the canopy (χ^2_3= 216440, P<0.001) and most intense in the low southern canopy (216.90 ± 2.93 lux) and least intense in the low northern canopy (133.96 ±1.16 lux).

Phenology of parasitoid emergence

In sleeve cage experiments conducted in 2008, male *A. polychrosidis* emerged before females (Fig. 3). There was a significant sex:date interaction (χ^2_1= 18.51, P<0.001) that showed males eclosed earlier than females under natural conditions in the field (Fig. 3). The number of *Diadegma* retrieved in sleeve cages was lower than *A. polychrosidis* and there was no difference in the number of males and females recovered over time (sex:date interaction, χ^2_1= 2.29, P=0.13). *Sympiesis* spp. were pooled and not separated by sex and capture numbers were low over the entire sample period.

Apanteles polychrosidis that emerged from rolled leaflets (n=3175) in the laboratory displayed a significant female-biased sex ratio (χ^2_1=10.79, P=0.001). There was a significant interaction between the sex of *A. polychrosidis* and the sample date that described adult eclosion (χ^2_1=7.40, *P*=0.0065). As was observed in the field (Fig. 4-3), males emerged first, dominated the first ten days of eclosion, peaked at 11-12 July, 2009 and dropped to zero by 23-24 July 2009 (Fig. 4-4). Females began to eclose 8-9 July 2009, dominated samples by 13-14 July 2009 and continued to eclose in low numbers until 26 July, 2009 (Fig. 4-4).

Courtship and mating behaviour of Apanteles polychrosidis

Courtship behaviour preceded all mating encounters observed (n=7). Males faced females and fluttered their wings, hopped toward the female and pulsed their antennae downward, followed by contact of the antennal tips with the female. After antennal contact, males would mount the abdomen of receptive females from behind. Courtship behaviour was followed with copulation that lasted, $\bar{x}$=49.25 ± 8.73 S.E. (n=7, range 36-80) seconds. Of the five males observed, one did not mate, two mated once, one twice and one thrice.

Acceptance of another host by Apanteles polychrosidis reared from C. fraxinella

One generation of *A. polychrosidis* was successfully reared on *C. rosaceana*. Ovipositing female *A. polychrosidis* use their antennal tips to drum *C. rosaceana* larvae and frass, which appears to initiate a sequence of ovipositor stabs that ends with ovipositor insertion into the host larva.

Discussion

A complex of parasitoids utilizes *C. fraxinella* in its expanded range as a host. *Apanteles polychrosidis* remains the most common parasitoid of *C. fraxinella* (Pohl *et. al.* 2004) twelve years after the initial introduction of its host to Edmonton. In 2001-2003, the parasitoid complex on *C. fraxinella* consisted of *A. polychrosidis* and a few isolated accounts of *Pteromalus phycidis* (Ashmead) (Hymenoptera: Pteromalidae), *Zagrammosoma americanum* Girault and *Elachertus argissa* (Walker) (Hymenoptera: Eulophidae) (Pohl *et al.* 2004) thus the parasitoid complex (2008 to 2011) has increased in complexity. Parasitism of *C. fraxinella* by *Zagrammosoma americanum* and *Elachertus argissa* (Pohl *et al.* 2004) are not supported in the current study but *P. phycidis* remains associated with *C. fraxinella* in low numbers. The current complex includes in order of frequency recovered: *A. polychrosidis*, a *Diadegma* sp., three species of *Sympiesis* and *P. phycidis*. *Diadegma* sp. and the *Sympiesis* spp. have not previously been recorded as parasitoids of *C. fraxinella.*

The rate of *A. polychrosidis* parasitism initially declined after the introduction of *C. fraxinella* from 23% in 2001, 7% in 2002 and 2% in 2003 (Pohl *et al.* 2004). In contrast, the parasitism rate by *A. polychrosidis* in the current study increased from 36.5% in 2008 to nearly 57% in 2009 followed by a decrease to 10% in 2010. The low parasitism rate in 2010 reflects a late spring snowstorm after leaf flush and many rainy periods that may have impacted the parasitoid population either directly (Fink and Völkl 1995) or indirectly through interference with host-parasitoid synchrony (Godfray 1994; Van Nouhuys and Lei 2004). The high parasitism rates in 2008-2009 suggest that *A. polychrosidis* has the potential to naturally suppress populations of *C. fraxinella*. The rate of parasitism by the other parasitoids in the complex remains consistently ≤ 1 %.

A similar complex of parasitoids utilizes *Caloptilia azaleela* (Brants) and *C. leucothoes* Kumata (Lepidoptera: Gracillariidae) leaf miners on Rhododendron (Ericaceae) in which *Apanteles* cf. *xanthostigma* (Hymenoptera: Braconidae) is the dominant parasitoid of *C. azalella* (Sugiura 2011). The primary parasitoids in the Rhododendron-*Caloptilia* system are also facultatively parasitized by two idiobiont hyperparasitoids, *Acrolyta* sp. and *Sympiesis dolicogaster* Ashmead (Hymenoptera: Eulophidae) which parasitize fifth instar and pupae of *C. azaleella. Sympiesis sericeicornis,* recorded in this study, is also a primary parasitoid on other leaf-miner larvae in the family Gracillariidae (Doganlor and Bierne 1980; Giradoz *et al.* 2006; Hagley 1985; Casas 1989). It also hyper-parasitizes the primary parasitoid of the spotted tentiform leafminer, *Phyllonorycter blancardella*, (Fabr.) (Lepidoptera: Gracillaridae) an *Apanteles* sp. (Hagley 1985). *Pteromalus phycidis* is also a hyper-parasitoid of other *Apanteles* sp. (Elliott *et al.* 1986) but was not recorded as a hyperparasite in the current study.

Caloptilia fraxinella prefers to oviposit in the lower canopy (Evenden 2009) but there is no evidence from the current study that this behaviour is dictated by light intensity. Most within-tree flight activity by females occurs after dusk and before dawn (Chapter 3) so light intensity may not play a role in ovipositional preference. Light intensity did not affect ovipositional preferences of another night-flying Gracillariid (Connor 1996). The distribution of early instar hosts or variable light intensity and temperature within the canopy did not affect the distribution of *A. polychrosidis* or *Diadegma.* However, most *A. polychrosidis* and *Diadegma* were found at the larval migration stage. Decreased light intensity reduces host location efficiency in another braconid parasitoid, *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) while flight activity in *Diadegma insulare* (Cresson) (Hymenopetera: Ichneumonidae) increases with light intensity (Idris and Grafius 1998). Temperature may have no effect on parasitoid foraging (Fink and Völkl 1995) and even though temperature within the canopy of green ash was significantly different, the differences were less than 1 ºC. *Sympiesis* sp.2 and *S. sericeicornis* were captured in traps on the north side of trees and may prefer lower light intensity and temperature. *Sympiesis sericeicornis* does not fly when wind speed exceeds 2 m/s (Casas 1989) so wind speed, which we did not measure, may also affect the distribution of these eulophids. *Pteromalus phycidus* may prefer to forage in the higher light intensities and temperature of the upper southern canopy even though the density of *C. fraxinella* hosts was lowest in the upper canopy because light intensity influences other pteromalid species (Smith and Rutz 1991).

Apanteles polychrosidis is the dominant parasitoid of *C. fraxinella* in its expanded range in Western North America. There was no evidence of *A. polychrosidis* parasitism of *C. fraxinella* eggs in dissections. Other *Apanteles* spp. parasitize their hosts at early and midinstars (Shaw and Huddleston 1991) and few are known to be egg parasitoids. *Apanteles polychrosidis* and *Diadegma* sp. are the only two endoparasitoids that parasitize *C. fraxinella*, recovered in this study. Thus the four endoparasitoid larvae found in dissections are likely one of these species. *Cotesia vestalis* has three distinct larval instars: first instar caudate-mandibulate, second instar with posterior anal vesicles and vermiform third instar (Yu *et al.* 2008) as do most *Apanteles* species (Shaw and Huddleston 1991) and dissections in the current study revealed three distinct larval instars.

Parasitism rates determined from dissected larvae were almost negligible and the majority of adult *A. polychrosidis* and *Diadegma* were trapped during the transition from leaf-mining to leaf-rolling when *C. fraxinella* is vulnerable. Similar numbers of female *Diadegma* sp. and *A. polychrosidis* are trapped while *C. fraxinella* migrate to new leaflets but *A. polychrosidis* has a much higher rate of parasitism. *Diadegma* and *A. polychrosidis* are both endoparasitoids and *A. polychrosidis* may out-compete *Diadegma* larvae within the host in multiparasitism events either by direct conflict between species (Salt 1961), or by greater resistance to host defense by *A. polychrosidis* larvae (Godfrey 1994). Campoplegine and microgastrine first instar larvae both have large mandibles to fight (Strand 2000). Also, *A. polychrosidis* superparasitizes *C. rosaceana* with up to 26 eggs per host but only one larva survives four days after oviposition (Cossentine *et al.* 2005) so intraspecific larvicide must occur in this species and interspecific larvicide could also occur.

Apanteles polychrosidis is a generalist parasitoid previously recorded from lepidopteran larvae in the family Tortricidae. *Apanteles polychrosidis* parasitizes the leaf-rolling species, *Choristoneura rosaceana*, on apple (Cossestine *et al.* 2004) and raspberry (Li *et al.* 1999; Schuh and Mote 1948) and other tortricid leaf-rollers; *Archips rosanus* (L.), *Spilonota ocellana* (Den. & Schff.) (LaGasa *et al.* 2000) *Platynota idaeusalis* (Walker) (Biddenger *et al.* 1994) and *Endopiza viteana* Clemens (Seaman *et al.* 1990) in North America. It also successfully parasitizes another leafroller, *Pandemis limitata* (Robinson) (Lepidoptera:

Tortricidae), when larvae are exposed without the leaf roll in laboratory experiments (Cossentine *et. al.* 2005). It utilizes several other *Choristoneura* species in North America that do not exhibit a leaf-rolling habit such as the spruce budworm, *C. fumifera* and the jack pine budworm, *C. pinus* (Huber *et al.* 1996). None of these other recorded hosts of *A. polychrosidis* are leaf miners and it is unlikely that this parasitoid would switch its host-search tactics to utilize the concealed larvae in leaf mines. In the current study, most adult *A. polychrosidis* were recovered from sticky traps during the migration of *C. fraxinella* from leaf-mining to leaf-rolling habits which suggests that parasitism occurs when larvae are exposed. Use of exposed *C. fraxinella* does not require *A. polychrosidis* to change its host-search tactics to accommodate oviposition in concealed leaf-miner larvae.

The protandrous emergence of *A. polychrosidis* and the tendency of male wasps to remain near their eclosion sites on ash trees (Wist: unpublished) suggest that males wait at eclosion sites for females to emerge. Indeed, *A. polychrosidis* mate soon after female eclosion in the laboratory and several other *Apanteles* species mate within the first hour of female emergence (Arakaki and Ganaha 1986; Tagawa and Kitano 1981). A courtship ritual occurs before *A. polychrosidis* mate that may be influenced by pheromonal and visual stimulation of males (Askari and Coppel 1978; Wesoloh 1977). Not all *Apanteles* species exhibit protrandry as synchronous emergence of the sexes occurs in *A. flavipes* but males still wait at female emergence sites for mating opportunities (Arakaki and Ganaha 1986). Mating soon after emergence suggests that *A. polychrosidis* females are monandrous like other *Apanteles* spp. (Ridley 1989) while males are capable of multiple mating, like many other microgastrine males (Matthews 1974). The mean copulation time of *A. polychrosidis* recorded in the current study is 49s which exceeds the 20s required for efficient sperm transfer, needed by *A. melanoscelus* (Ratzeburg) (Wesoloh 1977) and is longer than the mean mating time of 15s by *A. flavipes* (Arakaki and Ganaha 1986). Multiple mating in quick succession by some *A. polychrosidis* males and a skewed female sex ratio suggests that early emerging males that mate multiply gain a reproductive advantage over later emerged males as most female braconid wasps mate only once (Matthews 1974).

Most microgastrine braconids overwinter as endoparasitoids within larval hosts or as cocooned prepupae (Shaw and Huddleston 1991). *Apanteles polychrosidis* must use another host than *C. fraxinella* to overwinter because *C. fraxinella* overwinter as adults (Pohl *et al.* 2004). In Pennsylvania, *A. polychrosidis* overwinters in *Platynota idaeusalis* larvae (Biddenger *et al.* 1994) and in British Columbia *A. polychrosidis* emerge from late instar, overwintered *C. rosaceana* larvae (Cossentine *et al.* 2004). *Choristoneura rosaceana* may be an overwinter host for *A. polychrosidis* as it occurs in Edmonton (E.H. Strickland Virtual Entomological Museum, University of Alberta), and its larvae are readily parasitized by the summer generation of *A. polychrosidis* in the laboratory.

Since the introduction of *C. fraxinella* to Western Canada, the parasitoid complex has changed. *Apanteles polychrosidis* still dominates the parasitoid complex of *C. fraxinella* and is the most likely candidate to exact biological control in the expanded range of its host. Host distribution, light and temperature do not affect the distribution of parasitoids within the tree canopy. The majority of parasitism by *A. polychrosidis* occurs while *C. fraxinella* are exposed as fourth instar larvae during migration to new leaflets thus this parasitoid did not adopt a new host location strategy to utilize a concealed host. Protandrous emergence, followed by mating when females emerge and successful parasitism of an alternate larval host suggest that *A. polychrosidis* overwinters within larval hosts (Shaw and Huddleston 1991) before it parasitizes *C. fraxinella* each spring. With its high parasitism rate, *A. polychrosidis* may be a significant natural biological control to suppress *C. fraxinella* populations in the expanded range of this leaf-miner.

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Table 4-1. Percent parasitism of *Caloptilia fraxinella* by identified members of the parasitoid complex

Year	*Apanteles polychrosidis*	*Diadegma* sp.	*Sympiesis* spp.	*Pteromalus phycidis*	Total number of rolls
2008	36.50*	-	-	-	-
2009	58.88*	1.18	0.98	0.28	10,067
2010	9.94	0.90	0.62	0.26	23,863
2011	-	0.059	0.012	0.0038	17,159

"*" indicates that percentages of *Apanteles polychrosidis* were calculated from leaflet rolls sampled *in situ*. *Apanteles polychrosidis* was not sampled in 2011 and the remainder of the parasitoid complex was not sampled in 2008. Other percentages were calculated as the total number of each parasitoid species emerged from field collected *Caloptilia fraxinella* pupae divided by the total number of *C. fraxinella* pupae collected in that year and reared in the laboratory.

Figure 4-1. (A) Emergence hole (arrow) of *Apanteles polychrosidis* (*Ap*) in parasitized cone-rolled leaflet of *Caloptilia fraxinella*. *A. polychrosidis* cocoon (c). (B) Cone-rolled leaflet with *C. fraxinella* emergence window (arrowhead) and pupal exuvium (p). Note the serpentine mine on the leaflet surface. (C) Fourth instar larval *C. fraxinella* (arrowhead) on a partially rolled green ash leaflet. (D) *Diadegma* sp. cocoon (c) with remains of *C. fraxinella* cocoon. (E) Female (top) and male (bottom) *Diadegma* sp. (F) Five *Sympiesis* sp. 1 (top), a facultative hyperparasitoid, emerged from one *A. polychrosidis* pupa (*Ap*). Cocoon (c). (G) Entrance hole (arrowhead) of a larval *Sympiesis* sp. 1 in the cocoon of *A. polychrosidis*. (H) Emergence hole (arrowhead) from *C. fraxinella* cocoon and emerged adult *Sympiesis* sp. 1. (I) Larval *Sympiesis* sp. 1 (arrowhead) in abdominal cavity of *C. fraxinella* pupa. (J) Adult (a), pupal exuvium (p) and cocoon (c) of *C. fraxinella*. All scale bars represent 1 mm.

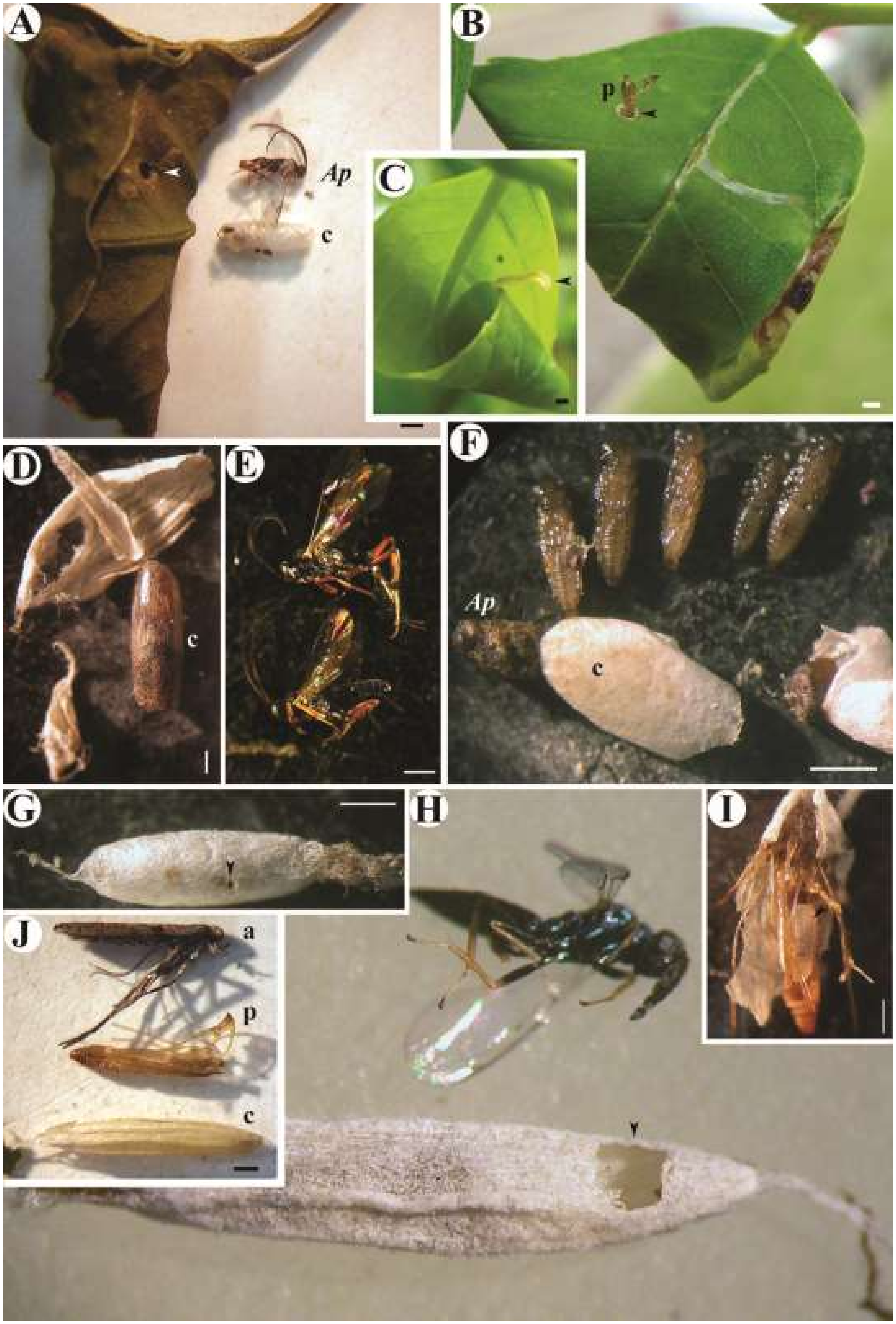
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Figure 4-2. Mean (± S.E.) numbers of parasitoids per sample date (left axis) caught in green ash trees matched with the damage caused and life stages present of their host *Caloptilia fraxinella* by sample date and tree phenology in 2008 at Edmonton, Alberta. L1 is first instar *C. fraxinella*, L2 is second instar, L3 is third instar, and L4 is fourth instar and percentages at each stage are indicated in brackets. Mean number of migrating *Caloptilia fraxinella* larvae per sample date are represented by diamonds and scaled to the right axis.

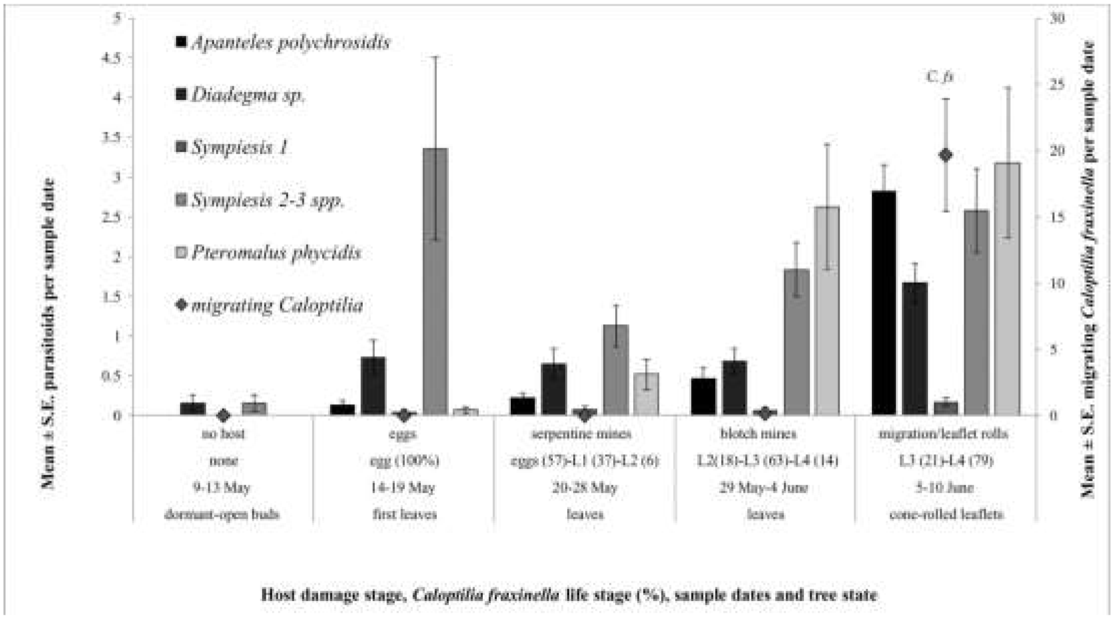


Figure 4-3. Mean (± S.E.) number and sex of insects emerged from *Caloptilia fraxinella* in rolled leaflets assessed with sleeve cage traps per sample date in 2008 at Edmonton, Alberta.

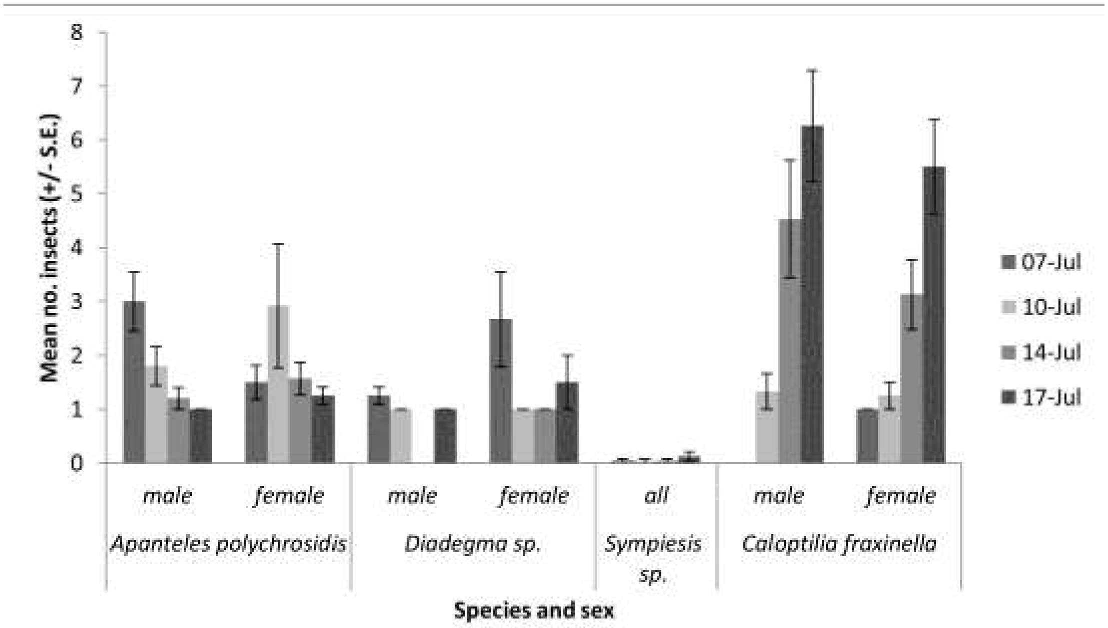
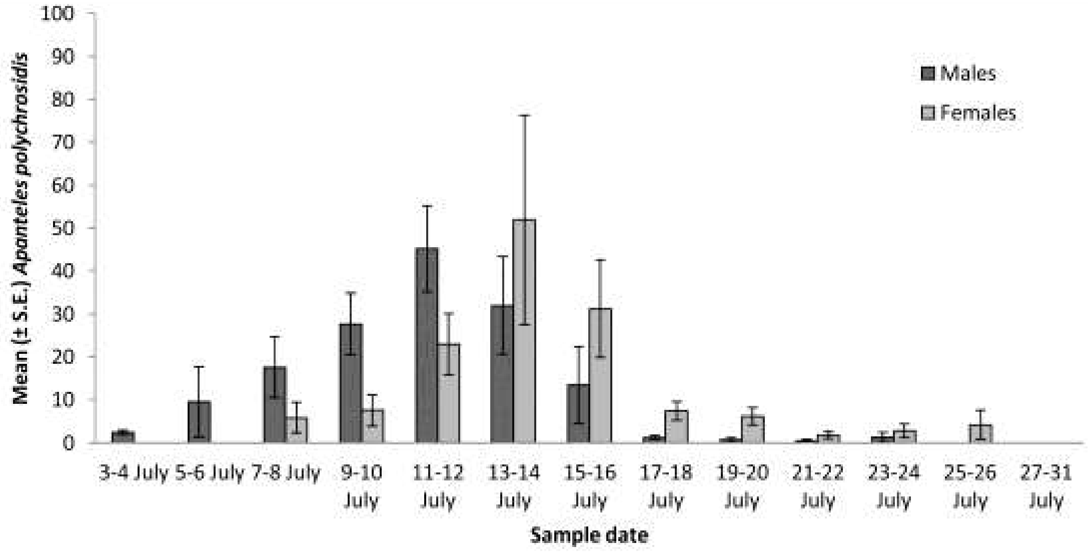


Figure 4-4. Mean number per site (± S.E.) of *Apanteles polychrosidis* emerged from *Caloptilia fraxinella* by sex and emergence date in rolled leaflets held under laboratory conditions in 2009.



Chapter 5: The effect of host tree and herbivore host-density on parasitism

Introduction

In tri-trophic interactions, the response of the plants to herbivore foraging activity is often well understood but the plant can also influence activity of the third trophic level, natural enemies of the herbivore (Barbosa et al. 2001; Lill et al. 2002). Differential parasitism of the same insect herbivore can be mediated by the host food plant of the herbivore (Vinson 1976; Martin et al. 1981; Roland 1986; Geervliet et al. 2000; Lill et al. 2002; Liu & Jiang, 2003). Indeed, the plant species may contribute more to parasitism success (Barbosa et al. 2001; Lill et al. 2002; Liu and Jiang 2003) and the community of active parasitoids (Lill et al. 2002) than does the herbivore host itself. There are several mechanisms (Barbosa et al. 2001; Lill et al. 2002) that could contribute to differential parasitism rates of herbivores on different host plants including: (1) differences in herbivore-induced plant volatiles (HIPV) released from plants that attract and retain parasitoids, (2) differences in plant structure that make the plant more or less suitable as parasitoid habitat, (3) activity of multiple species of herbivores on different plants, and (4) differences in herbivore density on the different plants followed by density-dependent foraging by the parasitoid.

Differential parasitism mediated by the host food plant can manifest in different numerical responses of the parasitoid to changes in density of the herbivore (Barbosa et al. 2001). Numerical responses describe changes in parasitoid density in response to density of herbivore hosts (Solomon 1949). Three numerical responses are often discussed between herbivore and parasitoid density (Lessells 1985; Stiling 1987; Fernandez-Arhex and Corley 2003): (1) positive density dependence in which parasitism increases with increasing host density, (2) lack of a density dependent response in which parasitoid density is not linked to host density, and (3) negative or inverse density dependence in which parasitoid density decreases with increasing host density. The mechanisms driving these relationships are related mainly to four parasitoid traits; searching (host location) capacity, host handling time, identification and acceptance of the host, and female parasitoid egg complement (Holling 1959). Searching capacity of the parasitoid is the mechanism mediating the numerical response of parasitoids most closely linked to the herbivore's host plant (Fernandez-Arhex and Corley 2003).

Long range host searching by parasitoids is primarily mediated by semiochemicals which are volatile organic compounds (VOCs) released by the host's food plant (Visser 1986; Vet and Dicke 1992; Mumm and Dicke 2010). Differential parasitism is often correlated with semiochemical differences among host food plants (Benrey et al. 1997; Liu & Jiang 2003) and in well studied tri-trophic systems, herbivore-damaged plants recruit more parasitoids than undamaged plants (Turlings et al. 1990; Rose et al. 1998). Specialist parasitoids, that use only one herbivore host, are more sensitive to cues released by plants fed upon by the specific herbivore they attack than to plants attacked by non-hosts, mechanically-damaged plants, or undamaged plants (Dicke et al.1990; Turlings et al.1990; 1991a,b; De Moraes et al. 1998). Generalist parasitoids are not as dependent on volatile cues specifically induced by herbivore feeding as specialists and can respond to undamaged or mechanically damaged plants (DeMoraes et al. 1998; Rose et al. 1998). Plants respond to insect herbivory with increasing the quantity and/or changing the composition of volatiles released (Turlings et al. 1995; Shiojiri et al. 2010; and for a review see Dicke and van Loon 2000). A change in the volatile profile released by plants due to changes in herbivore density could result in differential parasitism of herbivores that feed on several food plants (Romeis et al. 1997). In agricultural systems, VOC-mediated differential parasitism is well documented (Liu and Jiang 2003; Lou et al. 2006; Poelman et al. 2009; Degen et al. 2012) but this ecological question is rarely studied outside of an agricultural setting, for example, on deciduous trees (Barbosa et al. 2001).

The ash leaf-coneroller, *Caloptilia fraxinella* (Ely), (Lepidoptera: Gracillariidae) is an invasive moth in the Western Canadian Prairies that feeds on leaflets of ash trees, *Fraxinus* spp. (Oleaceae) in the larval stage (Pohl et al. 2004; Evenden 2009). Young larvae mine leaflets and older larvae roll leaflets into characteristic "cones" where pupation occurs. The main hosts of *C. fraxinella* in Western Canada are green, *F. pennsylvanica* and black, *F. nigra* ashes although they have also been collected feeding on white, *F. americana* and blue *F. quadrangulata* ashes (CNC National Collection, Ontario and Quebec) in their native range. A sedentary leaf-mining habit prevents larvae from escaping parasitism which can be important in the development of density dependent parasitism (Stiling 1987).

Apanteles polychrosidis Viereck (Hymenoptera: Braconidae) is the most abundant parasitoid of *C. fraxinella* in its extended range (Pohl et al. 2004; Wist and Evenden 2013). *Apanteles polychrosidis* is a solitary, generalist, internal koinobiont (allows host to develop) parasitoid that oviposits into larval life stages of lepidopteran hosts. The parasitoid kills its host in the final larval instar and pupates in the leaflet rolled by *C. fraxinella*. Soon after summer emergence from *C. fraxinella*, *A. polychrosidis* mates (Wist and Evenden 2013) and must seek out another host in which its offspring would overwinter. *Apanteles polychrosidis* is known to parasitize other lepidopteran hosts with a leaf-rolling habit including *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (Cossentine et al. 2004) that occurs sympatrically with *C. fraxinella*.

A tri-trophic interaction that consists of a native generalist parasitoid that shifted hosts to an invasive leaf miner which feeds on two closely related, introduced host trees, green ash and black ash, is studied here. This tri-trophic relationship is recently established with the earliest record in 2002 following the introduction of *C. fraxinella* in 1999 (Pohl et al. 2004). *Apanteles polychrosidis* is the only species in the parasitoid complex of *C. fraxinella* with the potential to control populations of this invasive leafminer, as parasitism rates of 60% occur in some seasons while parasitism rates of other species rarely exceeds 1% (Wist and Evenden 2013). An understanding of the host location mechanism by *A. polychrosidis* is necessary to enhance parasitism for control of this invasive leaf miner (James 2003; 2005).

This system lends itself to the study of differential parasitism as horticultural ash plantings consist of both species of ash that are available for foraging herbivores and parasitoids. As *A. polychrosidis* is a generalist parasitoid (Cossentine et al. 2004; 2005), it should be attracted to general plant VOCs (DeMoraes et al. 1998; Rose et al. 1998) and not specific HIPVs induced by feeding by *C. fraxinella*. Four hypotheses tested using this tri-trophic system are, (1) parasitism differs with the herbivore's host plant species and (2) there is a numerical response of parasitoids to the density of their herbivore host, (3) parasitism depends on differential responses of the parasitoid to plant VOCs is tested with laboratory behavioral experiments to elucidate the mechanisms that dictate successful host location in this system and (4) plant tissue is the origin of these signals.

Materials and Methods

Study system

The current study was conducted on horticultural plantings of ash, *Fraxinus* spp., when they were approximately 8-10 years old. Ash trees in Edmonton are planted outside of their natural range (Farrar 1998). Study trees were confined to boulevard and park trees in which the majority of the canopy could be sampled from the ground or with ladders. There is no within-canopy difference in the abundance of female *A. polychrosidis* (Wist and Evenden 2013) so a host tree was considered as the parasitoid patch in this study.

Calculation of host density and parasitism

Leaf miner density and percent parasitism were assessed on a range of 1-7 trees (mean 3.09 ± 0.13 SE) of each ash species at each of seven (2008) and eight (2009) sites separated by at least

1 km in Edmonton AB, Canada (53º34 N 113º31 W). Additional sites (n=2) in 2008 and (n=9) in 2009 augmented testing on green ash only. Horticultural black and green ash at each site was planted at approximately the same time.

Leaf miner density per tree was estimated by randomly selecting ~ 50 leaves throughout the canopy from each tree (381 ± 11.3 leaflets/tree) and scoring each leaflet as infested or uninfested by *C. fraxinella*. The percent parasitism was estimated on the same trees by inspecting one hundred rolled leaflets and scoring them as un-parasitized (containing the leaf miner cocoon) or parasitized (containing an *A. polychrosidis* cocoon). The number of parasitized leaflets divided by the total number of rolled leaflets sampled per tree gave the percentage of parasitism per tree.

Differential parasitism

A generalized linear mixed effects models (GLMM) were used to test for effects of ash species and year of the study on leaf miner density. Site was specified as random and the error distribution specified as negative binomial in the leaf miner model to account for overdispersion (Crawley 2007). A threeway ANOVA tested for effects on *A. polychrosidis* parasitim which used the Gaussian error distribution. The parasitism data approximated a normal distribution when plotted against a theoretical normal line and passed a Shapiro-Wilk's test of normality for each species (green ash, df= 70, P = 0.805; black ash, df = 39, p=0.547). A graph of the residuals did not reveal any abnormal patterns so linear models were deemed appropriate to illustrate the numerical response of parasitism. The threeway ANOVA model tested the main effect of leaf miner density (continuous variable), ash species, site and year of study on parasitism. Interactions between leaf miner density, ash species, site and year were included in the models to determine if the relationship of parasitism to host density differs on each ash species. Non-significant interaction terms were removed until the best fit model (AIC) remained. Statistics were conducted with R 2.13 (R Development Core team 2012) using the Rcmdr library (Fox et al. 2011).

A linear regression model was used to test the numerical response of parasitism to host density on the two species of ash trees. Data from all sites and both years were used with each sampled tree scored for parasitism (y axis) and host density (x axis). Linear regressions and graphs were produced with the Rcmdr library (Fox et al. 2011) in R 2.13.

Olfactometer experiments

Olfactometer bioassays were used to test the response of parasitoid wasps to cues associated with the two ash species with or without leaf miner activity. *Apanteles polychrosidis* cocoons were collected from green ash trees in July 2010 and 2011, removed from their leaflet rolls and allowed to emerge in a cage (BugDorm, MegaView Science, Taichung, Taiwan) held under the natural photoperiod at room temperature (23ºC). *Apanteles polychrosidis* mate as soon as the later emerging females exit their cocoon (Wist and Evenden 2013) so all experimental females were assumed to have mated prior to experiments. Parasitoids were fed 10% honey water *ad libitum* and were used within four days of emergence. Females were sensitized for host location prior to experimentation by exposing them to larvae and excrement of another larval host, *C. rosaceana* to initiate host location behavior in females (Rose et al. 1997; 1998; Wei and LeKang 2006). Pre-exposure to *C. fraxinella* larvae would have been difficult with the very low number of host larvae of the second generation at the time of *A. polychrosidis* emergence. Female parasitoids displaying these behaviors (surface antennation coupled with forward locomotion) that had not oviposited in *C. rosaceana* hosts were transferred individually using an aspirator to the opening of a y-tube olfactometer (2.5 cm internal diameter x 17 cm main body and 26 cm arms) lit from above with a 15 watt (1.4 W/m^2) white incandescent bulb. Charcoal filtered, humidified air flowed at 0.5 L/min down each arm of the olfactometer. The olfactometer was angled upwards from horizontal by 45° (Delury et al. 1999). The arms of the olfactometer were covered with white cardboard to reduce the influence of visual cues in selection of olfactometer arms. Each female parasitoid was given 10 mins to respond and was used only once. A choice was recorded as either movement towards the control (c) or treatment (t) arm when the parasitoid travelled 26 cm into an arm of the olfactometer and entered the ante-chamber before the treatment source. If no clear choice was made after ten mins, the parasitoid was removed and counted as a non-responder.

Several olfactometer experiments were conducted to compare cues associated with both ash species with or without herbivore presence as compared to a clean-air control. The first set of experiments compared parasitoid response to five leaflets from either a black or a green ash that were leaf-mined and rolled by *C. fraxinella* larvae (containing leaflet material, excrement and larvae) to a clean-air control (n=50 for black ash, n=62 for green ash). The second set of experiments compared parasitoid response to five leaflets from either a black or green ash that were mechanically damaged by ten cuts to each leaflet with a razor blade simulating mining larvae to a clean-air control (n=57 for black ash, n=62 for green ash). The third set of experiments compared parasitoid response to five undamaged leaflets of black or green ash to a clean-air control (n=50 for black ash, n=62 for green ash). Due to the univoltine phenology of *C. fraxinella* (Evenden et al. 2007) it is impossible to obtain moths for oviposition on naive plant material at the same time that parasitoid wasps can be collected in the field. A second generation of *C. fraxinella* in Edmonton, presumably from a sub-population that does not enter reproductive diapause, does occur in the rare instance that ash trees produce new leaves in mid-summer. Therefore, leaf-mined plant material was collected from this second generation at one horticultural planting where green and black ash both produced new leaves from “water suckers” at the base of the trees and used either on the day of collection or the following day after storage of the leaf overnight in a refrigerator with the petiole in a vial of water (2011). The experimental day was compared in green and black ash leaf-mined experiments to determine if the proportion of response by female wasps to each treatment was affected by overnight storage of leaflets. The proportion of *A. polychrosidis* that chose the treatment in the leaf-mined experiments was the response variable in two (one each for green and black ash) generalized linear models (R 2.13) with experimental day as the explanatory variable and with the binomial (logit link) family of errors. Mechanically-damaged and undamaged leaflets had to be obtained from one-year-old greenhouse-grown seedlings (Jeffries Nurseries, Portage La Prairie, Manitoba) to avoid a systemic effect of *C. fraxinella* herbivory on field-collected leaflets. Seedlings were planted individually in pots containing Sunshine #4 potting mix, placed into a growth chamber under summer conditions (16L:8D, 24.5°C, 50 %RH), and watered as needed. Parasitoid response to plant material was tested in blocks of ten to each treatment and halfway through each block the source ends of the olfactometer were switched to avoid a positional bias. Leaflets were handled with metal forceps. Olfactometers were cleaned with acetone and baked overnight at 150 ºC after each block of ten parasitoids was tested.

Statistics were conducted with non-responders removed (sample sizes after non-responders removed were: green undamaged n=34, black undamaged n=23, green mechanical damage n=30, black mechanical damage n=21, green leaf-mined n=40, and black leaf-mined n=23). Response of female parasitoids to the treatment *vs*. control arm of the olfactometer was assessed with Chi-squared tests (R 2.13).

Electrophysiological analysis of Apanteles polychrosidis antennae to green ash VOCs

To test the hypothesis that female *A. polychrosidis* detect VOC cues emitted from herbivore-infested plant material, volatile emissions were collected from experimentally manipulated seedlings in three treatments; (1) undamaged seedlings, (2) seedlings damaged by leaf-mining

C. fraxinella, and (3) mechanically damaged seedlings. Headspace VOCs were collected from whole seedlings to capture the volatile emissions parasitoids encounter during host location. Extracts of these emissions were tested with electroantennographic detection (EAD) analyses using *A. polychrosidis* antennae as the detector. Extracts of VOCs released from greenhouse-grown seedlings infested with *C. fraxinella* were used to stimulate antennae from wasps that had eclosed from rolled leaflets. An attempt to repeat these treatments with infested black ash seedlings failed so electrophysiological response of the parasitoid was tested only to treatments on green ash.

One year old green ash seedlings (Jeffries Nurseries, Portage La Prairie, Manitoba) were planted as described for the olfactometer experiments above. Seedlings were approximately the same size (~50-60 cm tall) and had similar amounts of foliage. Six seedlings were experimentally infested with *C. fraxinella* through introduction of 10 mated females into each of two insect cages (BugDorm 60x60x60cm, Megaview Science, Taichung, Taiwan), that contained three seedlings at bud flush. Eggs were laid and leaf-mining initiated on three of the six seedlings. Volatile organic compounds were collected from all three seedlings together once larvae had entered their 2nd instar, approximately 72 h after eggs hatched. Mechanical damage was accomplished by tracing serpentine “mines” with a razor blade on the upper surface of ten leaflets per seedling for each of three seedlings. Volatile organic compounds were collected during the first 8 h (daytime) after damage and again for 8 h during the day at 72 h after the initial mechanical damage to coincide with seedlings in the leaf-mined treatment. Aeration of a group of three undamaged seedlings occurred at the same time as the leaf-mined and damaged treatments. Each time VOCs were captured, a single control sample of the air within the growth chamber, was collected simultaneously. Volatile organic compounds released from the green ash seedlings were collected by enclosing the crown of three of the variously treated seedlings with a Look™ oven bag sealed around the stem of the seedlings. Charcoal-filtered air was drawn through each bag at 1 L/min using an aquarium pump modified to pull air. The drawn air was captured onto Porapak-Q in an absorbent tube (Porapak-Q, OD 6 mm, length 110 mm; absorbent: front layer 150 mg, back layer 75 mg; separated by glass wool) (SKC Inc., PA, USA distributed by Supelco, Bellefonte, Pennsylvania, USA) for 8h during the daytime.

Captured VOCs were extracted from the front and back layers of Porapak-Q beads with 1 ml pentane (Fisher, >99.6% purity) and analyzed using gas chromatography (GC) on a Hewlett Packard 5890A gas chromatograph equipped with a DB-5, column (each 30 m x 0.32 mm ID; J&W Scientific, Folsom, CA, USA) with a temperature program where 50 ºC was held for one min, then increased by 20 ºC /min to 280 ºC, and held for five min. The back layer of Porapak-Q in each sample was also tested and no VOCs “broke through” the front layer of Porapak-Q in the 8 h of sampling. The control samples did not collect significant amounts of VOCs. There were few qualitative differences among the treatments and the extracts with the highest peak areas were pooled across treatments for gas chromatographic-electroantennographic detection (GC-EAD) analysis with the antenna of either a female or male *A. polychrosidis* used as the EAD detector. The column was the same type as that used in the GC analysis above.

One hundred and sixty, *A. polychrosidis* pupae field-collected from green ash were shipped to Dr. Regine Gries at Simon Fraser University where adult parasitoids emerged and were allowed to mate before their antennae were removed. Wasp antennae were excised with fine forceps and positioned so that the base of each antenna was placed into the opening of a glass capillary electrode (1 x 0.58 x 70 mm, last 5 mm bend ca 45º) (A-M Systems, Carlsborg,WA, USA) filled with saline solution. The tip of each antenna, one from at least three individuals of each sex, was removed with fine spring microscissors (Fine Science Tools, North Vancouver, BC, Canada) and the cut antenna placed into the recording glass capillary electrode (Gries et al. 2002). The headspace extracts from the green ash seedlings were pooled to increase the sample volume and used to stimulate the cut antennae. Compounds from ash extracts that elicited an antennal response were identified using mass spectrometry attached to GC (Varian Saturn Ion Trap) with a column of the same type used for GC-EAD and GC analysis and a temperature regime of 50 ºC for five minutes, then increased by 10 ºC /min to 280 ºC for five minutes. Peaks were identified by comparison of their retention indices (RI) (relative to alkane standards; Van den Dool and Kratz 1963) with those of authentic standards (hexanal, Sigma; *z*-3-hexenal, oxidized from OH; *z*-3-hexenol, Aldrich; *z*-3-hexenyl-acetate, acetylated from OH; Ocimene, International Flavours and Fragrances; linalool (+/-), Sigma; methyl salicylate, Aldrich; *E*-β farnesene, Bedoukian; α-farnesene, Treatt; bourbonene, isolated from natural oil; dendrolasin, tentatively identified; *E*-4,8-dimethyl-1,3,7-nonatriene, synthesized by Dr. Regine Gries according to standard procedures (Leopold 1986; 1990)). The quantity of each electrophysiologically active compound in extracts from the variously treated trees was calculated from the peak areas of the GC traces.

Results

Differential parasitism

The overall leaf miner density on both ash species was significantly higher in 2009 (106.12 ± 5.64 cone rolled leaflets per tree, n=84) than 2008 (65.55 ± 6.29 cone rolled leaflets per tree, n=33) (Fig. 5-1, Table 5-1). Green and black ash did not differ in leaf miner density in either year of the study (Fig. 5-1, Table 5-1). Parasitism was significantly lower in 2008 (53.39 ± 3.45%, n=33) than 2009 (63.83 ± 2.14%, n=84) and parasitism of *C. fraxinella* was significantly higher on black ash than on green ash in both years (Table 5-1, Fig. 5-1). Since year was not significant it was removed from the model. The interaction between leaf miner density and tree species and between leaf miner density and site did not affect parasitism unless site was considered (Table 5-1), thus the relationship of parasitism to leaf miner density is different on the two ash species at each site. There was also a significant interaction between tree species and site, which indicates that parasitism on each species differs by site (Table 5-1). The main effects of leaf miner density, tree species and study year significantly influenced the percentage of *C. fraxinella* parasitized by *A. polychrosidis* (Table 5-1).

Numerical response of parasitism to host density

The significant effect of leaf miner density on parasitism is seen in the slope of the regression lines and the interaction of leaf miner density and ash species reveals an opposite relationship between parasitism and leaf miner density on black ash and green ash (Fig. 5-2). Regression analyses revealed an inverse relationship between leaf miner density and parasitism on green ash and a positive relationship on black ash (Fig. 5-2).

Olfactometer experiments

In all treatments, approximately half of the tested wasps did not make a choice for either treatment or control and were excluded from statistical analyses. The proportion of *A. polychrosidis* females that chose the leaf-mined treatment over the control did not differ among experimental dates in the green ash experiments (χ^2_3 =0.831, P=0.842) nor in black ash experiments (χ^2_2 =0.807, P=0.668) regardless of treatment of ash leaflets. On black ash, *A. polychrosidis* chose leaf-mined leaflets (containing larvae and excrement) over the clean air control (χ^2_1 = 5.26, P=0.021) but showed no preference for mechanically damaged (χ^2_1 = 0.048, P=0.827) or undamaged black ash leaflets (χ^2_1 = 1.96, P=0.162) compared to the clean air control (Fig. 5-3). There was no difference in the response by parasitoids to green ash leaflets mined by the herbivore compared to a clean air control (χ^2_1 = 0.024, P=0.875). Parasitoids preferred undamaged (χ^2_1 = 4.24, *P*=0.04) or mechanically damaged green ash leaflets to the clean-air control (χ^2_1 = 5.76, *P*=0.016) (Fig. 5-3).

Volatile analysis: comparisons between green ash treatments

Initial GC analysis did not show any qualitative differences among the variously treated green ash seedlings and the samples from the four treatments were pooled for GC-EAD analysis (Table 5-2). Thirteen electrophysiologically -active compounds were identified that elicited significant antennal peaks (Fig. 5-4) and eleven of these were identified (Table 5-2). The initial mass-spectrophotometer analysis before pooling for GC-EAD revealed trends for quantitative differences of the electrophysiologically-active compounds among the treatments (Fig. 5-4, Table 5-2). Treatments could not be compared statistically because samples were pooled prior to GC-MS analysis. Antennae of male wasps responded to the same compounds as females (Fig. 4) with smaller amplitudes of response to the green leaf volatiles (GLVs), *z* 3-hexenal, *z* 3-hexenol, *z* 3-hexenyl acetate and farnesenes.

Discussion

Differential parasitism is mediated by host plant species

Differential parasitism of *C. fraxinella* by *A. polychrosidis* occurs on two ash species infested by the leaf miner. Parasitism is greater on black ash than green ash and is partly driven by the population densities of the leaf miner on each ash species. Differential parasitism mediated by the plant host species of the herbivore also occurs in several other braconid parasitoids (Benrey et al. 1997; Geervliet et al. 2000; Lui and Jiang 2003).

There are several potential mechanisms that may cause the differential parasitism of *C. fraxinella* observed in this study (Barbosa et al. 2001; Lill et al. 2002). This study provides evidence for two of the four proposed theoretical mechanisms (Barbosa et al. 2001; Lill et al. 2002): The density of the herbivore host affects response of parasitoids on both ash species but in opposite directions; and differences in the volatile profiles of the plant species differentially induces parasitoid behavior. The other two proposed mechanisms, that parasitism is enhanced by multiple species of herbivores and differential parasitism is mediated by differences in plant structure, were not studied. However, parasitism of two other gracillariid leaf miners, *C. azaleella* and *C. leucothoes* (Lepidoptera: Gracillariidae), is mediated by leaf surface morphology in which trichome density on the leaves of one rhododendron species reduces parasitism by *Acryrocharoides* sp. (Hymenoptera: Eulophidae) (Sugiura 2011). There are no apparent differences in the surface morphology of the upper surface of leaflets of the two ash species as both green and black ash have glabrous adaxial surfaces.

Numerical responses (Solomon 1949) of parasitoids to host density, in addition to parasitism rates of *C. fraxinella,* differ on the two species of ash. The numerical response measured as the relationship between parasitism and leaf miner density (Hassell 2000; Fernandez-Arhex and Corley 2003) was different depending on the species of ash upon which *C. fraxinella* occurs. On black ash, parasitism is positively dependent on leaf miner density, and on green ash, the relationship is inversely density-dependent. There is density-dependent parasitism by *Cyzenis albicans* (Fall.) (Diptera: Tachinidae) of winter moth *Operophtera brumata* (L.) (Lepidoptera: Geometridae) on oak but no relationship between parasitism rate and winter moth density on apple (Roland 1986). Two other microgastrines (Hymenoptera: Braconidae) track their larval hosts solely by the identity of the host plant but independent of the density of their host; *Cotesia plutellae,* on different cabbage varieties (Lui and Jiang 2003) and *Cotesia glomerata* on the preferred food plant of *Pieris brassicae* (Benrey et al. 1997; Geervliet et al. 2000).

The inverse relationship between parasitism and *C. fraxinella* density on green ash suggests that *A. polychrosidis* is less efficient at parasitism of *C. fraxinella* as leaf miner density increases on green ash. High host density can swamp the effectiveness of parasitoids that are unable to forage efficiently enough to suppress herbivore populations (Lessels 1985) through mutual interference with conspecific parasitoids (Chong and Oetting 2006; Mahmoudi et al. 2010) attracted by high host densities. Parasitoid swamping might have occurred at high densities of the leaf miner on green ash due to limitations of parasitoid egg-load or foraging time constraints (Lessels 1985). Parasitoids foraging at high host densities may also be overwhelmed by high levels of herbivore-induced stimuli that can cause foraging mistakes and limit successful location of unparasitized hosts and subsequently reduce parasitism (Holling 1961; Jenner 2003; Weaver et al. 2005). However, the density of leaf miners on green ash was not significantly different from black ash which suggests that other mechanisms are responsible for the opposite parasitism relationships.

Inverse density dependence between host and parasitoid densities suggests a type II or III functional response by the parasitoid at high host densities on green ash. These functional responses often result from an increase in host handling time by parasitoids with increased herbivore density (Hassell 2000; Connor and Cargain 1994). Inversely density dependent relationships between herbivore density and parasitism rates like that observed on green ash, occur in a number of host-parasitoid relationships. Twenty-three percent of 171 insect host-parasitoid systems were inversely density dependent, 52% were density independent and 25% were positively density dependent (Stiling 1987) like the relationship observed on black ash in this study. Parasitoids of other lepidopteran leaf miners in the gracillariid family exhibit both types of relationships found in the current study (Stiling 1987). For example, parasitism of *Phyllonorycter* spp. leaf miners on deciduous oak trees in Japan is independent of leaf miner density (Sato et al. 2002) whereas parasitism of *Phyllonorycter* spp. on oak and beech in the U.K. is positively density dependent (Miller 1973). Positive density dependence and independence from density were found in two distinct populations of citrus leaf miner, *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) (Legaspi et al. 2001). Parasitism of

Cameraria hamadryadella is inversely density-dependent to leaf miner density (Connor and Beck 1993; Connor and Cargain 1994). One potential influence on parasitism rates that has not been eliminated in this study is variation in growth rate of *C. fraxinella* on the two ash species (Chapter 3) that could cause differences in exposure time to the parasitoid (Benrey and Denno 1997).

Parasitoid responses to host plant volatiles

Although *A. polychrosidis* is a generalist parasitoid, with host records from herbivore hosts in multiple Lepidopteran families and from several host plants, its response to different sources of volatile cues in lab experiments in the current study could help to explain the differential parasitism and numerical response observed on the two ash hosts in the field. Caterpillar-infested plants selectively attract parasitoids with herbivore-induced changes to their volatile profiles (DeMoraes et al. 1998; reviewed in Arimura et al. 2005; Dicke 2009). *Apanteles polychrosidis* females are attracted to leaf-mined leaflets of black ash that contain damaged leaflets, live hosts and excrement. On green ash, female parasitoids orient to undamaged and mechanically-damaged leaflets but not leaf-mined leaflets. The undamaged leaflets however, were excised from plants and could be releasing VOCs in response to stem but not leaflet damage. Wasps that respond to host-damaged leaflets on black ash may concentrate their activity and decrease the foraging time of successful host location. *Apanteles polychrosidis* seems to be attracted to, or retained longer (Bruce et al. 2008), on black ash trees by leaf miner induced plant volatiles. Larvae and excrement were also present in green ash leaf-mined leaflets tested in olfactometers, thus it is likely that infested black ash releases a qualitatively or quantitatively different blend of VOCs than green ash that could attract a greater number of parasitoids to the foraging patch. Green ash does produce a quantitatively lower amount of VOCs than black ash (Pureswaran and Poland 2009). In green ash trials, more *A. polychrosidis* make a clear choice in the olfactometer and this could suggest that experimental wasps were biased to prefer VOCs of green ash due to a “chemical legacy” from green ash VOCs that may have permeated their cocoons (Corbet 1985) although cocoons were removed from leaf rolls after collection before wasp emergence in this study. *Cyzinis albicans* (Fallén) (Diptera: Tachinidae) parasitism of winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae) shows a similar pattern in which parasitism rate is linked to leaf damage by its host on oak but not on apple with a difference in chemical profiles suggested as the mechanism (Roland 1986). It is possible that HIPVs on green ash are not optimally attractive to foraging parasitoids, perhaps due to the overall lower quantity of VOCs from green ash (Pureswaran and Poland 2009) and recruit a limited number of parasitoids to green ash trees. A low density of wasps may become overwhelmed at high host densities and result in the inverse-density dependent relationship observed on green ash in the field. In contrast, parasitism is high at both low and high host densities on black ash with a small positive response to host density. This field result, and the attraction of parasitoids to leaf-mined black ash leaflets in olfactometer experiments, suggests that HIPVs may act as a signal to *A. polychrosidis* that hosts are present and may convey host density information. Other braconid parasitoids, two specialists and one generalist, can determine host density through HIPV cues (Geervliet et al.1998; Shiojiri et al. 2010; Girling et al. 2011).

There is precedence for plant VOC-mediated differential parasitism by other braconids (Hymenoptera: Braconidae). The commonly held paradigm is that specialist parasitoids (that use one or a few host taxa) respond primarily to HIPVs (Vet and Dicke 1992; DeMoraes et al. 1998; Rose et al. 1998). However, generalist parasitoids are also attracted to HIPVs (Roßbach et al. 2005; Bruce et al. 2008) and specialists, especially those with hosts on cruciferous plants, can be attracted to undamaged plant profiles (Bukovinszky et al. 2005; Roßbach et al. 2005). Differences among the volatile profiles of herbivore host plants mediate differential parasitism by specialist braconids *C. plutellae* (Liu and Jiang 2003), *C. kariyai* (Fukiwara et al. 2000) and *C. glomerata* (Benrey et al. 1997; Gols et al. 2008). *Cotesisa glomerata* shows a preference for hosts on certain plants (Poelman et al. 2009) and prefers mechanically damaged and herbivore damaged plants over intact plants (Benrey et al. 1997). The specialist *C. plutellae* can distinguish plants infested with its host from mechanically damaged plants (Geervliet et al. 1996) and reacts in a positive density-dependent manner to increased number and quantity of HIPVs (Girling et al. 2011).

Specialist parasitoids also can respond to constitutive VOCs from the plant alone where their host typically feeds (Bruce et al. 2008; Bukovinszky et al. 2005; Roßbach et al. 2005) but this appears less common. Parasitoids that are generalist feeders such as *A. polychrosidis* should be more plastic in their response and not as dependent on the use of HIPVs from a specific plant or induced by a specific host (Vet and Dicke 1992; DeMoraes et al. 1998; Rose et al. 1998).

These experiments illustrate that the generalist parasitoid, *A. polychrosidis*, shows characteristics of both specialist and generalist parasitoids in its different responses to VOCs from the two host plants in the olfactometer. *Apanteles polychrosidis* functions more as a generalist when attracted to VOCs from undamaged and mechanically wounded but not leaf-mined green ash. Although not tested statistically, mechanical wounding of green ash resulted in the highest level of VOC emission measured from the variously treated green ash and may be due to more severe damage to plant tissue by the mechanical damage than that inflicted by leaf-mining larvae. In contrast, *Apanteles polychrosidis* behaves more like a specialist on black ash in female response to HIPVs (Vet and Dicke 1992), as only the leaflets with active *C. fraxinella* leaf-mines attract wasps. The generalist braconid wasp *C. marginiventris* is attracted to green leaf volatiles and terpenoids released by artificially-damaged cotton plants while the specialist braconid *Microplitis croceipes* is not. Both braconids are highly attracted to cotton plants damaged by caterpillars (Rose et al. 1998) and so the non-response of *A. polychrosidis* to leaf-mined green ash is unusual. However, an interesting explanation that warrants further experimentation might be that *C. fraxinella* can suppress the release of HIPVs on green ash (Schwartzberg et al. 2011) but not on black ash. Naïve *C. marginiventris* are most attracted to maize plants that release high quantities of green leaf volatiles compared to ones that release lower quantities (Hoballah et al. 2002) and its sister species, *C. glomerata* is less attracted to mutant plants in which green leaf volatile release is inhibited (Shiojiri et al. 2006). These results suggest that the green leaf volatiles released by damage to ash, like *z*-3 hexenol (Wei et al. 2007), *z*-3 hexanal and *z*-3 hexenyl acetate, might be the most important cues for generalist parasitoids such as *A. polychrosidis* in host location. Methyl salicylate is also implicated in parasitoid attraction and its concentration is correlated with higher parasitism of fall armyworm, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), by *C. marginiventris* on maize (Degen et al. 2012).

Parasitoids that attack other leaf miner species respond to specific HIPVs (Sugimoto et al. 1988a, b; Dicke and Minkenberg 1991; Petitt et al. 1992; Keller and Horne 1993; Lengwiler et al. 1994; Finidori-Logli et al. 1996). For example, the antennal-response by the specialist parasitoid *M. croceipes* is more pronounced to herbivore-induced VOCs than constitutive VOCs. *Apanteles polychrosidis* responds to general plant VOCs as most of the electrophysiologically -active compounds identified here from green ash are commonly found in the headspace volatile profiles of other plants (Paré and Tumlinson 1999; Dudareva et al. 2006).

Volatile signal received by **<u>Apanteles polychrosidis</u>**

GC-EAD revealed that the antennae of *A. polychrosidis* males and females sense thirteen individual compounds in the volatile profile of green ash. Gas chromatography revealed qualitative and quantitative differences in these electrophysiologically-active volatiles among the treatments on green ash but did not provide a clear answer to which, if any, VOCs are specifically herbivore-induced. Mechanical wounding of green ash increases production of VOCs which were more attractive to female parasitoids than the clean air control in olfactometer experiments. Mechanical damage alone can induce systemic release of VOCs (Mithöfer et al. 2005; Connor et al. 2007). The electrophysiologically active volatiles however, were all host plant VOCs which supports the hypothesis that VOCs used in host location by the parasitoid are plant derived. The leaf-mining habit allows larvae to conceal their excrement and bodies (Vet and Dicke 1992), thus plant VOCs with or without herbivory may be a more reliable and stronger signal for parasitoids of leaf-mining hosts. Many of the electrophysiologically active compounds recorded in the current study also are known to stimulate the antennae of other parasitoids and attract both specialist and generalist braconid parasitoids that are closely related to *A. polychrosidis*. The alcohols and aldehydes shown to elicit a response in *A. polychrosidis*, *z*-3-hexenal, *z*-3-hexenol, ester *z*-3-hexenyl acetate and the terpenoids, linalool, *E* 4,8-dimethyl-1,3,7-nonatriene and *E-β* farnesene attract other braconid parasitoids in the genera *Cotesia* and *Microplitis* (Turlings et al. 1991b; Rose et al. 1998; Schnee et al. 2006). The ester, methyl salicylate, is also commonly released by plants in response to insect herbivory and attracts a braconid aphid parasitoid (Sasso et al. 2009) and several other hymenopteran parasitoids of lepidopteran larvae (James and Price 2003; Simpson et al. 2011). Linalool, *z* 3-hexenal and α-farnesene released by both leaf-mined and mechanically damaged plants attract *Opius dissitus* Muesebeck (Hymenoptera: Braconidae), a leaf miner parasitoid (Wei and Kang 2006; Wei et al. 2007). Bourbonene and dendrolasin are floral volatiles that have not been reported in other parasitoid systems but are present in the pheromone blend of several ants (Hayashi and Komae 1980; Francis et al. 2005). The identification of VOCs detected by *A. polychrosidis* allows for further research to enhance their recruitment for control of the leaf miner (James 2003; 2005; James and Price 2004; Simpson et al. 2011).

Summary

This study has tested potential mechanisms to explain the differential responses by female parasitoids to leaf miner hosts in the field. Parasitism of *C. fraxinella* by *A. polychrosidis* occurs differentially and with different numerical responses of the parasitoid to herbivore density on the two ash species. Parasitism of *C. fraxinella* on black ash is positively density dependent and on green ash is inversely-density dependent. These differences in parasitism may be mediated by differences in the volatile signals used by *A. polychrosidis* in long distance host location as occurs in other braconids (Hoballah et al. 2002; Degen et al. 2012). *Apanteles polychrosidis* responds as a generalist parasitoid on green ash where it is attracted to volatiles of the plant alone but as a specialist on black ash where it is attracted by HIPVs. Finally, the volatile signal received by *A. polychrosidis* consists of plant-derived and not specifically herbivore-derived compounds. These VOCs can be further tested for attraction of *A. polychrosidis* in an effort to retain parasitoids and enhance control of the invasive leaf miner. This study illustrates differential parasitism by a native generalist parasitoid on an invasive herbivore on two introduced host plants and provides evidence that differential attraction to plant VOCs mediates parasitism.

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Table 5-1. ANOVA table of the main effects and interactions of the generalized linear mixed effects model (with the variable site as random) of *Caloptilia fraxinella* density and the linear model of *Apanteles polychrosidis* parasitism on green and black ash (Tree species) in Edmonton.

Response variable	**Predictor**	**DF**	χ^2	***P* value**
Leaf miner density	Tree species	1	0.19	0.662
	Year	1	15.87	**<0.001**
	Tree species*Year	1	2.02	0.156
Parasitism	Leaf miner density	1	10.51	**0.002**
	Tree species	1	19.37	**<0.001**
	Site	9	6.17	**<0.001**
	Year	1	11.38	**0.001**
	Leaf miner density*Tree species	1	0.25	0.617
	Leaf miner density*Site	9	0.086	0.564
	Tree species*Site	8	2.29	**0.030**
	Leaf miner density* Tree species*Site	7	2.49	**0.023**

Table 5-2. Release rate of compounds from variously treated green ash, *Fraxinus pennsylvanica* that elicit an antennal (EAD) response in mated female *Apanteles polychrosidis*. The concentration of compounds marked with "- -" was too low to measure.

Green ash treatment					
EAD active volatiles (ng/µl per h)					
Compound	**Undamaged**	**Leaf-mined**	**Mechanical damage 8h**	**Mechanical damage 72h**	**GC Retention time**
z-3 Hexenal	0.12	--	0.21	--	2.28
z-3 Hexenol	0.22	--	0.3	0.17	2.88
z-3 Hexenyl acetate	0.42	0.15	0.61	0.5	4.29
Ocimene	2.41	0.49	1.51	0.71	4.7
Linalool	0.17	--	0.36	0.62	5.19
E 4,8 Dimethyl 1,3,7 nonatriene	2.5	0.56	1.59	5.61	5.31
Methyl salicylate	0.47	0.61	0.95	2.13	6.04
E-β Farnesene	0.089	--	0.18	0.14	8.26
α-Farnesene	0.53	0.24	1.12	1.64	8.36
Dendrolasin	0.055	0.078	0.22	0.28	8.83

Figure 5-1. Effect of tree species on mean (±S.E.) density of *Caloptilia fraxinella* and mean (±S.E.) percent parasitism by *Apanteles polychrosidis* in Edmonton in 2008 and 2009. "*" represents significance at α=0.05 (GLMM, LM).

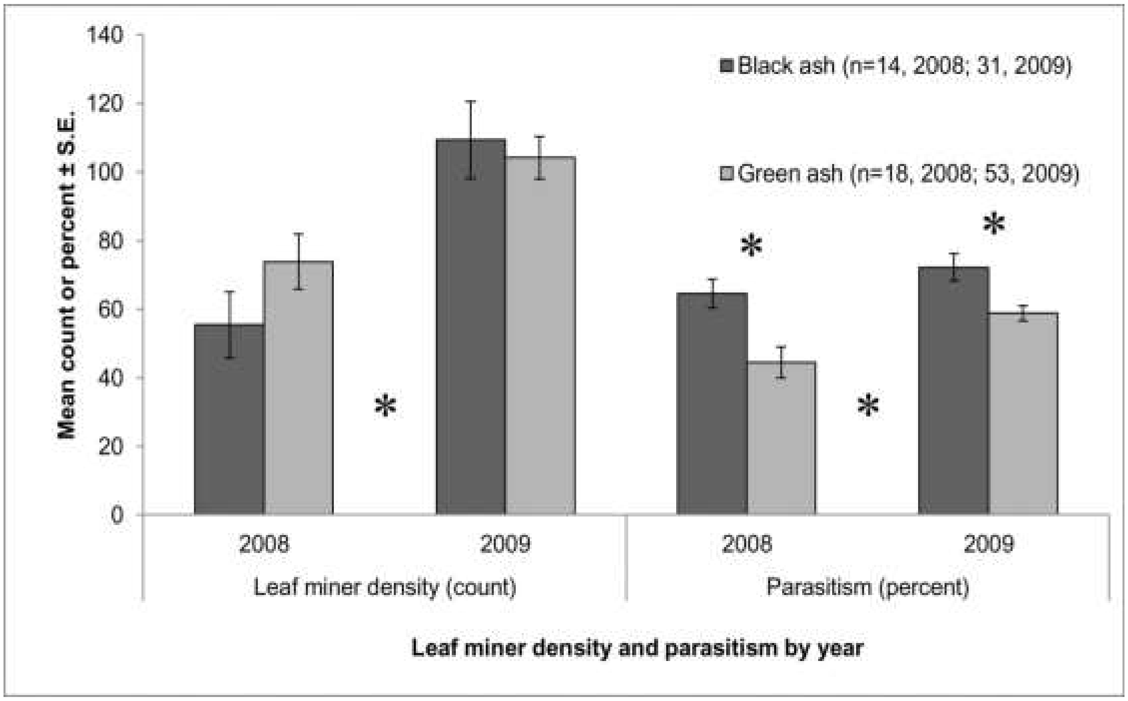


Figure 5-2. Linear regression of percent *Apanteles polychrosidis* parasitism on host density of *Caloptilia fraxinella* by ash species (black ash, circles; green ash, triangles).

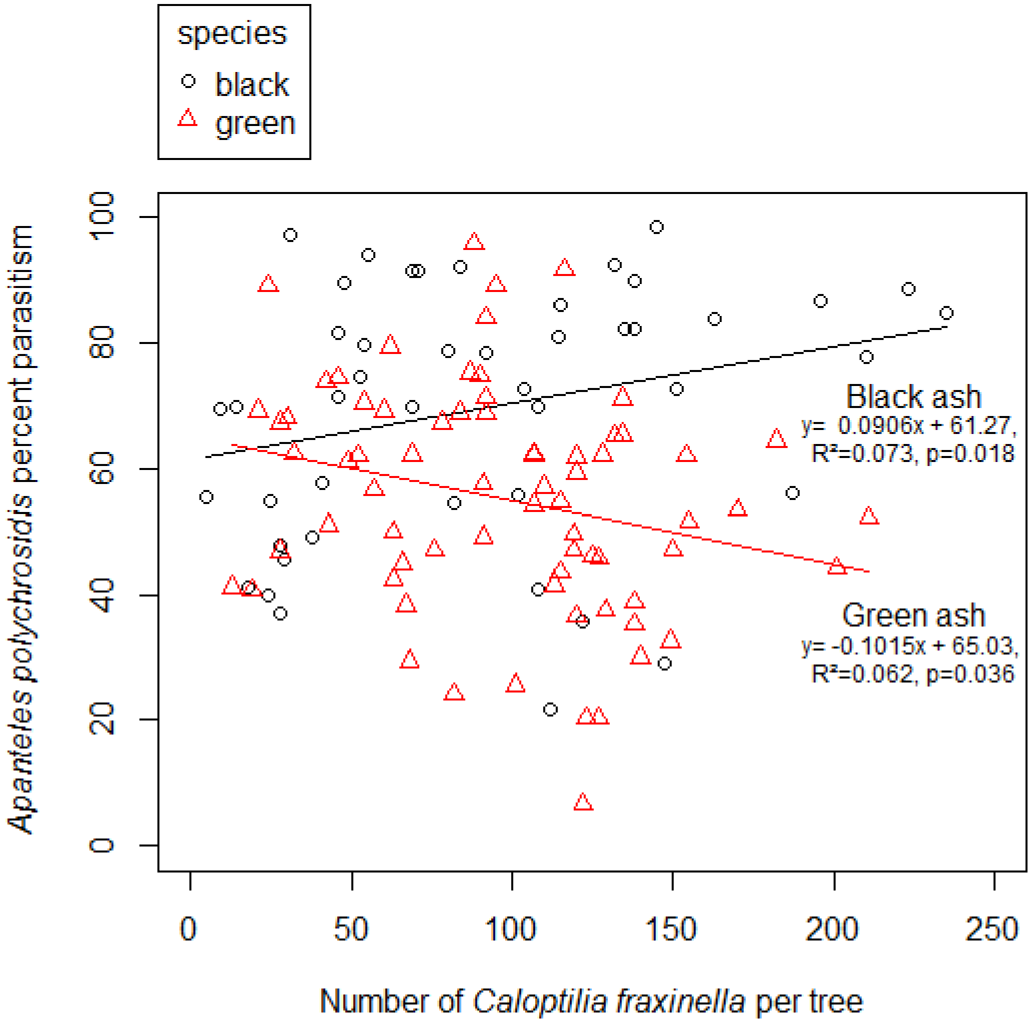


Figure 5-3. Choices made by female *Apanteles polychrosidis* in olfactometer experiments with leaf-mined, mechanically-damaged and undamaged black and green ash leaflets compared to a clean air control. "*" indicates a significant different between treatment and control (α=0.05, Fisher's Exact Test).

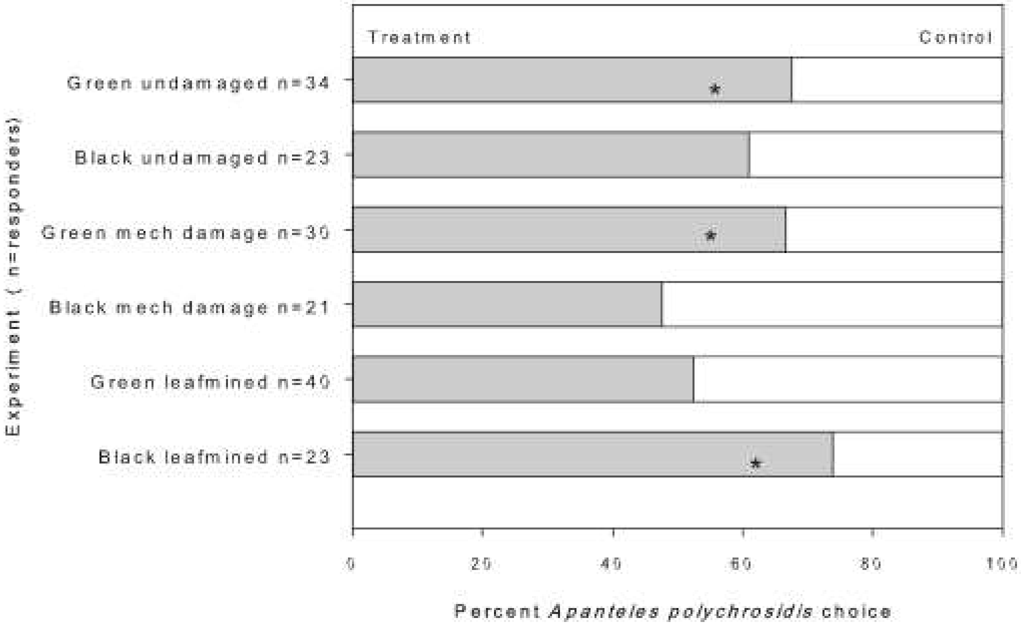
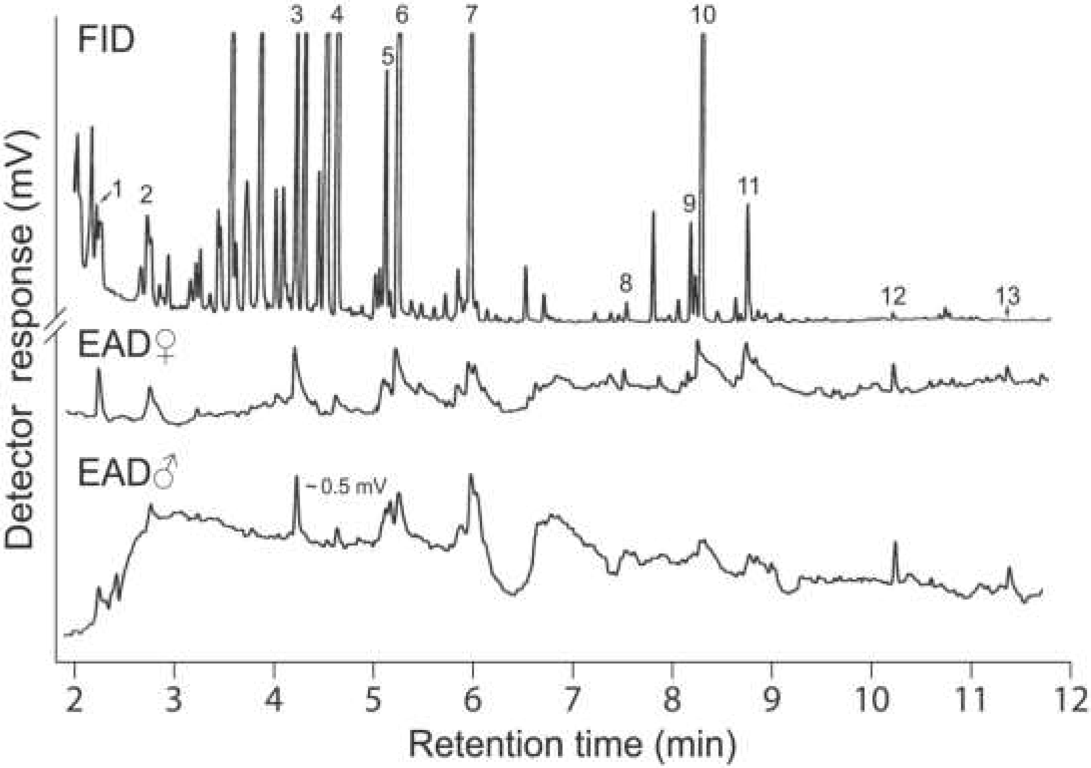


Figure 5-4. Gas chromatogram (upper graph) and the antennal responses recorded by electroantennography of *Apanteles polychrosidis* females (♀, middle graph) and males (♂ lower graph) to the combined headspace volatiles of leaf-mined, mechanically damaged and undamaged green ash seedlings. The electrophysiologically -active volatile organic compounds are: (1) *z*-3-hexenal (2) *z*-3-hexenol (3) *z*-3-hexenyl-acetate (4) ocimene (5) linalool (6) *E* 4,8-dimethyl-1,3,7-nonatriene (7) methyl salicylate (8) bourbonene (9) *E*-*β*-farnesene (10) α-farnesene (11) dendrolasin (12, 13) unknown (no peaks in MS).



Chapter 6: General Conclusion

This study investigated a newly formed tritrophic interaction with the ultimate goal to control populations of the invasive leaf miner, *Caloptilia fraxinella*. It was inspired by the opportunity to control the population of a newly introduced, invasive insect and it led to a diversified study of the chemical relationships in the tritrophic system of *Fraxinus* trees, *Caloptilia fraxinella* moths, and *Apanteles polychrosidis* wasps. The oviposition preference of *C. fraxinella* among its host plants was unknown and this study, coupled with the performance of larvae on host plants revealed that in its expanded range, this moth makes poor oviposition choices for its offspring. *Caloptilia fraxinella* was practically unknown until it appeared in Edmonton, and the chemical ecology of most leaf miners in the gracillariid family is poorly studied, except for the citrus leaf miner (Stelinski and Czokajlo 2009) and the horse chestnut leaf miner (Svatoš et al. 2009). This study not only increases the general knowledge of host location through chemical cues by gracillariid females but reveals the plastic nature of female response to two similar but distinct blends of VOCs that distinguishes each host plant. In addition, the complex of parasitoids that use *C. fraxinella* as hosts in its expanded range had only been vaguely hinted at (Pohl et al. 2004) and no information about parasitoid associations is available in its native range. These knowledge gaps allowed for an exploration of the adoption of a new host by native parasitoids and the link between VOC-mediated host location and differential parasitism of hosts on two ash trees.

VOCs in Caloptilia fraxinella host location

The importance of VOCs in host location of *C. fraxinella* is evident in each experiment conducted in the current study (Plate 6-1a). Female *C. fraxinella* are physiologically capable of VOC mediated-host location after emerging from overwintering locations before natural leaf flush. These weeks before leaf flush give a window of control in which a VOC lure based on ash volatiles could be effective to attract and kill female *C. fraxinella* before they oviposit. Wind tunnel experiments reaffirm the importance of VOCs in host location. Control plastic seedlings do not elicit any host location behaviour by females; unless the whole headspace collected from ash seedlings (Fig A-1) or lures made from a blend of synthetic VOCs perceived by the antennae of females were presented along with a plastic seedling (Chapter 3). Several common plant VOCs from the headspace of ash elicited electrophysiological activity on the antennae of mated, female *C. fraxinella.* Four of these VOCs are common between black and green ash and it is likely that these four, in their unique ratios (Chapter 3), not only mediate host location to trees but allow female *C. fraxinella* to discern the species identity of ash trees.

Synthetic blends of electrophysiologically -active VOCs from black ash and green ash both elicit upwind oriented flight in wind tunnel bioassays so the attractive volatiles must be present in ratios close enough to mimic the signal of an ash tree. Deployment of a lure with synthetic VOCs in the field is the final test of VOC-mediated host location and a key step towards development of an attracticide but neither lure caught a significantly greater number of *C. fraxinella* females than the unbaited control traps. Discrepancies between attraction to VOC-based lures in lab and field bioassays is common (Ansebo et al. 2004; Coracini et al. 2004; Yang et al. 2004; 2005; Cha et al. 2008; Knudsen et al. 2008; Anfora et al. 2009; Svatoš et al. 2009; Loeb et al. 2011) possibly due to interactions with background odours (Knudsen et al. 2008) or ineffective trap design in field studies (Cha et al. 2008). This study is in agreement with the majority of research on kairomonal attraction of moths; that synthetic host-plant VOCs rarely attract many moths under field conditions, even if the number of moths caught on traps baited with synthetic lures is significantly higher than the unbaited control (Anfora et al. 2009). Optimization of the synthetic lures may be warranted (Cha et al. 2008) but kairomonal lures based on host plants may not be able to function except as a tool to monitor the presence of pest insects (Anfora et al. 2009) or to synergize and increase the attractiveness of pheromone lures (Light et al. 1993). The current state of research into creating lures based on kairomones to control female moths is summed up by Svatoš et al. (2009); "The prospect of using host plant attractants to manipulate females is far from being implemented".

Preference-performance link

Experiments in this thesis tested the "Mother knows best" (Gripenberg et al. 2010), "preference-performance" (Jaenike 1978) or "optimal oviposition" hypothesis (Thompson 1988) (Plate 6-1 b). Experiments tested host preference and acceptance at multiple scales. Had oviposition preference been studied in isolation from host location preference the preference-performance link would have been a clear negative relationship. In only two experiments did female *C. fraxinella* prefer green ash over black ash: a seedling oviposition experiment and a wind tunnel bioassay, and both of these experiments incorporate flight behaviour. There is evidence from these experiments that the preference-performance link may be positive if moths are permitted to fly as part of the tested host preference behaviour. However, the number of female *C. fraxinella* captured in tree canopies at leaf flush was greater in black ash than green ash at the same sites which suggests that black ash is also preferred under the most natural conditions tested. The preference-performance hypothesis is not supported as black ash is the preferred host for oviposition despite it being an inferior host for *C. fraxinella* larvae in comparison to green ash. Larvae reared on black ash had lower survival and slower development than those reared on green ash in both field and laboratory experiments. The similar densities of cone-rolled leaflets on black and green ash (Chapter 5) is likely a function of the greater mortality of *C. fraxinella* larvae on black ash. These results also do not support the predictions of the enemy-free space hypothesis (Jeffries and Lawton 1984), in which an inferior host plant for larval development is preferred for oviposition as a refuge to avoid natural enemies. Parasitism of *C. fraxinella* by *A. polychrosidis* is consistently higher on black ash than green ash (Chapter 5).

Parasitoid complex

The parasitoid complex associated with *C. fraxinella* has changed since it was first described (Pohl et al. 2004). All of the parasitoids of *C. fraxinella* in its expanded range are generalists and probably shifted their host use from other lepidopteran hosts when *C. fraxinella* became a plentiful resource. The ichneumonid, *Diadegma* sp., recovered in the current study, was not originally documented in the initial parasitoid complex of *C. fraxinella* in Edmonton (Pohl et al. 2004). Although this parasitoid could not be identified to species, it successfully oviposited and developed in another lepidopteran host from a different family (Plutellidae) (unpublished) and therefore must be a generalist parasitoid. *Apanteles polychrosidis* remains the dominant parasitoid in the complex of species using *C. fraxinella* in its expanded range (Plate 6-1 c). The percentage of parasitism of *C. fraxinella* by *A. polychrosidis* has increased since 2002 (Pohl et al. 2004), and it appears to be the only parasitoid with the capacity to control populations of *C. fraxinella* (Chapter 4). *Apanteles polychrosidis* has only previously used hosts that are exposed on the leaf surface (Cossentine et al. 2004) and did not modify its host use behaviour to utilize *C. fraxinella* but was most commonly found in ash trees when *C. fraxinella* larvae were exposed during their transition from leaf mining to leaf rolling. This finding helps to explain how this generalist parasitoid so successfully exploited *C. fraxinella*. *Diaegma* sp. adults were also most commonly found associated with exposed *C. fraxinella* larvae but capture of chalcid and pteromalid parasitoids when *C. fraxinella* were present as leafmining instars suggests that these ectoparasitoids in this complex can use leafmining hosts, in addition to hosts concealed in leaflet rolls (Chapter 4). Parasitoid complexes that contain braconids, chalcids (Pottinger and LeRoux 1971; Maier 1982; Girardoz et al. 2006) and ichneumonids (Sugiura 2011) occur on other gracillariid leafminers and the similarity may be attributed to these parasitoids foraging for hosts in similar ecological niches (Pottinger and LeRoux 1971). Invasive leaf miners such as *C. fraxinella* are often colonized by generalist native parasitoids (Godfray 1995; Urbaneja et al. 2002; Amalin et al. 2002, Grabenweger et al.2004) and in some cases, like with *A. polychrosidis*, one parasitoid exerts substantial parasitism pressure (Langor et al. 2002).

VOCs in Apanteles polychrosidis host location

Thirteen plant derived VOCs from green ash elicit antennal activity in *A. polychrosidis* (Chapter 5) in contrast to the six VOCs that are electrophysiologically active in its host *C. fraxinella* (Chapter 3). All six VOCs active on *C. fraxinella* antennae also elicit electrophysiological activity on *A. polychrosidis* antennae. The larger number of VOCs that *A. polychrosidis* perceives, especially the three GLVs, may allow for plasticity in VOC-mediated host location to hosts on various unrelated plants which is more typical of a generalist parasitoid (Ngumbi et al. 2010; McCormick 2012). *Apanteles polychrosidis* was not attracted to the black ash VOCs that attracted *C. fraxinella* in the wind tunnel (Fig. A-2).

Host density and parasitism

Although female *C. fraxinella* consistently lay more eggs on black ash than green ash (Chapter 2), black ash elicits less host location behaviour from female *C. fraxinella* in wind tunnel bioassays than green ash (Chapters 2, 3). The performance of larval *C. fraxinella* on black ash is poor as fewer larvae survive on black ash in experiments compared to green ash and those that do are subject to higher parasitism by *A. polychrosidis.* However, the density of *C. fraxinella*, measured as the number of conerolled leaflets per tree, on black ash and green ash are similar (Chapter 5). The findings of the current study suggest that this similarity in density at the coneroller stage on the two ash hosts could be due to more *C. fraxinella* females ovipositing on black ash (Chapter 2) resulting in a greater number of eggs laid on black than green ash that compensates for the lower survival of larvae on black ash. In the field larval performance experiment (Chapter 3) more eggs were laid on black ash leaflets than green ash.

When *C. fraxinella* invaded the urban forests of Western Canada and became established, *A. polychrosidis,* which is widespread in the Nearctic region (Fernández-Triana and Huber 2010), probably switched hosts to exploit the new resource. There is no evidence that *A. polychrosidis* followed or came with *C. fraxinella* from Eastern Canada but this possibility cannot be discounted completely with *A. polychrosidis* common in Eastern Canada and widespread in the Neartic realm (Fernández-Triana and Huber 2010). Several VOCs that are commonly induced by herbivory, such as methyl salicylate, α-farnesene and *E* 4,8 dimethyl 1,3,7 nonatriene (Dudareva et al. 2006), are perceived by the antennae of *A. polychrosidis.* These three VOCs are also induced by mechanical damage to green ash and increase between 8 h and 72 h after initial damage (Chapter 5).

Apanteles polychrosidis is well established on *C. fraxinella* in Edmonton but differentially parasitizes its host based on the ash species that *C. fraxinella* is feeding on (Plate 6-1 d). More *C. fraxinella* are parasitized on black ash than green ash. In addition, the response of parasitism to host density is also different (Plate 6-1 d). On green ash in the field, parasitism by *A. polychrosidis* is negatively density dependent. An increase in *C. fraxinella* density does not increase parasitism, which suggests that on green ash, host location might be mediated by plant VOCs not induced by herbivory or that the parasitoid does not perceive host density through the VOC signal. This result is supported by olfactometer bioassays in which *A. polychrosidis* is attracted to VOCs of undamaged and mechanically damaged green ash and not leaflets mined by *C. fraxinella*. This finding suggests that constitutively available VOCs, and not HIPVs, mediate host location of *A. polychrosidis* to *C. fraxinella* on green ash. On black ash, the opposite situation occurs in which only leaf-mined leaflets are attractive to the parasitoid and this is reflected in the field by positive density dependence of parasitism on *C. fraxinella* density on black ash. This study contrasts with others that observed dependence of parasitism on host density in gracillariids (Miller 1973; Connor and Beck 1993; Connor and Cargain 1994; Legaspi et al. 2001; Sato et al. 2002) by providing evidence for a mechanism to explain the differential parasitism of hosts. In another tritrophic system, differential parasitism of a gracillariid on two host plants is explained by the presence or absence of glandular trichomes (Suguira 2011). In the *C. fraxinella* tritrophic system, differential attraction to plant VOCs mediates parasitism. This study contributes another tritrophic system to the sparse literature (Barbosa et al. 2001) on VOC-mediated differential parasitism on decidous trees.

Future directions

Caloptilia fraxinella

The *Fraxinus*/*C. fraxinella*/*A. polychrosidis* tritrophic system still exists in the urban forests of Western prairie cities as *A. polychrosidis* has not eradicated *C. fraxinella*. Future avenues of research include the optimization of the blend of VOCs to attract gravid *C. fraxinella* females. To understand which VOCs are essential for host location, VOCs could be serially removed from the full VOC-blend or individually added to the main constituent of the blend (Cha et al. 2008). Using this method, single VOCs might be identified that are repellent, or that are particularly attractive. Once an attractive VOC blend is identified, field trials with a high VOC signal to compete with background VOC "noise" from other plants should be conducted. This attractive VOC blend can then be blended with an insecticide to create an attracticide (Evenden et al. 2008) that can be tested for efficacy against gravid female *C. fraxinella* in the time period between mating and leaf flush on ash trees. Individual, commercially available, synthetic VOCs released from an analogue of known ash bark VOCs (Fig A-3, Table A-1) and green leaf volatiles from ash leaves (Fig A-4, Table A-2) have been field tested with no successful attraction of *C. fraxinella*.

The second generation of *C. fraxinella* on new ash foliage in the summer could be a fruitful avenue of research. Until this study it was thought that *C. fraxinella* was univoltine with an obligatory reproductive diapause (Evenden et al. 2007). A small second generation occurs in early July on new foliage of water suckers at the base of stressed and dying ash trees. Wind tunnel experiments with newly eclosed moths in summer suggest that up to 80% of virgin females will call for mates when exposed to VOCs from green ash trees (unpublished). Although it is not known if newly eclosed calling females release a similar titre of pheromone as the females active after overwintering in the spring, mating pairs of newly emerged adults do occur in the field (personal observation) and laboratory (Dr. Joelle Lemmen, University of Alberta, personal communication). The factors that keep this subpopulation from entering reproductive diapause should be investigated.

Apanteles polychrosidis

The current work has not identified VOCs that mediate attraction of *A. polychrosidis* to *C. fraxinella.* If the compounds that elicit behaviour could be identified, attraction of *A. polychrosidis* could be manipulated to enhance parasitism of *C. fraxinella* (James 2003a; James 2003b; James 2005; James and Grasswitz 2005; Turlings and Ton 2006). Deployable synthetic versions of these VOCs might recruit parasitoids to an area, retain the parasitoids already present by suppression of their patch-leaving tendency or maintain host searching to increase the number of hosts used in an area (Gross et al. 1975). However, these same cues may also attract ash herbivores and hyperparasitoids of *A. polychrosidis.* The community-level impacts of release of VOCs should be investigated (Kaplan 2012; Xiao et al. 2012).

The technique to estimate host density and parasitism on trees used in this study can be further employed to explore landscape effects on the density of *C. fraxinella* and parasitism by *A. polychrosidis*. Landscape effects such as proximity to known overwintering sites may drive the density of *C. fraxinella* and *A. polychrosidis* on ash trees. There are likely factors besides the differential response to VOCs discovered in this study that impact the parasitism response of *A. polychrosidis* on *C. fraxinella.* As the current study revealed only low R^2 values for the regressions of *A. polychrosidis* parasitism on *C. fraxinella* density, there may be additional elements that were not collected that could further explain the parasitism response to leaf miner density (Chapter 5). The overwintering hosts of *A. polychrosidis* in Edmonton are not currently known and may be a key factor in determining wasp population density in the spring. Wasps that emerge from *C. fraxinella* in the summer mate immediately after eclosion from pupae and readily oviposit into obliquebanded leafroller larvae which are known to serve as overwintering hosts in other parts of the range of *A. polychrosidis* (Cossentine et al. 2004). Most micrograstrine braconids overwinter within larvae (Matthews 1974; Shaw and Huddleston 1991) and if the overwintering host of *A. polychrosidis* is identified, the population dynamics of the overwintering host could be linked to the amount of *A. polychrosidis* parasitism on *C. fraxinella*.

The induction of VOCs by herbivore and mechanical damage on both black and green ash needs to be further elucidated. There is an interesting trend that suggests that on green ash, *C. fraxinella* could be capable of suppression of HIPVs. The comparison with black ash could not occur in the current study because *C. fraxinella* failed to establish leaf mines on experimental black ash seedlings. If leaf mining could be established on black ash seedlings, or head space volatiles collected from trees in the field, the comparison of black ash and green ash VOCs could support the results of the field experiment of the effect of host density on parasitism (Chapter 5).

An interesting experiment considers the poor performance of *C. fraxinella* larvae on black ash (Chapter 2) and their density as calculated through comparison of leaflet rolls to normal leaflets (Chapter 5). The density of *C. fraxinella* on black ash and green ash is not significantly different and the increased mortality of larvae on black ash must be compensated by increased oviposition. The increased oviposition observed in single leaflet and leaf bioassays and the greater number of *C. fraxinella* captured in black ash trees (Chapter 2) suggest that greater oviposition on black ash than green ash is likely but needs to be tested under field conditions by comparison of egg densities. The cause of the poor performance of *C. fraxinella* on black ash would also be an interesting research topic.

Ideally, a dual control system that manipulates parasitoids and attracts female *C. fraxinella* will be employed in urban municipalities to control the population levels of *C. fraxinella*, reduce its aesthetic damage to horticultural ash trees, increase the beauty of the urban forest, and fulfill the ultimate goal of population control of the ash leaf-coneroller. If the mechanism in black ash that confers resistance to *C. fraxinella* were found then it could be exploited to develop resistant ash trees.

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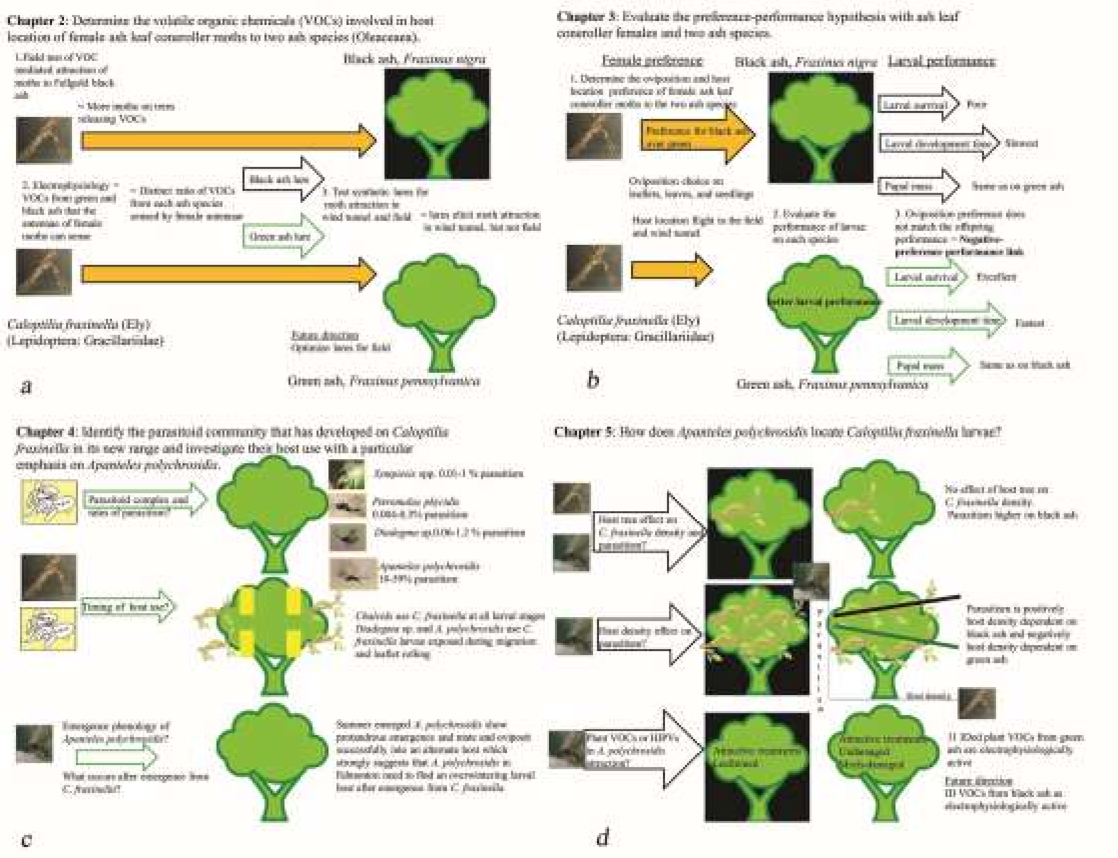
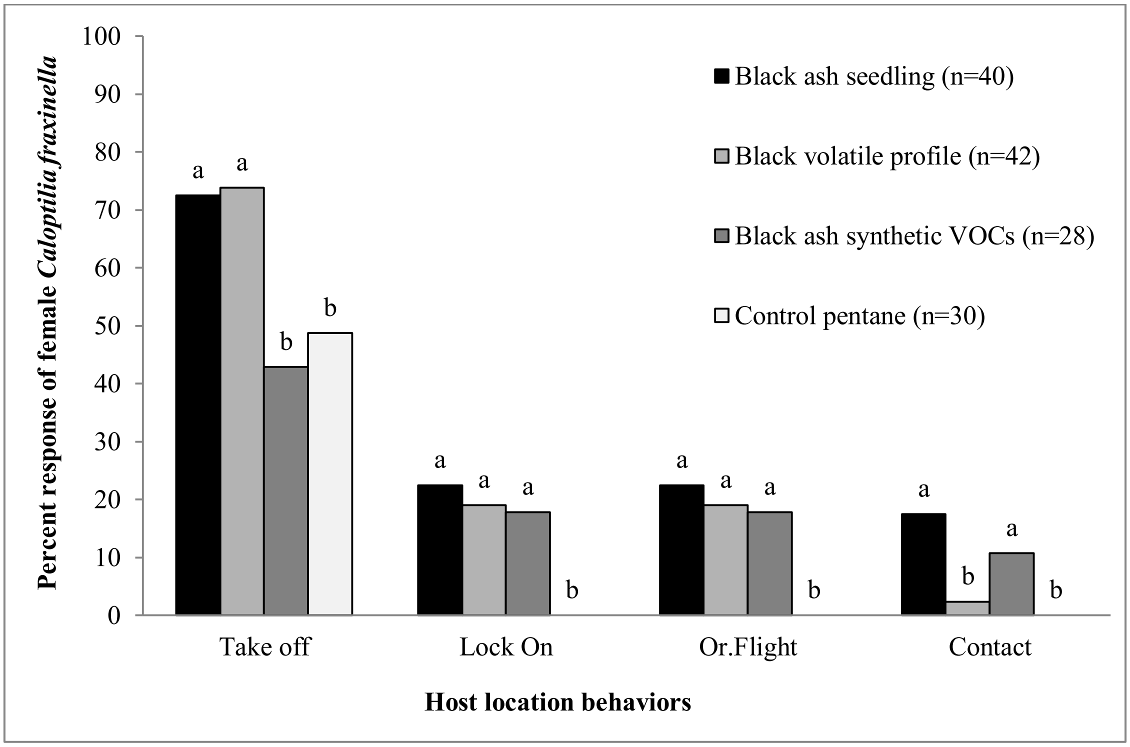
Plate 6-1. Conceptual review of thesis questions and results. a) Chapter two examined the volatile organic compounds (VOCs) involved in host location for oviposition by female *Caloptilia fraxinella*. b) In Chapter three the oviposition and host location preference of female *C. fraxinella* for black ash and green ash was determined. The performance of larvae on each ash species was examined and compared with the preference of female moths in a test of the preference–performance hypothesis. c) Chapter four identified the parasitoid complex, their rates of parasitism and the phenology of their use of *Caloptilia fraxinella* larvae with *Apanteles polychrosidis* as the dominant parasitoid. d) In chapter five the relationship of the parasitism rate of *A. polychrosidis* to the density of *C. fraxinella* wass explored on black and green ash and the VOC cues used in host location were investigated.



Appendix

Figure A-1. Response of spring field collected, mated female *Caloptilia fraxinella* to black ash seedlings, the volatile profile of black ash in solvent, a synthetic blend of five electrophysiologically active volatile organic compounds in solvent, and a blank control (2010). Statistically significant differences among treatments are represented by different letters (Fisher's Exact Tests with 4x4 contingency tables among treatments for each behaviour and 2x2 contingency tables between treatments).

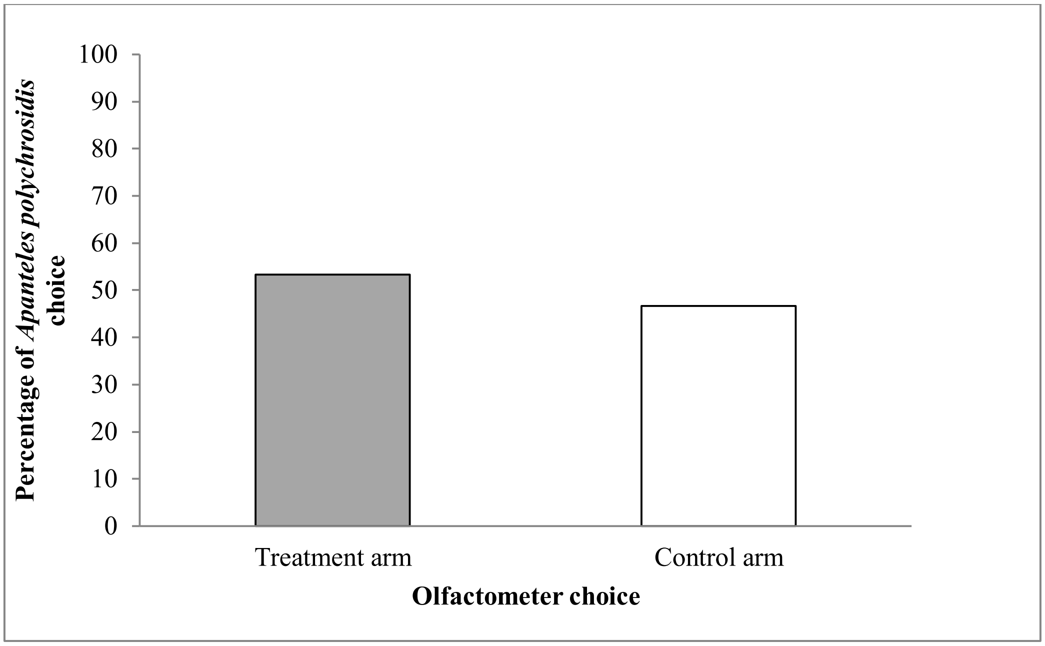


Black ash: volatile host location in a wind tunnel

The incidence of take-off behavior was significantly different among groups (*P*=0.0157). Take off was not different between the black ash seedling and the whole volatile profile (*P*=1) the seedling and the control (*P*=0.0799) or between the synthetic VOCs and the control (*P*=1). There was more take-off behavior to the seedling than to the synthetic VOCs (*P*=0.0228) and the control (*P*=0.00787), and more take off to the whole volatile profile than the synthetic VOCs (*P*=0.0126) and blank control (*P*=0.0486). The host location behaviours of lock on and oriented flight (Fig. A1) by female *C. fraxinella* to the volatile profile of black ash and the synthetic VOCs were not statistically different from the moth's response to black ash seedlings (*P*=0.765). Incidence of contact was less when a plastic seedling was paired with the black whole volatile profile than with the synthetic black mix and the black ash seedling (*P*=0.017) (Fig. A1). Contact was not significantly different between black ash and the black synthetic treatment (*P*=0.51). There was no lock on, oriented flight or contact with lures or plastic seedlings in control treatments (Fig. A1).

Response of Apanteles polychrosidis in a Y- tube olfactometer to a blend of five synthetic VOCs

Figure A-2. Response of *Apanteles polychrosidis* (n=15 responders, 5 NR) in a Y- tube olfactometer to a blend of five synthetic VOCs in pentane that mimics the identity and ratio of VOCs from black ash perceived by antennae of *Caloptilia fraxinella* females. There is no difference in choices between olfactometer arms (*P*=1, Fisher's Exact test).



Experiments with single ash leaf VOCs and bark mimicking VOCs

Abstract

Individual, commercially available, synthetic VOCs released from ash leaves and an analogue of known ash bark VOCs were field tested with no successful attraction of *C. fraxinella*.

Introduction

Essential oil steam distilled from the manuka or "tea tree" tree, *Leptospermum scoparium* (Myrtaceae), was purified into an insect lure called Malure™ (Merchant Ag/Response Inc.-Ohio, Illinois) after Manuka oil was found to be attractive to the emerald ash borer, *A. plannipennis* (Crook et al. 2008). Manuka oil shares four sesquiterpenes in common with *Fraxinus* species (α-cubebene-, α-copaene, *trans*-β- caryophyllene-, α-humulene) (Crook et al. 2008) and thus is also considered a mimic of the bark volatiles of an ash tree. Four individually presented ash green leaf volatiles and manuka oil, an ash bark analogue, are tested in field experiments.

Materials and methods

Experiments A3 and A4 tested commercially available compounds as potential attractants for female *C. fraxinella* in field trapping experiments. Experiment A3 tested a manuka oil product, called Malure ^{TM} (Merchant Ag/Response Inc., Ohio, Illinois), which shares four sesquiterpenes common to ash bark (Crook et al. 2008). Three volumes of Malure ^{TM} (0.4 ml, 2 ml and 4 ml) in polypropylene vials and a blank control (empty vial) were tested at eight sites with historically high coneroller populations. Treatments were hung from the centre of sticky panel traps (45 cm x 67 cm) (Contech Enterprises Inc., Delta, BC) tied on the south side of large, boulevard green ash trees approximately 4.5 m from the base of the tree beneath the canopy. Panel traps were positioned in the field on 22 May, 2008 at leaf flush to target overwintered moths as they orient to ash trees for oviposition. Traps were checked weekly and all *C. fraxinella* moths were counted. After approximately one month (20 June 2008), traps and lures were replaced and the moths caught during the first trapping period were separated by sex. Traps remained in the field over the emergence period of the next generation of *C. fraxinella* to target moths eclosing from ash trees until mid-August. Trap capture was counted weekly except for a two-week period between *C. fraxinella* generations. Generalized linear mixed effect models (GLMM) were used to compare the number of trapped female and male *C. fraxinella* per treatment during the two trapping periods with site specified as a random factor followed by *post hoc* Tukey's HSD to identify differences among treatments (R 2.13, R Development core team 2012, package Multcomp) using the Poisson family of errors and the lme4 package (Bates et al. 2011).

Experiment A4 tested individual ash green leaf VOCs alone and together (Rodriguez-Saona *et al.* 2006; de Groot *et al*. 2008) for attraction to *C. fraxinella* before and after bud flush. Six wing traps (Contech Enterprises Inc., Delta, BC) with removable inserts baited with each treatment were hung in the mid-canopy of green ash trees at ten sites with historically high coneroller populations approximately four weeks (5 May 2008- 2 June 2008) before expected bud flush in late-May and for four weeks after bud flush (2 June 2008- 30 July 2008). Traps were baited with one Bubble cap (Contech Enterprises Inc., Delta, BC) of the four most abundant green leaf VOCs from Manchurian and green ash (Rodriguez-Saona *et al.* 2006, de Groot et al. 2008); hexanal, (*E*)-2-hexenal (13.0 mg/day), (*Z*)-3-hexenol, (*E*)-2-hexenol (3.8mg/day), bubble caps containing all four VOCs, and a blank control with no VOCs. Bubble caps were replaced once at leaf flush after four weeks in the field. Sticky inserts were changed once per week and moths were enumerated and separated by sex. A generalized linear mixed effect model (GLMM) analyzed the number of moths caught per treatment with sex of moths and VOC treatment as independent factors in the model (R 2.13, R Development core team 2012). This analysis was followed by two GLMMs to compare 1) the number of trapped female and 2) male *C. fraxinella* per treatment. GLMMs had the factors "site" and "week" specified as random factors and the Poisson family of errors with a log link was used with the lme4 package (Bates et al. 2011). All GLMMs were followed by *post hoc* Tukey's

HSD to identify differences among treatments (R 2.13, R Development core team 2012, package Multcomp)

Results

In experiment A3, more *C. fraxinella* moths were captured on panel traps during the spring oviposition period than after eclosion of the summer generation (Fig. A3, Table A1). There was a significant effect of Malure™ treatment [m1] on the number of female and male moths trapped that appeared to show a level of repellency of Malure™ to *C. fraxinella*. Traps baited with the highest dose of Malure™ (4 ml) captured fewer female moths than traps baited with the lowest (0.4 ml) Malure™ dose and the unbaited control traps. The number of females captured on traps baited with the intermediate 2 ml dose was also lower than that in the unbaited control and 0.4 ml lure baited traps (Fig. A3, Table A1). The pattern was similar for male *C. fraxinella* as more males were captured on unbaited control traps than on traps baited with the high 4 ml dose. The significant treatment*sample date interaction obscures the differences among treatments in the spring trapping period (Table A1). The pattern of repellency was similar when all of the captured moths (Total number) which included males, females and moth that could not be separated by sex because their terminal abdominal segment was obscured by the glue on the panel traps. Fewer moths were captured at the high 4 ml dose than all other doses while the intermediate dose caught fewer moths than the control but the same amount as the low 0.4 ml dose (Fig. A3, Table A1). The treatment*sample date interaction was significant however so the relationship among treatments was not consistent across both sample periods.

In experiment A4, VOC treatment had a significant effect on the number of moths captured with more males caught on traps than females (Fig. A4, "Sex", Table A2). A significant sex* treatment interaction (Table A2) indicated that treatments were differentially attractive to male and female moths (Fig. A4). The unbaited control traps attracted more males than other treatments and there was no difference in the number of females trapped among treatments (Fig. A4).

Discussion

Two field experiments localize the source of the VOC signal to leaves and one further explores if single, GLVs from the leaves mediate host location of *C. fraxinella* females to ash trees. Commercially available, synthetic green leaf VOCs identified from the leaves of Manchurian and green ash (Rodriguez-Saona et al. 2006; de Groot et al. 2008) and an ash bark analogue, Malure™ (Crook et al. 2008) did not attract significantly higher numbers of *C. fraxinella* than the unbaited control. These four GLVs elicit antennal activity in the emerald ash borer (Rodriguez-Saona et al. 2006), but when presented singly or as a blend, none of these attracted female *C. fraxinella*. The sesquiterpenes in Malure™; α-cubebene, α-copaene, *trans*-carophyllene and α-humulene (Crook et al. 2008), simulate ash bark, but do not attract *C. fraxinella.* These VOCs were tested prior to the GC-EAD experiment where VOCs electrophysiologically-active to *C. fraxinella* were identified from green and black ash. None of these field-tested VOCs were electrophysiologically active in female *C. fraxinella* (Chapter 2).

The highest doses of Malure™ (2ml and 4ml), however, were repellent to male and female *C. fraxinella* with the highest catches of *C. fraxinella* on traps baited with the lowest Malure™ dose (0.4 ml) and the control. Two unidentified sesquiterpenes in the VOC profile of black ash elicit a small antennal response in female *C. fraxinella* and these sesquiterpenes could serve to repel moths away from bark and towards leaves.

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Table A-1. ANOVA table of the main effects and interactions of the generalized linear mixed effects model of the total number of *Caloptilia fraxinella* and the number of males and females caught on panel traps baited with different concentrations of Malure™ in Edmonton (2008). The variable site is random. Degrees of freedom are shown in subscripted brackets.

		Total no. moths		No. males		No. females	
Effect	***Post hoc* contrasts**	**Test stat**	***P* value**	**Test stat**	***P* value**	**Test stat**	***P* value**
Treatments		$\chi^2_{(3)}=137.87$	**<0.001**	$\chi^2_{(3)}=75.1$	**<0.001**	$\chi^2_{(3)}=49.0$	**<0.001**
Sample date		$\chi^2_{(1)}=3119.41$	**<0.001**	$\chi^2_{(1)}=11773$	**<0.001**	$\chi^2_{(1)}=604.1$	**<0.001**
Treatment * Sample date		$\chi^2_{(3)}=26.36$	**<0.001**	$\chi^2_{(3)}=13.9$	**=0.003**	$\chi^2_{(3)}=6.3$	=0.884
	4 ml *vs.* 2 ml	$z_{(1)}=3.60$	**=0.002**	$z_{(1)}=2.18$	=0.126	$z_{(1)}=1.22$	=0.613
	4 ml *vs.* 0.4 ml	$z_{(1)}=3.10$	=0.010	$z_{(1)}=0.92$	=0.789	$z_{(1)}=4.3$	**<0.001**
	4 ml *vs.* control	$z_{(1)}=6.82$	**<0.001**	$z_{(1)}=3.8$	**<0.001**	$z_{(1)}=6.2$	**<0.001**
	2 ml *vs.* 0.4 ml	$z_{(1)}=0.53$	=0.952	$z_{(1)}=1.31$	=0.555	$z_{(1)}=3.1$	=0.01
	2 ml *vs.* control	$z_{(1)}=3.55$	=0.002	$z_{(1)}=1.83$	=0.255	$z_{(1)}=5.0$	**<0.001**
	0.4 ml *vs.* control	$z_{(1)}=4.06$	**<0.001**	$z_{(1)}=3.05$	**=0.012**	$z_{(1)}=1.93$	=0.215

Table A-2. ANOVA table of the main effects and interactions of the generalized linear mixed effects model of the total number of *Caloptilia fraxinella* and the number of moths caught on wing traps baited with four single green ash leaf VOCs and a combination of all four VOCs in Edmonton (2008). The variable site is random. Degrees of freedom are shown in subscripted brackets.

		No. moths	
Effect	***Post hoc* contrasts**	**Test stat**	***P* value**
Treatment		$\chi^2_{(5)}$=75.2	**<0.001**
Sex		$\chi^2_{(1)}$=155.9	**<0.001**
Treatment * Sex		$\chi^2_{(5)}$=22.0	**<0.001**

Fig. A-3. Mean ± S.E. number of *Caloptilia fraxinella* females, males and sex unknown on panel traps baited with three volumes of Malure™ (n=8 sites) caught in spring and summer 2008.

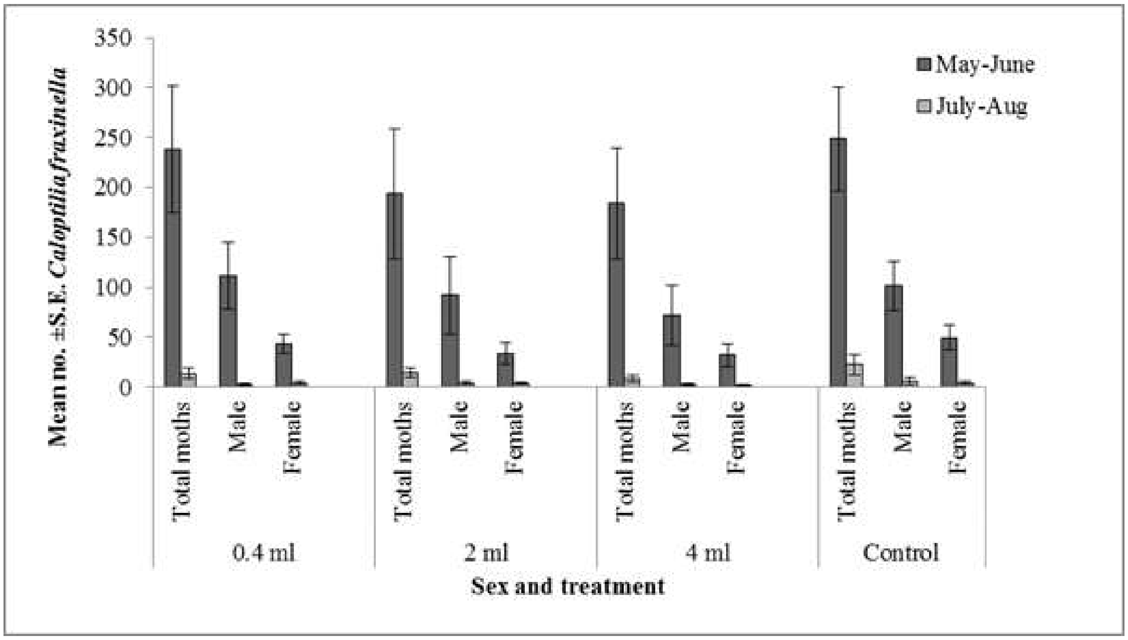

Fig. A-4. Mean ± S.E. number of female and male *Caloptilia fraxinella* moths caught in six bark volatile treatments from ten sites in Edmonton, Alberta, 2008. Statistically significant differences among treatments are represented by different letters (Uppercase: females, lower case: males).