CHEMICAL ECOLOGY OF BLUEBERRY SPANWORM, *ITAME ARGILLACEARIA* (PACKARD) (LEPIDOPTERA: GEOMETRIDAE)

by

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To her, who's persevered sense of humour, continued support, encouragement and patience through this whole process even through times of great difficulty.

I dedicate this dissertation to, sweetest and toughest lady I have ever had, my wife, Nilu Wijesingha. Thank you for all your love and laughs.

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Abstract

Blueberry spanworm, *Itame argillacearia* (Packard), is an important defoliator of wild (syn. 'lowbush') blueberry in north-eastern North America. Identification of *I. argillacearia* sex pheromone(s) could be useful for monitoring, mating disruption or mass trapping, as a way to improve integrated pest management programs for this pest. Thus, the main goal of this study was to identify sex pheromone(s) of *I. argillacearia.* The courtship, mating and oviposition patterns of *I. argillaceara,* was studied in the laboratory. GC/MS, GC/EAD and EAG analysis of pheromone gland extracts, in combination with y-tube experiments and two field trapping studies confirmed the chiral-alkenyl-epoxide, (3R,4S)-epoxy-(Z,Z)-6,9-17:H and tri-ene, (Z,Z,Z)-3,6,9-17:H as the major female-produced sex pheromone components. Contech Wing traps® placed at canopy level were effective for capturing male *I. argillacearia* moths.

List of Abbreviations Used

cm:	centimeter (1 cm = 1 x 10^{-2} m)
C.I.:	confidence interval
DAT:	days after treatment
df:	degrees of freedom
et al:	and others
F:	F-test statistic
g:	gram
h:	hour
ha:	hectare (1 ha = 10,000m^2)
Inc.:	incorporated
IPM:	integrated pest management
kg:	kilogram (1 kg = 1 x 10^3 g)
km:	kilometer (1 km = 1 x 10^3 m)
kpa:	kilopascals
L:	liter
L:D:	light: dark
m:	meter
mg:	milligram (1mg = 1 x 10^{-3} g)
mm:	millimeter (1 mm = 1 x 10^{-3} m)
mL:	milliliter (1 mL = 1 x 10^{-3} L)
n:	sample size
P:	P – value
ppm:	parts per million (1 ppm = 1 mg L^{-1})
R^2:	regression coefficient of determination
RH:	relative humidity
SEM:	standard error of the mean
α:	level of significance
^oC:	degree Celsius
™:	trademarked
®:	registered
µg:	micrograms (1 µg = 1 x 10^{-6} g)
χ^2:	chi-square

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Chapter One: Introduction

1.1. Wild Blueberry Industry

Wild blueberry(syn. "lowbush blueberry"), *Vaccinium angustifolium* Aiton (Ericaceae), is an important horticultural crop that is native to eastern and north-central North America (Prior et al. 1998, Yarborough 1998). Six species in the genus *Vaccinium* constitute what is referred to as wild blueberry: *V. angustifolium* Ait., *V. myrtilloides* Michx., *V. boreale* Hall and Alders, *V. darrowii* Camp., *V. tenellum* Ait., and *V. pallidum* Ait.) (Vorsa 1997), with *V. angustifolium* being the most abundant species in Maine and Canada (Smagula et al. 1997).

Vaccinium angustifolium is different from most crops because growers do not plant the crop, but instead manage wild stands that spread naturally by clonal propagation through a network of underground rhizomes (Barker and Collins 1963, Hall et al. 1972, Kinsman 1986). It is a small shrub that is often one of the first plants to colonize freshly disturbed areas (Kinsman 1986). Since managed wild blueberry fields are composed of many distinct clones of plants, fields are often not uniform in appearance (Hall et al. 1979, Collins et al. 1995). Each clone has genetically different attributes, including leaf and flower color, sprout emergence, bloom time, and resistance to pests (Collins et al. 1996, Barry et al. 2003). While the low sweet wild blueberry, *V. angustifolium* Ait., makes up approximately 80-95% of most fields, the remainder are sour-top or velvet leaf (*V. myrtilloides* Michx.) (Hall et al. 1979, Vander Kloet 1981). The plants grow wild in areas of sandy, gravely, well-drained soils with acidity levels of 4.2-5.0 that are generally unsuitable for other types of agriculture.

Wild blueberries are usually harvested on a two year cycle with the first year being a vegetative “sprout” phase and the second year being a fruiting “crop” phase (Yarborough and Collins 1997). Agricultural practices including pruning, fertilizing, eliminating competing vegetation, and controlling pests and diseases, can dramatically increase production. Pruning is accomplished by mowing or burning fields in the spring or fall every other year. Pruning with fire offers many advantages that mowing does not (Yarborough 1998), including reduced incidence of insect, diseases, and weeds (Kinsman 1986). However, many growers currently prune by mowing to decrease environmental pollution and greenhouse gas emissions, because of challenges in obtaining burn permits, and rising fuel costs. Pruning by mowing has environmental and economic benefits, but has resulted in increased pest outbreaks in some regions (Crozier 1995). Herbicides, fungicides, and insecticides are commonly used to suppress vegetation and weeds, diseases and insect pests, respectively (Vander Kloet 1981).

Although it is small in size, the wild blueberry industry is a big part of Canada’s horticultural commodity. Canada is the second-largest producer and exporter of wild blueberries, after the United States. Blueberry crops cover almost half of land areas in fruit and nut production in Canada (Robichaud 2006, Statistics Canada 2012). Over the past five decades, improved management coupled with increased trade, globalization and awareness of the healthful properties of blueberries has resulted in continual economic and agricultural expansion for the wild blueberry industry. Together with highbush blueberries, which are predominately produced in British Columbia and

Ontario, Canadian blueberry production in 2011 exceeded 123,860 metric tons covered 69,974 ha of land, generated a farm gate value of $203 million (Statistics Canada 2012).

The growth in the blueberry industry may be due partly to an increased demand for anti-oxidant rich fruit by a health-conscious public (Smith et al. 2000). They are also low in calories, high in fibre and nutrients, and may contribute to overall health since they appear to have anti-inflammatory activity and may reduce neurodegenerative disease and blood cholesterol levels (Zheng and Shiow 2003, Willis et al. 2005).

1.1.1. Insect Pests of Wild Blueberry

Wild blueberry producers usually contend with insect pest pressures using insecticide applications (Ramanaidu et al. 2011). Insect pests limit fruit yields and quality of blueberries in Canada (Yarborough 1998). In wild blueberry fields, several insect species have been identified which cause damage in blueberry fields and reduce yields through destruction of the fruit buds, berries, and leaf tissue. These insect pests include blueberry leaftier (*Croesia curvalana* (Kearfott)), blueberry spanworm (*Itame argillacearia* (Packard)), chain spotted geometer (*Cingilia catenaria* (Drury)), blueberry flea beetle (*Altica sylvia* (Malloch)), and blueberry fruit fly (*Rhagoletis mendax* (Curran)). Blueberry spanworm is the primary defoliator in many parts of Nova Scotia (Yarborough and Collins 1997).

1.2. *Itame argillacearia* (Blueberry Spanworm)

1.2.1. Biology

Itame argillacearia ranges from Quebec to Nova Scotia, and in the United States from Maine to West Virginia (Wagner et al. 2001). *Itame argillacearia* also feeds on highbush blueberries and cranberries (Turner and Liburd 2007).There have been a number of documented outbreaks of this insect pest, the largest of which occurred in Maine in 1981 (Forsythe and Flinders 1982).

The insect overwinters as an egg in leaf litter around the base of the plant. Egg (Figure 1) hatch usually occurs in late May. Early instar larvae feed on developing flower buds, blossoms and/or emerging shoots, while later instars will feed upon foliage. Larvae (Figure 1) tend to be most active in the upper canopy foliage at night, moving to the lower parts of the plant canopy or leaf litter during the day (Drummond and Groden 2000, Booth et al. 2007). Larvae develop through four instars, ranging from 3-20 mm long. First instar larvae are tan or grey with black spots. Mature larvae are yellow-orange with a series of black spots along the body (Crozier 1995).

Figure 1. Life stages of *Itame argillaceria*

Itame argillacearia has one generation per year and *I. argillacearia* larvae usually attack both pruned vegetative and harvestable fields. If an *I. argillacearia* outbreak is severe, 'burning like' localized patches can be observed throughout the fields. Blueberry

plants can be completely defoliated during severe outbreaks (Collins et al. 1995, Crozier 1995). Larval feeding continues until late June or early July at which time they usually migrate into the soil to pupate (Figure 1). Approximately two weeks later in late July adult moths (Figure 1) emerge (Crozier 1995).

Female *I. argillacearia* moths emit a sex pheromone(s) that is attractive to male moths, 2-3 h after commencement of photophase, for their reproductive success (Alford and Diehl 1985, Boo et al. 1998). Adults mate in late July to early August and mated females lay eggs on leaves or on the ground which do not hatch until the following year spring (Crozier 1995, Drummond and Groden 2000). Adult *I. argillacearia* moths have grey-brown wings (Figure 1). Females have dark spots on the wings, whereas the males are mostly uniform in color. The wingspan is about 23-29 mm (Crozier 1995).

1.2.2. Pest Status and Management of *Itame argillacearia* in Wild Blueberry

Larvae of *I. argillacearia* can be a key pest of the wild blueberry agro-ecosystems (Crozier 1995). In Nova Scotia, the recommended action threshold for *I. argillacearia* larvae, in fruit bearing fields is 12 larvae per 25 sweeps, and 7 larvae per 25 sweeps in sprout fields (Crozier 1995, Howatt 2005), although the accuracy of these thresholds is unclear. Currently, most growers rely on chemical insecticides for *I. argillacearia* control (Ramanaidu et al. 2011). Chemical controls for *I. argillacearia* have included (past and/or present): Dylox 420L® (trichlorfon), Decis 5 EC® (deltamethrin), Sevin XLR Plus® (carbaryl) and Imidan 50WP® (phosmet), Delegate WG® (spinetoram), Success 480 SC® (spinosad), Assail 70 WP® (acetamiprid), Decis 5 EC® (deltamethrin), and Altacor®

(chlorantraniliprole). With the exception of Dylox 420L® (trichlorfon), all products remain registered for control of *I. argillacearia* on blueberries (Ramanaidu et al. 2011). Few other practical pest management tools have been developed to control this insect pest.

Itame argillacearia larvae are parasitized by a number of naturally occurring wasps, viruses and entomopathogenic fungi. These are present during the spring when larvae are present. Parasitic wasps of families Diapriidae, Chalcidoidea, and Cynipoideamay attack *I. argillacearia* larvae (Karem et al. 2006). Naturally occurring entomopathogens, such as *Beauveria bassiana* [Balsamo-Crivelli] Vuillemin and *Metarhizium anisoplia e*[Metschinkoff], also exist and can infect *I. argillacearia*. In addition, natural predators include the beetles *Harpalus rufipes* De Geer (Coleoptera: Carabidae), *Calosom acalidum* Fabricius (Coleoptera: Carabidae) and Opiliones (Arachnidia) may also be important predators (Drummond et al. 2008) and several species of tachinid parasitoids (Diptera) may attack *I. argillacearia* larvae(Drummond and Groden 2000). Unfortunately, these natural enemies are often not able to adequately regulate *I. argillacearia* when larval populations are high (Ramanaidu et al. 2011).

1.3. Insect Chemical Ecology

1.3.2. Insect Semiochemicals and Sex Pheromones

Insect “semiochemicals” (Gk. *semeon*, “a signal”) are volatile or non-volatile chemicals that mediate and regulate interactions between organisms (Law and Regnier 1971).

Semiochemicals are subdivided into allelochemicals and pheromones depending on whether the interactions are inter- or intraspecific. Allelochemicals (Wilson and Bossert 1963) are chemicals that are significant to individuals of a species different from the source species (Nordlund and Lewis 1976). Pheromones (Gk. *phereum*, "to carry"; *horman*, "to excite" or stimulate) are released by one member of a species to cause a specific reaction by another member of the same species (Butenandt et al. 1959). Pheromones may be further classified on the basis of the type of behavior affected, such as sex pheromones, aggregation, alarm, trail and social pheromones (Jutsum and Gordon 1989).

The semiochemical groups most widely explored are the sex pheromones, which are produced and released by one sex to attract or excite the opposite sex for mating. Moths' sex pheromones usually consist of either single or multi-component blends of unsaturated hydrocarbons and/or epoxides with enantiospecificity often found to be critical to bioactivity (Ando et al. 2004).

Chemists have been making rapid advances in the techniques of isolation, identification, and synthesis of these highly active and naturally occurring sex pheromones. Based on those natural pheromones from insects studied in the laboratory, synthetic pheromones analogues have now been produced. The majority of these synthetic sex pheromones analogues or attractants belong to the order Lepidoptera (Pedigo and Rice 2006).

1.3.2. Use of Insect Pheromones in Insect Pest Management

The most significant impact of the chemical ecology research in the applied sciences, has been in the field of insect pest management (Pedigo and Rice 2006). Since the first identification of a sex pheromone, that of the silkworm moth, *Bombyx mori* (Lepidoptera: Bombycidae) (Butenandt et al. 1959), sex pheromones and attractants of about 1,600 moth species have been chemically identified (El-Sayed 2008). A great deal of research has been undertaken on using synthetic pheromones in insect pest management programs. Pheromone lures, placed in insects traps, are used to monitor the presence and abundance of insect pest populations (El-Sayed 2008). Traps, or pheromone dispensers within crops, have also been used in a variety of ways to control many pest species.

Insect pest population monitoring and management using sex pheromone baited traps has long been a part of many insect pest control programs (Seabrook and Dyer 1983). Monitoring is important for the efficient use of conventional insecticides. The importance of insect monitoring is accentuated in view of increasing problems associated with the use of conventional insecticides (Seabrook and Dyer 1983, Drummond et al. 2008). A means of detecting and predicting insect pest outbreaks earlier would be very useful, and a trapping system involving sex pheromones may provide improved detection sensitivity and sampling efficiency. Currently, the primary uses of pheromones for insect monitoring in agricultural systems are to determine: emergence of the first insects of the season, timing of spring and summer sprays, pest

species density, where the pest species are coming from and population trends within a season and from year to year (Seabrook and Dyer 1983, Drummond et al. 2008).

Synthetic sex pheromone components released in the field individually or as mixtures can disrupt male orientation and mating, or be used in mass trapping and monitoring in several lepidopteran species (Critchley et al. 1985, Flint et al. 1988, Webb et al. 1988). How synthetic pheromones interrupt normal orientation is uncertain, but the most probable mechanisms invoke adaptation and habituation, competition between point sources of formulation and females, and a camouflage of a female's pheromones plums by the formulation (Underhill et al. 1987). Use of semiochemicals to detect and predict insect outbreaks can provide improved detection sensitivity and sampling efficiency (Seabrook and Dyer 1983, Flint et al. 1988), which often leads to better control and reduced use of insecticides (Ando et al. 2004). The most common strategy for control by the use of semiochemicals is to attract, trap, and kill the pest insects (Witzgall et al. 2010).

The only two pests of the blueberry industry for which monitoring traps have been used are the blueberry fruit fly and blueberry leaftier. Lonergan et al. (1989) were able to identify the blueberry leaftier sex pheromone blend. Ponder and Seabrook (1991) subsequently found that polyvinyl chloride lures releasing the pheromone could effectively capture male blueberry leaftier. Polavarapu and Seabrook (1992) showed that pheromone traps were not only useful for monitoring this pest, but also had potential in mass trapping. Virtually all growers use Pherocon® AM sticky boards to

monitor blueberry fruit fly populations. However, these sticky boards are loaded with an ammonium acetate as a bait enhancer and do not utilize fly pheromones.

1.4. Study Rationale

Alford and Diehl (1985), in one of the only peer-reviewed studies on *I. argillacearia*, showed that female moths emit attractive sex pheromones and suggested that a better understanding of sex pheromone(s) from this insect could assist growers through improved monitoring, mass trapping, or mating disruption. The government of Canada has identified control options for *I. argillacearia* as one of the key issues in wild blueberry production (Bussmann 2008). Currently, pest management and monitoring tools for blueberry spanworm are limited. Although, there are many successful examples of employing semiochemicals in other agricultural systems, however, it is surprising that studies targeting the identification and/or use of pheromones to manage insect pests in wild blueberries, one of the most prominent agricultural commodities in Canada, have received little attention.

The increasing uses of insecticides and concern over fruit and environment contamination have stimulated research into non-insecticidal insect control. Management of this insect pest still relies heavily on chemical insecticides (Ramanaidu et al. 2011), and sweep netting for larvae is the only monitoring technique currently available for this pest (Howatt 2005, Drummond et al. 2008). Although sweep netting is effective, because blueberry spanworm larvae are most active at night (Crozier 1995) estimates of larval densities may not be accurate. Furthermore, some growers used the

sweep nets ineffectively or are unable to correctly identify blueberry spanworm larvae. In addition, sporadic population fluctuations make spanworm infestations difficult to predict, and since action thresholds for this insect are low (Crozier 1995), population thresholds are often exceeded before they are detected. Detection is often more problematic in vegetative fields that are essentially bare ground in late spring. Such fields tend to be monitored less often by growers, often resulting in significant bud damage and reduced fruit yields in the following crop year. Hence, there is a place for alternative monitoring and management tools to minimize and optimize pesticide inputs.

Incorporation of species-specific sex pheromone lures into traps has provided an inexpensive and indispensable tool for disruption of male orientation and mating, and/or insect pest sampling and detection in agriculture and forestry, including many species of Lepidoptera (Carde and Minks 1995). Alford and Diehl (1985) previously suggested that pheromone traps could provide a useful tool to growers in population management for this pest, although the putative attractants have yet to be fully identified for this species. The primary goal of the present study was to identify the female-produced sex pheromone of *I. argillacearia*, which could potentially be used in traps for monitoring or mass-trapping. I also attempted to study the courtship, mating behavior and oviposition patterns of *I. argillacearia* under laboratory conditions.

1.4.1 Objectives and Hypotheses

The primary purposes of this thesis research was to study the attractiveness of male *I. argillacearia* to female moths and synthetic compounds, and to identify the molecular structure(s) of sex pheromone components produced and released by *I. argillacearia* females that result in male moth aggregation and/or mating. This entailed examination of male moth behavior in laboratory bioassays, field trapping experiments, and chemical analyses of female moth pheromone glands using GC/MS and GC/EAD recordings of male antennal response when exposed to the female pheromone and synthesis of identified pheromone components.

The objectives and hypotheses of this research were to:

I. Observe courtship, mating and oviposition behaviors *I. argillacearia* moths under laboratory conditions. I hypothesized that a *I. argillacearia* male would respond to a calling female by approaching her while fanning his wings and showing a sequence of mating events, and that females would lay eggs in no particular pattern.

II. Observe male behavioral response to calling female moths or their pheromone gland extracts in a Y-tube olfactometer. I hypothesized that *I. argillacearia* male moths would show more rapid attractiveness or flight simulation response to calling live female moths and to pheromone crude extracts or synthetic chemicals in a Y-tube olfactometer.

III. Identify the molecular structure(s) of sex pheromone components produced by *I. argillacearia* females that result in male moth antennal stimulation and attraction. It has been reported that Geometridae sex attractants or sex pheromones contain

mono- or di-unsaturated epoxides as components (Type 2 pheromones) (Millar et al. 1990, Millar 2000). Thus, I hypothesized that female sex pheromone glands contain male-attractive compounds that consist of single or multi-component blends of unsaturated hydrocarbons and/or epoxides with enantiospecificity.

IV. Determine the role of pheromone trap design and height in trapping *I. argillacearia* male moths. I hypothesized that a Delta® trap and trap placement just above the blueberry canopy would be most efficacious in trapping male moths.

Chapter Two: Courtship, Mating Behaviors and Oviposition Patterns of *Itame argillacearia* Under Laboratory Conditions

2.1. Introduction

In developing a pheromone-based monitoring system for insects pests, it is important to first understand the oviposition pattern of the pest (Silverstein 1981, Lingren et al. 1982, Krupke 1999). Although, there is basic value in knowing the mating and oviposition behavior (Quiring 1994), little attention has been devoted to learning in the context of courtship behavior in *I. argillacearia*. The calling behavior of female *I. argillacearia* was previously documented by Alford and Diehl (1985). However, the courtship behavior of male moths or sexual excitation for mating and the specific biological aspects of oviposition pattern by females have not been fully described.

Therefore, in this study, I attempted to examine the adult male mating behavior and female oviposition behavior, the possibility of multiple mating of females, the behaviors implicit in that mating, how many eggs females produced in her life time, the duration of egg laying and patterns of egg laying.

2.2. Materials and Methods

2.2.1. Insect Rearing

Late-instar *I. argillacearia* larvae were collected from blueberry fields in the late spring, intermittently over several days, using a 30 cm diameter sweep nets. Fields were located in Debert (45° 25′ 11.42″ N; 63° 30′ 26.55″ W) and Farmington (45° 33′ 25.5″ N; 63° 54′ 18.2″ W), Nova Scotia. Larvae were brought to the laboratory and rearing was

conducted in closed 1-L clear plastic containers containing fresh blueberry foliage that was replenished as needed. Insects were maintained in a growth chamber at 21 ± 3^0C, 65 ± 5% RH, under a light–dark regime of 16L:8D (8:00-24:00), until pupation occurred. All pupae were separated by sex (Butt and Cantu 1962) and placed in 1-L transparent plastic containers that were lined on the bottom with a filter paper. Moths later emerged and were maintained under the same environmental conditions as pupae. Two-day-old virgin adults were used in bioassays to provide uniformity for mating, except in experiments testing multiple mating intermittently over several days, where adults were older and were not virgin.

2.2.2. Courtship and Mating Observations

Adult courtship and mating behavior was observed by placing male and female moths in clear glass observation chambers (60 x 30 x 40 cm high; 21 ± 3°C and 60 ± 5% RH) covered with nylon mesh secured with a rubber band (Álvaro 2000, Klaus et al. 2007). One hour prior to the onset of the photophase, couples were transferred from rearing cages to an observation cage. Visual observations of sexual behavior were carried out beginning one hour before photophase and continued three hours into photophase. Release of sex pheromones ('calling') by female moths was determined by observation of ovipositor extrusion from the abdomen (Alford and Diehl 1985). After three hours observation, each individual were separately returned to the rearing cages for additional observations the next day, and observations were conducted for a total of four consecutive days. Courtship sequence, copulation behavior and the duration of

copulation were observed. Repeated observations were made on these patterns using several pairs of moths over a two-week period.

In addition to visual observations, video recording and photographs were taken of moth courtship and mating behavior sequences. Two manual video cameras were used to record behavioral sequences (Canon SX150, 14.1 megapixels, 12x optical zoom; and Sony Cyber-shot, DSC-W560, 14.1 megapixels, 4x optical zoom). Camera lenses were focused against the mating arenas and photography was done below, allowing detailed observations within 4 to 6 cm from the moths.

2.2.3. Oviposition Behavior

Two-day-old male and female virgin pairs were placed in a covered 1-L clear plastic containers. After copulation (approximately two hours long male was in the cages), the male was removed and the female was left in the container for the duration of their lifespan. One fresh blueberry stem was inserted into a floral pick containing water and placed close to an inner wall of each container. A 5% sucrose solution was provided *ad libitum* in a plastic tube (40 x 10 mm diameter) containing a 3.75 cm cotton wick. Observations were made upon ten virgin pairs (Greenberg et al. 2002). For all females, the daily numbers of the eggs laid and the location of oviposition was recorded over consecutive days during her life time (Tomas and Juhan 2000). The eggs were counted and destroyed from cages daily and were not track the eggs hatching. The blueberry stems and sucrose solution were replenished as needed.

For comparing the daily numbers of eggs laid, a repeated measures data analysis was done using the PROC mixed procedure in SAS 9.1.2 (SAS 2001) at α=0.05. Data with residuals not normally distributed were log ($\log_{10}$ x+1) transformed to satisfy data assumptions for analysis of variance. Back-transformed means and the difference of backtransformed upper and lower confidence intervals are presented.

2.3. Results and Discussion

2.3.1. Courtship and Mating Behavior

Itame argillacearia males exhibited a sequence of simple courting and mating behavioral responses which appeared to coordinate with the reproductive activities of females. Both sexes rested on the substrate with their wings flat and parallel to the substrate. When completely at rest, the female antennae projected backward, parallel to her body (Figure 2B). The behavioral sequence was initiated when asexually receptive female moths everted her sex pheromone gland in the posterior abdomen. This happens 1-5 minutes followed the onset of photophase. As previously documented by Alford and Diehl (1985), the female posture during calling occurred throughout the first 2-3 hours into photophase and was clearly different from the normal resting position (Figure 2B,C). Calling females elevated their abdomen off the surface, curved the abdominal tip downwards, and raised their wings. In most cases it was possible to observe the pheromone gland extruded from the tip of the abdomen (Figure2C).

Males showed no mate searching behavior before the initiation of photophase, with a resting female. The resting male, during the initial stage of photophase, would

occasionally fly off and keep its antennae motionless and not show any excitory behavior. Upon receiving the sex pheromone(s) signal, most males were stimulated and started to respond with characteristic behavioral sequences very similar to those previously reported for other Lepidoptera (Schneiderman et al. 1986, Klaus et al. 2007).

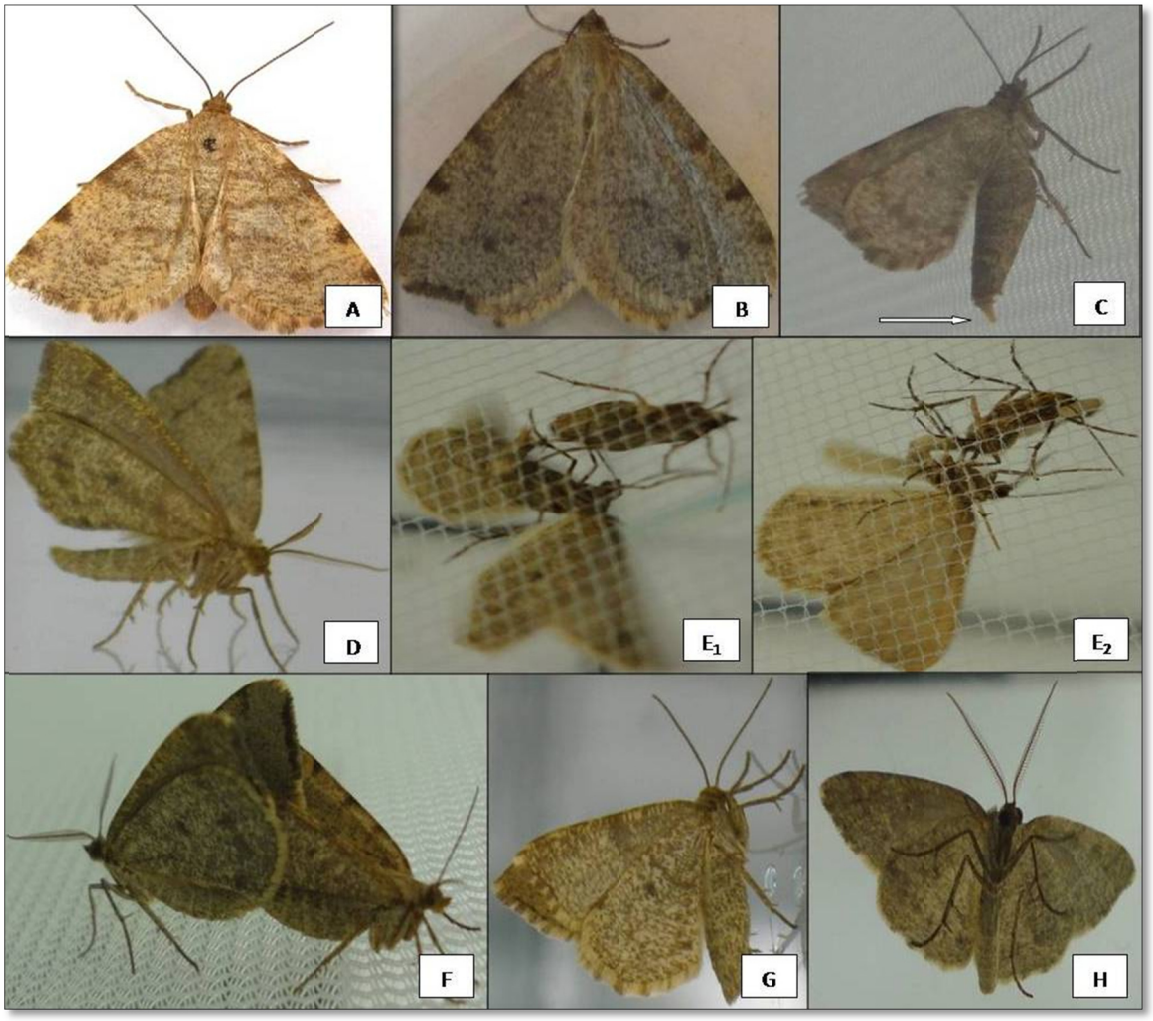

Figure 2. Behavioral sequence of events during successful courtship and mating of *I. argillaceria*: (A) resting male; (B) resting female; (C) female releasing sex-attractant/pheromone by everting her sex pheromone gland (arrow); (D) male first sexual excitement/activation; ($E_{1\text{-}2}$) approaching the calling female while rapidly fluttering wings and a copulation attempt; (F) successful copulation; (G) post-copulatory female; (H) post-copulatory male.

Stimulated males moved towards the female, exhibiting rapid wing fanning, up and down vertical movement of antennae and/or directed ambulation (Figure 2D). A few males flew rapidly, just below the mesh which covered the chamber, in a vagrant manner around the space of the chamber. Occasionally, males landed on the bottom, beating their wings swiftly a few times, and continued flying before a copulation posture was assumed. When by a female's side, a male would continue to rapidly flutter his wings while moving over or around the female in the upright position (Figure 2E). Sometimes males approached the female with his wings fluttering and thereafter immediately attempt to mate without walking over and around her.

Since females in the field are hidden in the blueberry canopy from potential mates, it would seem that olfactory (pheromones) cues would be most important for long-range attraction. Mating happens during early photophase (Alford and Diehl 1985), suggesting visual cues could be important to initiate copulation when males and females are in close proximity. Others have shown that the sequence of events of sexual excitation in Lepidoptera may depend on pheromone odor, eye contact between male and female (Tomislav et al. 2006).

After a male located the calling female, there was a pre-copulation courtship (Figure 2 $E_{1\text{-}2}$) in which the male assumed a position parallel to the female (Figure $2E_1$). He then moved his abdominal tip toward the distal end of the female abdomen (Figure $2E_1$), ending up approximately 90 degree to the left of the female during initiation copulation. The male then turned 180 degrees, always turning from the left side of the

female (figure. 3). The entire sequence from the initiation of copulation to the "resting copulation" stage at 180 degrees (Figure 3) each step lasted about two minutes.

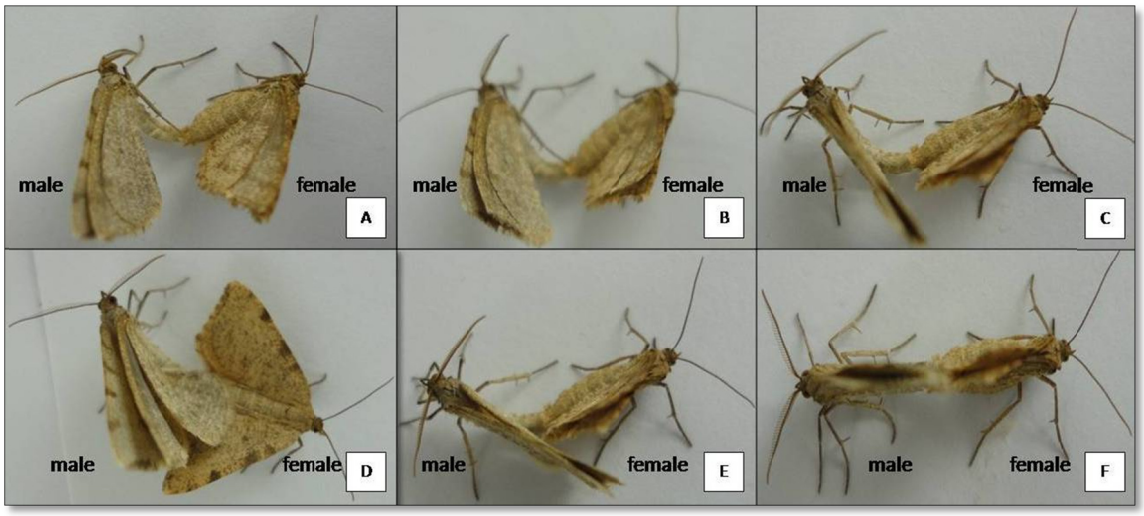

Figure 3. A copulation sequence in *I. argillaceria*: the male turns 180 degrees, always turning from the left side of the female (A-F).

Copulation took place early in the morning between 8 a.m. to 11 a.m. lasted between 60 and 90 minutes. The tail-to-tail copulation position (Figure 3F) is typical for most Lepidoptera. After copulation ceased, males had the tip of their abdomens tilted to the left. No couples mated twice during the observation period and males were more active than females during copulation bouts. Occasionally a female would fly away when a male approached.

Unsuccessful courtships often terminated with the female retracting her pheromone gland and moving away from the courting male by walking away, turning around, or jumping away. Once a female displayed these behaviors, the male's chances of subsequently succeeding in mating with her were low.

The sex behavioral responses of mated females to virgin males were monitored during the first two hours of photophase on four consecutive days. Mated females were quiescent and did not display calling behavior in the presence of males. This suggests that females mate only once or are not eager to copulate a second time. However, in the presence of virgin females, mated males were usually active and showed mate locating behavior, and mated a second time.

2.3.2. Oviposition Behavior Observations

Egg colour changed over time from a pale yellow or whitish green when first laid (Figure 4A), to light yellowish-green at one month (Figure 4B), and finally to a brownish color (Figure 4C). Eggs were elongate and oval and approximately 1x 0.65mm in length, with their surface covered with small tubercles arranged in polygonal groups.

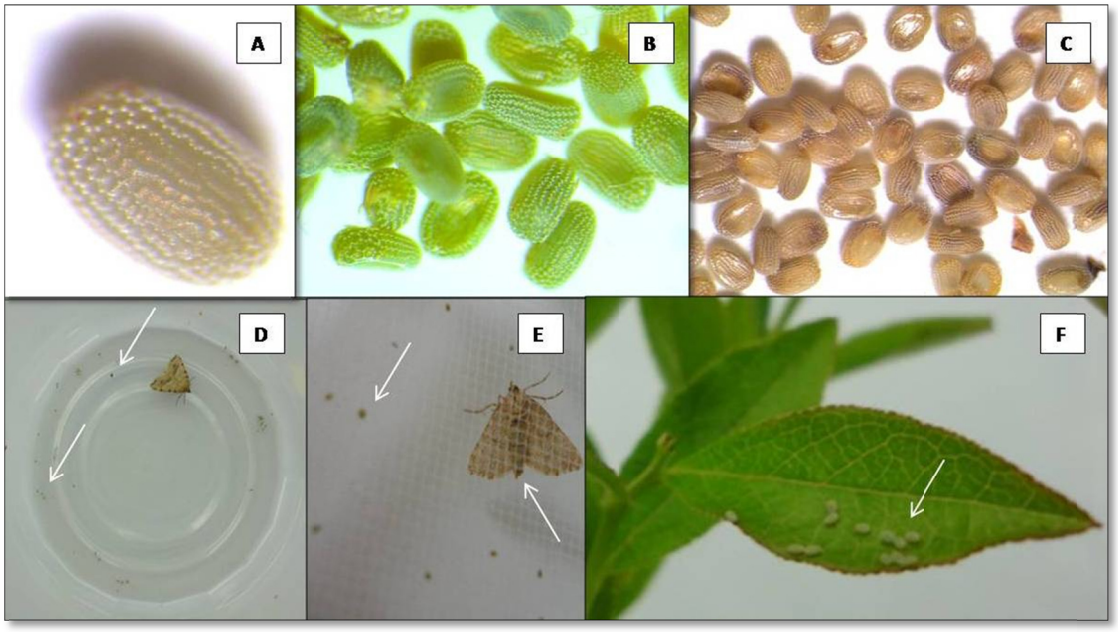

Figure 4. (A) 2 minute, (B) one month, and (C) seven months old *I. argillacearia* eggs (D,E), *I. argillacearia* eggs on blueberry foliage (F).

Upon mating, the majority of females initiated oviposition in the evening of that same day. Oviposition was recorded intermittently over the lifespan of female moths. All *I. argillacearia* females showed relatively similar egg laying responses. Egg laying rates strongly increased and peaked between the second and fourth days (F=211.67, df=17, 162, P<0.0001) (Figure 5).

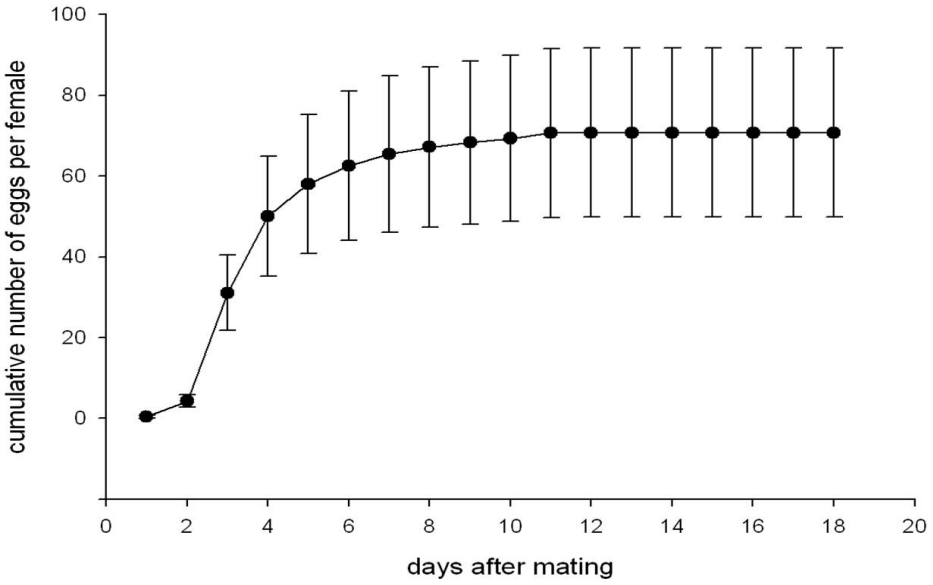

Figure 5. Cumulative number of eggs laid by *I. argillacearia* females after mating.

I did not identify any specific patterns of egg distribution in the container. Most eggs were laid in the bottom of the transparent plastic containers and were distributed along the wall of the container (Figure 4D,E). In general, the daily number of eggs laid was highly variable, especially two days after the initiation of oviposition, a similar pattern found in several Geometridae (Ayres et al. 1987, Toomas and Juhan 2000).Initial

oviposition rate may also be affected by the nutritional status of the female moth (Ayres et al. 1987, Toomas and Juhan 2000).

The basic biological aspects of oviposition pattern and sexual behavior of this pest have not previously been fully described. It is important to comprehensively examine life history in the interest of developing successful monitoring and management strategies for *I. argillacearia.* For example, knowledge of the egg production rate and laying pattern of females may be useful in predicting the number of offspring the following spring, helping to optimize the time and rate of insecticide spraying.

Chapter Three: Identification of the Sex Pheromone Components of the Female *Itame argillacearia*

4.1. Introduction

Much of the effort to develop eco-friendly monitoring for lepidopteran pests has focused on sex pheromones (Gueldner and Parrott 1978, Aldrich et al. 1988, Ho and Millar 2002, Ryall et al. 2010), which pose virtually zero risks to human and the environment. Given the success and importance of semiochemicals in management of agricultural and forest insect pests, it is surprising that more research on blueberry pest management has not much focused on insect chemical ecology. Sex pheromone blends of the blueberry leaftier have been identified (Lonergan et al. 1989), and can be useful for monitoring blueberry leaftier, and for mass trapping (Polavarapu and Seabrook 1992). Alford and Diehl (1985) previously showed that female blueberry spanworm moths emit a pheromone(s) that is attractive to male moths. They suggested that pheromone traps could provide a useful tool to growers in population management for this pest. The goal of the present study was to identify sex behavior modifying pheromone(s) utilized by *I. argillacearia.*

4.2. Materials and Methods

4.2.1. Insects and Pheromone Gland Extraction

Itame argillacearia late-instar larvae were collected from blueberry fields located in Debert (45° 25′ 11.42″ N; 63° 30′ 26.55″ W) and Farmington (45° 33′ 25.5″ N; 63° 54′ 18.2″ W), Nova Scotia, and brought to the laboratory. Rearing of larvae to adults was done using methods described in chapters two. Pheromone glands were manually

everted and excised from two-day old virgin females during the first 2-3 h of photophase (peak period of *I. argillacearia* pheromone release; Alford and Diehl 1985) by gently squeezing the abdomen and clipping the sex pheromone glands using microscissors (Fisher Scientific, Ottawa, Canada) into spectrograde n-hexane. Approximately one thousand female glands were excised (50 female glands per 50 µl hexane per vial). Extracted glands were held at -20^{0}C until analysis (Ryall et al. 2010). Before use for GC/MS or GC/EAD analysis, combined crude extracts were concentrated under a N_2 stream (Martin et al. 2004, Ryall et al. 2010) to obtain a concentration of approximately five female glands per 1µl hexane.

4.2.2. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Female gland extracts were analyzed by GC-MS on a Hewlett-Packard 7890A GC and a 5975C mass selective detector (MSD) in the electron impact (EI) mode (70 eV). The column (Agilent; J &W, 19091S-433; capillary: 30 m × 250 µm × 0.25 µm film) was used in the splitless mode with helium as carrier gas (Martin et al. 2004, Ryall et al. 2010). The injection port was at 250°C and the oven temperature was programmed from 70°C, held for 3 minutes and then increased at 15°C/min up to 220°C, and held again for 30 minutes (Martin et al. 2004, Ryall et al. 2010).

4.2.3. Gas Chromatographic-Electroantennographic Detection (GC/EAD), EAG Analysis

GC/EAD analyses of female pheromone gland extracts were done on *I. argillacearia* male antennae using a Varian CP-3380 single FID GC equipped with a split/splitless

injector (Martin et al. 2004, Silk et al. 2007, Ryall et al. 2010). The outlet was added to a purified and humidified air stream, directed over the excised male antenna. Antennae were excised close to the head and mounted using electrode gel (Spectra®-60 gel; Parker, USA) on EAD probe for electrical contact (Silk et al. 2007). The relative activity of the synthetic analogues was recorded by electroantennographic (EAD) bioassays using approximately 2ng of each stimulus placed on a strip of filter paper inside a glass pipette. EAD signals and flame ionization detector (FID) responses were simultaneously recorded. GC-EAD output was analyzed using Syntech recording and analysis software v. 2.6 (Syntech, The Netherlands). Injections were made in the splitless mode with helium as carrier gas and the injector held at 220°C. The column was a 30-m Supelco DB-5 (30 m × 0.30 mm × 0.25 µm film) temperature programmed from 70°C, held for 3 minutes then at 25°C/min up to 245°C (Silk et al. 2007, Ryall et al. 2010).

Electroantennographic (EAG) response of antennae from two-day old, virgin *I. argillacearia* of both sexes was tested with following five treatments: (1) pheromone crude extract; (2) female glands equivalent); (3) 3R,4S-epoxy-(Z,Z)-6,9-17:H ; (4) 3S,4R-epoxy-(Z,Z)-6,9-17:H; (5) (Z,Z,Z)-3,6,9-17:H; and (6) hexane control. A test series consisting of above treatments was randomly applied to the antennal preparations starting with the hexane control. The EAG recordings were obtained from at least 10 antennal preparations from each sex. Three puffs of stimulus was introduced into the air stream flowing over the antenna with approximately five seconds interval between each puff (Silk et al. 2007, Ryall et al. 2010).

The magnitude of each antennal response was measured using Syntech recording and analysis software v. 2.6 (Syntech, The Netherlands). Data that did not meet normality assumptions were log (x+1)transformed before analysis using the PROC MIXED ANOVA procedure at α=0.05 (SAS 2001).

4.2.4. Y-Tube Olfactometer Bioassays

The response of adult *I. argillacearia* males to volatiles emanating from live females, crude pheromone extract or synthetic chemicals were tested and compared in a Y-tube olfactometer which had 50^0 inner angle, a 40 mm diameter and 28 cm long glass arms (Analytical Research Systems Inc, Gainsville, FL). The olfactometer system used in this study has been previously described by Chen and Fadamiro (2007). The incoming air before entering into the Y-tube arm was filtered through activated charcoal and humidified with doubly distilled, deionized water. The filtered air was split through a plastic valve and regulated at 3.5 L/minute by using an in-line flow meter in each arm (Blackmer et al. 2004). Temperature and RH of the olfactometer air flow was maintained at 22 ± 2^oC and 80 ± 5%, respectively. A 60-W incandescent red bulb was positioned 70 cm above the Y-tube olfactometer setup. Before each trial, light intensity over each arm was measured with a light meter (Model 62344-944,800, VWR lab shop, VWR International, Batavia, IL), and the red bulb was adjusted until a light intensity of 66 ± 7.2 (± SEM) lux was achieved in each arm of the tube (Yang et al. 2011). The entire Y-tube olfactometer apparatus was placed in a fume hood. Before each experiment, the fume hood was run for approximately 30 minutes to ensure that moths were not

exposed to other non-experimental odors. Temperature and RH inside of the holding chamber were maintained at 22 ± 2^{o}C and 55 ± 5%, respectively.

Two-day old male and female moths were placed in separate rooms so that they were not exposed to test odors and each other before being used in the Y-tube olfactometer. Bioassays were performed 2-3 h after commencement of photophase (Alford and Diehl 1985, Boo et al. 1998). For each bioassay run, a randomly chosen male was placed in the stem of the Y-tube at a start line, set arbitrarily 5 cm from the base. Each male moth was given five minutes to respond to the treatment, and choice of the left or right arm of the Y-tube was noted when the insect passed the final choice line, arbitrarily set 8 cm from the tube branching point. Those remaining in the stem were recorded as no response (Du et al. 1998, Blackmer et al. 2004). For each bioassay, the number of moths that selected either Y-tube arm, the response time to a choice, and the number of attraction were determined.

Pheromone gland crude extracts (approximately 2 female glands equivalent) or synthetic chemicals (approximately 2 ng) were applied to 0.5 x 2 cm pieces of Whatman no. 1 filter paper. In tests, individual pieces of filter paper were placed in the incoming airstream of one of the Y-tube arms as appropriate for a given test. Hexane was used to dissolve the pheromone extract and synthetic compounds, and controls consisted of filter paper pieces treated with hexane solvent alone.

Epoxide and tri-ene compounds are sex attractants for several Geometridae (Cantelo et al. 1982, Ryall et al. 2010). Behavioral observations and laboratory and field bioassays suggests that the enantiomerically enriched epoxide, phenylacetaldehyde,

and unsaturated hydrocarbons components are also can play a role in mate location in at least nine species of Geometridae (Millar et al. 1990). These findings and personal preliminary analysis of pheromone crude extract of female *I. argillacearia* guided the treatment combinations used in the current study. The treatment combinations were as follows:

(1) live female vs hexane;

(2) pheromone crude extract vs hexane;

(3) phenylacetaldehyde vs hexane;

(4) (Z,Z,Z)-3,6,9-17:H, vs hexane;

(5) 3S,4R-epoxy-(Z,Z)-6,9-17:H vs hexane;

(6) 3R,4S-epoxy-(Z,Z)-6,9-17:H vs hexane;

(7) (Z,Z,Z)-3,6,9-17:H vs live female;

(8) pheromone crude extract vs live female;

(9) 3R,4S-epoxy-(Z,Z)-6,9-17:H vs live female;

(10) 3R,4S-epoxy-(Z,Z)-6,9-17:H vs pheromone crude extract;

(11) Hexane vs clean air.

Only virgin males were used for bioassays. Trials were replicated until treatments had a minimum of 60 individuals that responded. The null hypothesis was that blueberry spanworm male moths show no preference for either Y-tube arm (a response equal to 1:1). A Chi-square test was used to determine whether moth choice depended on treatment (SAS 2001).

4.3. Results and Discussion

4.3.1. GC/MS Analysis

Electron Impact (EI)-mass spectra and chromatographic peaks from GC/MS analysis of female sex pheromone glands extracts (approximately 10 virgin glands equivalent injected) confirmed the presence of (Z,Z,Z)-3,6,9-17:H and *cis*-3,4-epoxy-6,9:17:H, but the stereochemistry of epoxide was not defined (Millar et al. 1990, Millar 2000). The EI-mass spectra of these compounds, retention times and elution order were identical to those produced from the authentic synthetic materials, and confirmed against data previously reported (Millar et al. 1990, Millar 2000). The diagnostic ions were m/z 59, 79, 93, 178 and 234, M+ 250 (Millar et al. 1990, Millar 2000, Yamamoto et al. 2000)for the cis-epoxide adducts and m/z 67, 108, 178 and M+ 234 for the triene hydrocarbons.

Synthesis, purification, and chemical characterization of all three compounds – (1) 3S,4R-epoxy-(Z,Z)-6,9-17:H; (2) 3R,4S-epoxy-(Z,Z)-6,9-17:H; (3) (Z,Z,Z)-3,6,9-17:H – was done by Dr. Peter Silk's research group at Natural Resources Canada, Atlantic Forest Centre, Fredericton, Canada.

4.3.2. GC/EAD Analysis

Female pheromonal gland extract and 3R,4S-epoxy-(Z,Z)-6,9-17:H elicited a strong positive response from male antennae (Figure 8A,C) but 3S,4R-epoxy-(Z,Z)-6,9-17:H did not (Figure 8B). There was no EAD response for (Z,Z,Z)-3,6,9-17:H (Figure 8D).

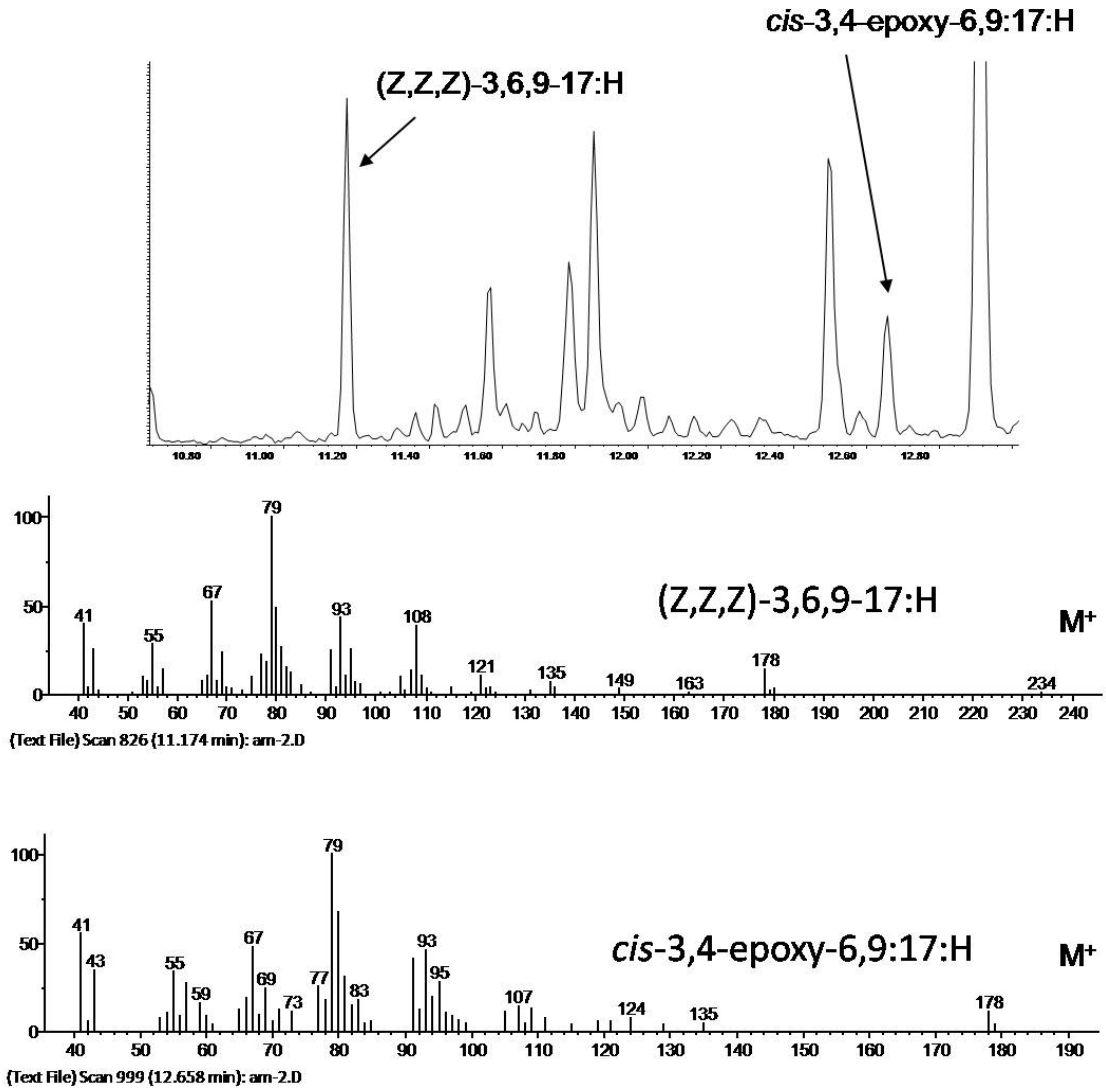

Figure 6. Gas-chromatogram and mass-spectrum of hexane extract of *I. argillacearia* virgin female sex pheromone glands.

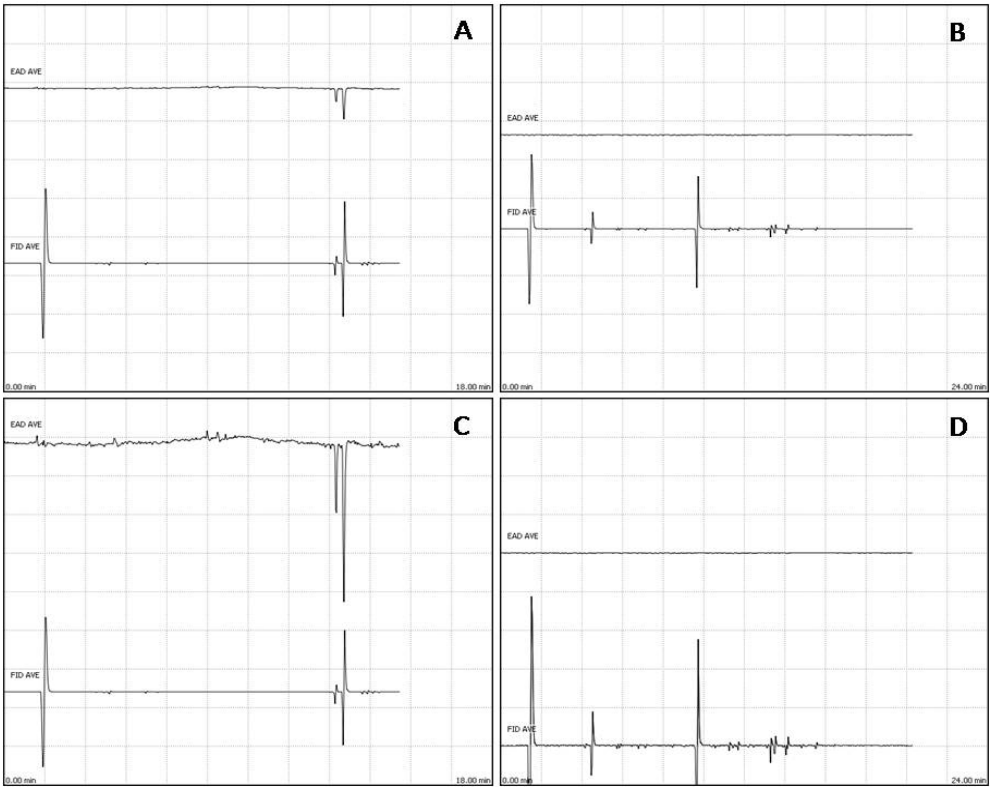

Figure 7. GC-FID/EAD responses of *I. argillacearia* male antennae to: (A) pheromone extract; (B) 3S,4R-epoxy-(Z,Z)-6,9-17:H; (C) 3R,4S-epoxy-(Z,Z)-6,9-17:H; and (D) (Z,Z,Z)-3,6,9-17:H.

4.3.3. EAG Analysis

EAG responses were separately compared for male and female antennae. There were significant differences in the magnitude of antennal response between treatments (F=23.63, df=4, 281, $P < 0.0001$) (Figure 9). EAG output showed that 3R,4S-epoxy-(Z,Z)-6,9-17:H elicited a significantly greater response than its enantiomer 3S,4R-epoxy-(Z,Z)-6,9-17:H. This suggests that the insect-derived epoxide is 3R,4S-epoxy-(Z,Z)-6,9-17:H. Male moths also responded significantly to (Z,Z,Z)-3,6,9-17:H (Figure 9).

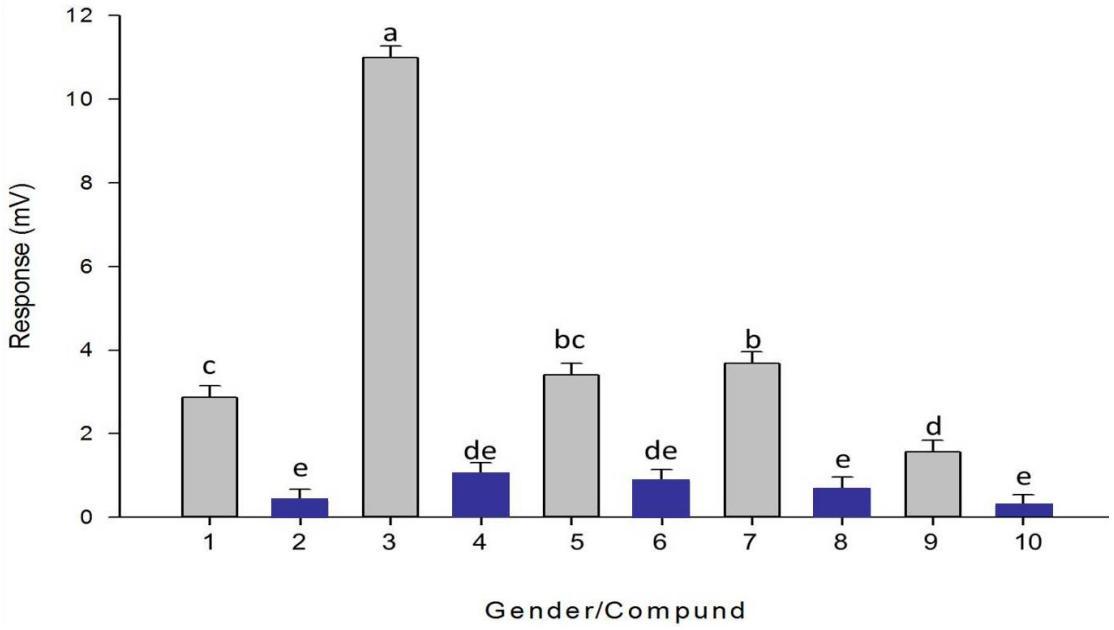

Figure 8. Mean (±SEM) electroantennogram (EAG) responses of *I. argillacearia* male (grey bars) and female (blue bars) antennae to: (1,2) phromone extract; (3,4) 3R,4S-epoxy-(Z,Z)-6,9-17:H; (5,6) 3S,4R-epoxy-(Z,Z)-6,9-17:H; (7,8) (Z,Z,Z)-3,6,9-17:H; and (9,10) hexane. Means followed by the same letter are not significantly different (LSD test, $P<0.05$)

The molecular structure of over forty sex pheromones have been identified for moths in subfamily Ennominae (Millar et al. 1990, Millar 2000, Yamamoto et al. 2000, Gibb et al. 2006). Polyenes and/or their corresponding mono-epoxides structural motif are predominant. In addition, compounds with odd numbers of carbons are common and C_{17} polyenes and mono-epoxides are restricted to this sub-family (Millar et al. 1990, Millar 2000) (Table1).

Table 1. Summary of attractant pheromones of subfamily Ennominae.

Species	Molecular structure of attractants	References
Itame brunneata (Thunberg)	(6Z,9Z,3S,4R)-epoxy-17:H	(Millar et al. 1990)
Itame occiduaria (Packard)	6Z,9Z,3R,4S-epoxy-17:H & 3Z,6Z,9Z-17:H	(Millar et al. 1990)
Itame pustularis (Guenée)	2-phenylacetaldehyde	(Peltotalo and Tuovinen 1986)
Mnesampela private (Guenée)	3Z,6Z,9Z-19:H & 1-hexadecanol & 1-octa decanol	(Martin et al. 2004)
Ennomos subsignaria (Hübner)	Z6-9S,10R-epoxy-19:H	(Ryall et al. 2010)
Probolea micaria (Herrich-Schiffer)	(6Z,9Z,3S,4R-epoxy-19:H) & 3Z,9Z,6R,7S-epoxy-19:H & 3Z,6Z,9Z-19:H	(Millar et al. 1990)
Itame wauaria (Linnaeus)	Z3Z13-18Ac & E3Z13-18Ac	(Cantelo et al. 1982)
Bistonrobustum (Butler)	3Z,9Z-6,7-epoxy-19:H &6Z,9Z-19 :H; 3Z,6Z,9Z-19:H	(Yamamoto et al. 2000)

It is likely that females of other species in this subfamily produce sex pheromones that are variations on this structural motif with odd-numbered carbon skeleton epoxides (Millar et al. 1990, Ryall et al. 2010).

As already suggested, a long chain epoxide and/or tri-ene could be the major component(s) of the *I. argillacearia* sex pheromone, corresponding to findings with other Geometridae (Underhill et al. 1987, Millar et al. 1990, Ryall et al. 2010). It was clear that, GC/MS results were well correlated with GC/EAD and EAG results. GC/MS,

GC/EAD and EAG results also provided strong evidence, *I. argillacearia* sex pheromone components are likely (3R,4S)-epoxy-(Z,Z)-6,9-17:H and (Z,Z,Z)-3,6,9-17:H. Field studies of these synthetic compounds, which would be the most practical way to clarify the pheromone components, are presented in the following chapter.

4.3.4. Y-Tube Olfactometer Bioassays

The results confirm those of Alford and Diehl (1985) who showed that blueberry *I. argillacearia* male moths respond to the calling females. In the current study, the Y-tube bioassays demonstrated a significant preference response of males to volatiles from live females, pheromone crude extract and 3R,4S-epoxy-(Z,Z)-6,9-17:H (Figure 9). This result corresponds to the results found in the EAG analysis, which showed excised *I. argillacearia* male antennae and highly stimulated by 3R,4S-epoxy-(Z,Z)-6,9-17:H.

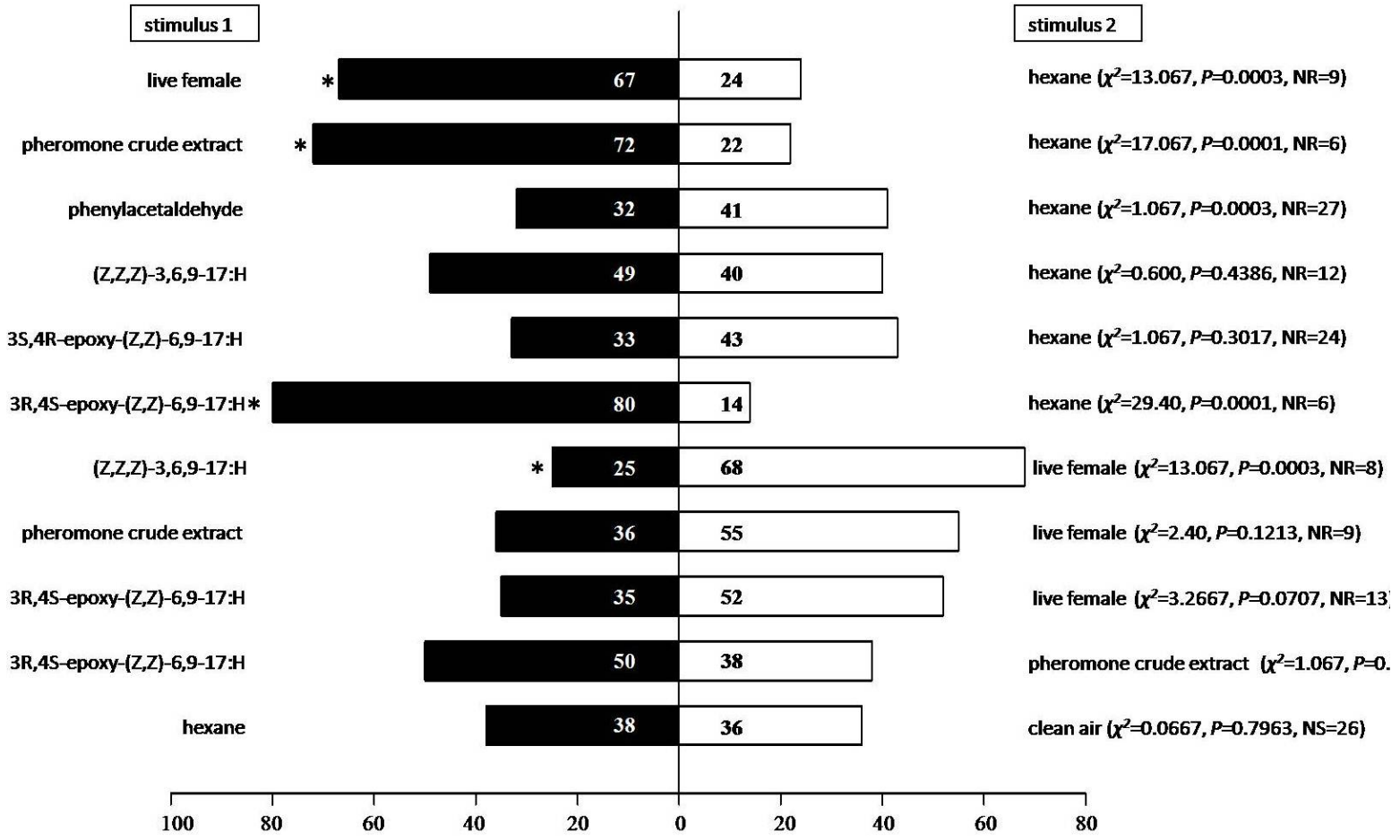

Figure 9. *I. argillacearia* male moth preference (percent) for different stimulus combinations in a Y-tube olfactometer. Asterisks indicate significant differences within each choice test (Chi-square test: P<0.05), NR: non responded moths (percent).

Males did not showed significant responses to filter paper treated with phenylacetaldehyde, 3S,4R-epoxy-(Z,Z)-6,9-17:H, or (Z,Z,Z)-3,6,9-17:H, and did not discriminate between 3R,4S-epoxy-(Z,Z)-6,9-17:H and live females or pheromone crude extract (Figure 9). Again, these results coincide well with those of the EAG. It has been reported that several Geometridae males were attracted to phenylacetaldehyde (Cantelo et al. 1982), but male *I. argillacearia* moths showed no attraction to this compound. A number of males did not respond to any stimulus, the reason of which is unknown.

For male moths that engage in flight response, Y-tube olfactometers may not be the best way to examine their response to synthetic chemicals (Boo et al. 1998, Booth et al. 2007). Orientation during flight may be an integral part of the behavioral sequence that leads the moths to the chemicals. Furthermore, due to the lack of space inside of the Y-tube olfactometers may be difficult to see actual flight behavior of moths (Blackmer et al. 2004). For a better understanding of these various behavioral interactions, we hope to develop a trapping system that may be effective in monitoring *I. argillacearia* male moth.

The results of the Y-tube bioassays provide evidence that *I. argillacearia* females emit pheromones that elicit significant responses on males. The results for the Y-tube and EAG experiments suggest that a long chain epoxides and/or tri-ene molecule could be the major component(s) of the *I. argillacearia* sex pheromone, corresponding to findings with other Geometridae (Underhill et al. 1987, Millar et al. 1990, Ryall et al.

2010). Field bioassays were carried out on *I. argillacearia,* and are presented in the following chapter.

Chapter Four: Field Trapping Studies with Synthetic Sex Pheromone Components for Blueberry Spanworm

5.1. Introduction

It was previously shown that female *I. argillacearia* emit a pheromone(s) that is attractive to male moths (Alford and Diehl 1985). The chemical identification of sex pheromone components in female *I. argillacearia* is described in chapter four of this thesis. Several other factors need to be considered if these pheromones are to be used in the field with traps for monitoring or management of the pest. For example, trap design and position, pheromone concentration, inter-trap distance, and trap height and color may influence trap captures (McNally and Barnes 1981, Hoyt et al. 1983). Inadequate trap design or position poses the risk of underestimating pest pressure. Conversely, trap designs that are overly attractive to moths species can also cause inefficiency of monitoring efforts due to capturing of non-target insects that might clog-up the trap and may be have repellant affect for incoming moths (Brown 1984, Knight 2001). Pheromone-baited sticky traps, such as the Delta trap® and Contech Wing trap®, have proven effective for trapping other moths species (Ponder and Seabrook 1988, Lonergan et al. 1989, Hillier 2001, Hillier et al. 2003). These are commercially available and are economical.

Three field experiments were conducted. The first two field experiments tested hypotheses concerning the chirality, concentrations and ratios of the *I. argillacearia* sex pheromone constituents identified in the previous chapter of this thesis. The objective of the other experiment was to compare the efficacy of Delta vs. Wing traps placed at two different heights at capturing *I. argillacearia* male moths.

5.2. Materials and Methods

5.2.1. Insects

I. argillacearia late-instar larvae were collected from a blueberry field located in Debert (45° 25′ 11.42″ N; 63° 30′ 26.55″ W) and Farmington (45° 33′ 25.5″ N; 63° 54′ 18.2″ W), Nova Scotia, and brought to the laboratory. Rearing of larvae through to the adult stage was done using methods described in chapters two and three. One day old, a virgin female moths were used as "lures" in traps in experiments as required (Alford and Diehl 1985).

5.2.2. Field Testing of Synthetic Sex Pheromone Components

Traps were deployed in the wild blueberry sprout fields located in Debert (45° 25′ 11.42″ N; 63° 30′ 26.55″ W), Nova Scotia, that was used for larvae collection, during the moth flight season, in 2012. Lures containing treatments were placed within Delta® traps (Contech, Delta, British Columbia) with sticky inserts to capture male moths. Traps were placed approximately 20 m apart (Cantelo et al. 1982, Hillier 2001, Hillier et al. 2002), and suspended from iron stakes at the blueberry foliage canopy level. The first field experiment was done with seven treatments: (1) 3R,4S-epoxy-(Z,Z)-6,9-17:H (50 µg); (2) 3S,4R-epoxy-(Z,Z)-6,9-17:H (50 µg); (3) (Z,Z,Z)-3,6,9-17:H (50 µg); (4) 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (50 µg each); (5) 3S,4R-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (50 µg each); (6) one day old virgin female; (7) blank control trap. Each treatment was absorbed on red rubber septa (Wheaton, NJ, USA). Traps were in the field from 9–26 July, 2012 and were emptied or replaced twice per week during this period.

A second field experiment attempted to establish a dose-response with 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H, the best treatment combination found in the first trapping experiment. Six replicates of five doses (0, 1, 10, 50, 100 µg) were used, along with two additional treatments: (6) 3R,4S-epoxy-(Z,Z)-6,9-17:H (45 µg) & (Z,Z,Z)-3,6,9-17:H (5 µg); and (7) 3R,4S-epoxy-(Z,Z)-6,9-17:H (5 µg) & (Z,Z,Z)-3,6,9-17:H (45 µg). Traps were in the field from 23 July to 3 August, 2012 and were emptied or replaced twice per week during this period.

In both experiments traps were arranged in a randomized complete block design with ten replicates in the first experiment and six replicates in the second field experiment. The field was segmented on the basis of the slope of the fields which was considered the blocking factor. Counts of male moths in traps were done twice per week (Hillier 2001, Hillier et al. 2002). Trap capture data were natural-log transformed ($\log_{10}$ X+1) to satisfy assumptions of normality and equal variances. Data were analyzed at α=0.05 using the PROC MIXEDANOVA procedure (SAS 2001). Backtransformed means and the difference of backtransformed upper and lower confidence intervals are presented, where significant differences were detected, Fisher's least significant difference (LSD) was used for multiple mean comparisons among treatments.

5.2.3. Evaluation of Trap Type and Height

Delta traps (Figure10B) and Contech Wing traps I (Figure10A) (Contech, Delta, British Columbia) baited with live female moths were used in this experiment. All traps contained sticky inserts. The experiment was done in the wild blueberry field located in

Debert (45° 25′ 11.42″ N; 63° 30′ 26.55″ W), Nova Scotia, from which larvae were collected. Traps were deployed 10-29 July 2012. A live female in a plastic container with 25 ventilation holes was placed inside each trap (Figure10E). Traps were placed at either ground level (0 cm from ground to the trap bottom) or at canopy height (20 cm from the ground). Captured moths were counted, removed and replaced with new traps with one day old new alive virgin female, every 2-3 days. Captured moths were sexed and identified as *I. argillacearia.*

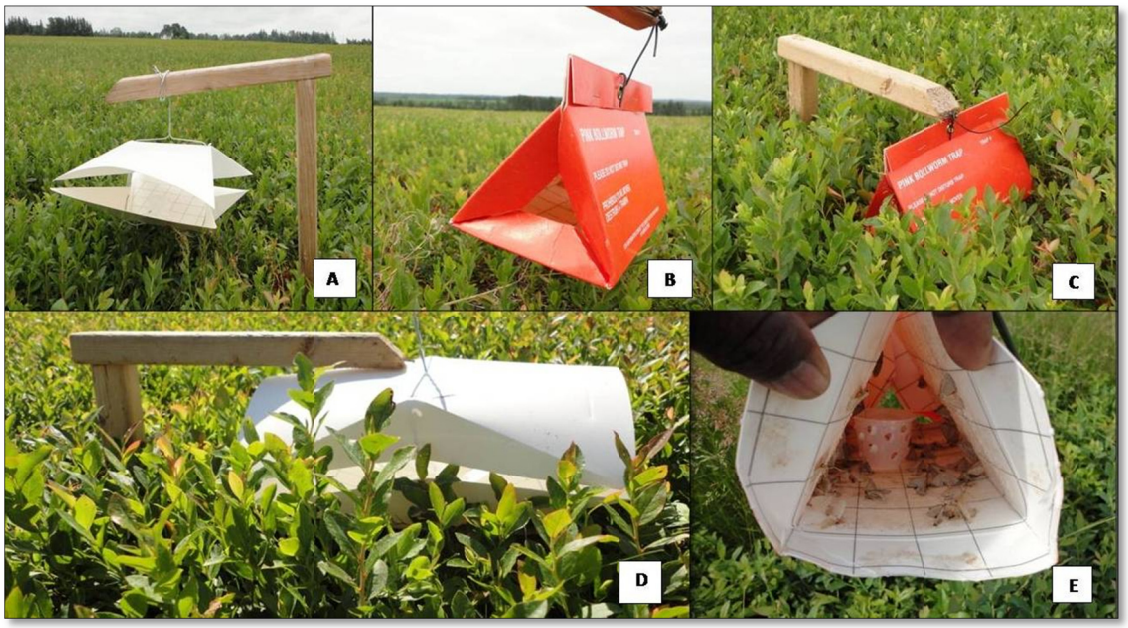

Figure 10. Trap types and positions used to capture male *I. argillacearia* moths: (A) Wing trap at canopy level; (B) Delta trap at canopy level; (C) Delta trap at ground level; (D) Wing trap I at ground level; (E) Delta trap at canopy level showing perforated container used to hold female moth and male moths stuck in the trap.

The experimental design was a 2 X 2 factorial ANOVA with trap type and height levels as the factors. Traps were deployed in a complete randomized block design with

five replicates, hung on wood stakes and placed 20 m apart (Hillier et al. 2002, Ho and Millar 2002). For all trap catches, normal distribution and constant variance assumptions on the error terms were verified by examining the residuals (Montgomery 2005). When trap type and/or height effects were marginally significant (0.05<*P*-value<0.1), means were separated with Fisher's least significant difference (LSD) test at α = 0.05. The PROC MIXED procedure in SAS was used for the analyses (SAS 2001).

5.3. Results and Discussion

5.3.1. Field Testing of Synthetic Sex Pheromone Components

An initial trapping experiment was conducted to determine the best treatment(s) for capturing *I. argillacearia* males. The trapping results were well correlated with GC/MS, GC/EAD and EAG results. There was a significant difference in captures of male moths for the different treatments (F=484.04, df=6, 280, P<0.0001) (Figure11). Furthermore, there were a significant block effect (F=12.11, df=9,280, P<0.0001) and treatment-block interaction (F=3.88, df=54, 280, P=0.0001) in captures of male moths. Significant block effects were observed for trap captures, indicating differences in response among the experimental replicates, may be a result of slope of the field, wind direction, forest edge or possible combinations. Not surprisingly, live females attracted the greatest number of males. 3R,4S-epoxy-(Z,Z)-6,9-17:H + (Z,Z,Z)-3,6,9-17:H caught more males than did 3R,4S-epoxy-(Z,Z)-6,9-17:H alone. 3R,4S-epoxy-(Z,Z)-6,9-17:H and (Z,Z,Z)-3,6,9-17:H were confirmed as the primary sex pheromones component with the (3R,4S) epoxide

enantiomer giving significantly higher trap captures when combined with the triene than the (3S,4R) enantiomer epoxide (P <0.05).

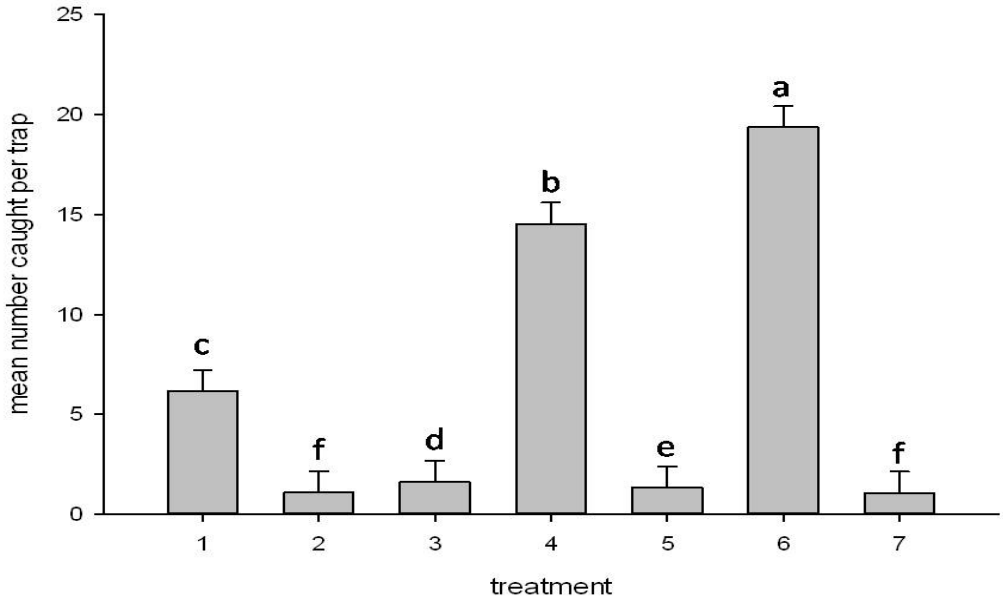

Figure 11. Mean (±SEM, n=10) trap catches of male *I. argillacearia* moths in traps with: (1) 3R,4S-epoxy-(Z,Z)-6,9-17:H (50 µg); (2) 3S,4R-epoxy-(Z,Z)-6,9-17:H (50 µg); (3) (Z,Z,Z)-3,6,9-17:H (50 µg); (4) 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (50 µg each); (5) 3S,4R-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (50 µg each); (6) live virgin female; or (7) blank trap. Means followed by the same letter are not significantly different (LSD test, P <0.05).

The second trapping experiment attempted to establish a dose-response of male *I. argillacearia* to increasing dosages of 3R,4S-epoxy-(Z,Z)-6,9-17:H + (Z,Z,Z)-3,6,9-17:H, over the range of 0 to 100 µg. Dose had a significant effect on the mean number of males caught per trap (F=69.24, df=6,126, P<0.0001) (Figure12). Increased trap captures were observed when pheromone doses increased, but only up to the 10 µg dose when the epoxide and triene components were presented in a 1:1 ratio. The ratio of the 3R,4S-epoxy and triene components also was important. When these were respectively

presented in a 9:1 ratio (45:5 µg), a 30% increase in moth captures was observed compared with the equivalent 10-100 µg treatments of both pheromone constituents. Furthermore, the block effect (F=3.29, df=5, 126, P=0.0079) and treatment, block interaction (F=1.14, df=30, 126, P=0.3061) were not significant difference in captures of male moths.

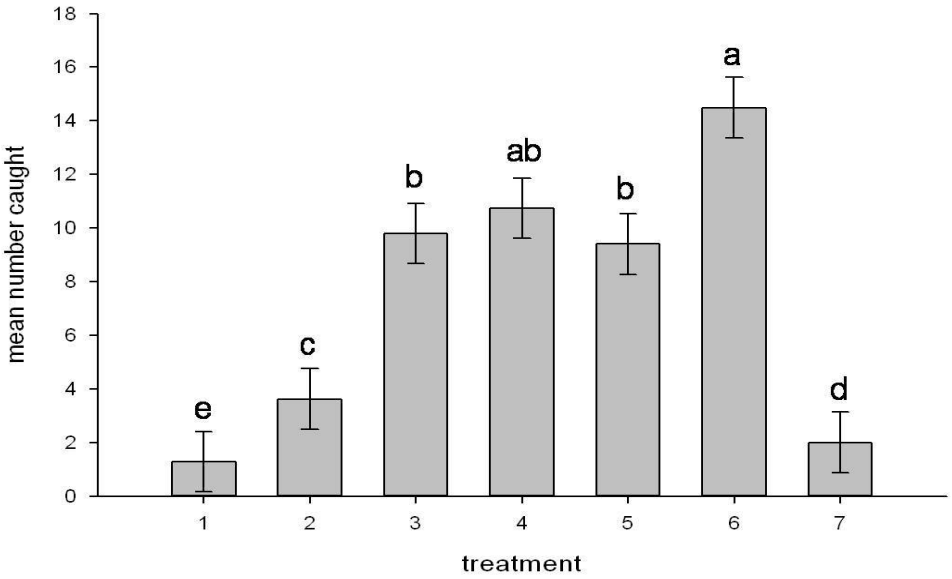

Figure 12. Captures of male *I. argillacearia* moths in traps baited with: (1) blank lure; (2) 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (1 µg); (3) 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (10 µg); (4) 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (50µg); (5) 3R,4S-epoxy-(Z,Z)-6,9-17:H & (Z,Z,Z)-3,6,9-17:H (100 µg); (6) 3R,4S-epoxy-(Z,Z)-6,9-17:H (45 µg) & (Z,Z,Z)-3,6,9-17:H (5 µg); (7) 3R,4S-epoxy-(Z,Z)-6,9-17:H(5 µg)& (Z,Z,Z)-3,6,9-17:H (45 µg). Means followed by the same letter are not significantly different (LSD test, P<0.05)

On the other hand, a 1:9 ratio (5:45 µg) of the 3R, 4S-epoxy and triene components resulted in a sharp decrease in moth captures (Figure12). Therefore, to be most effective in capturing male *I. argillacearia*, 3R, 4S-epoxy-(Z,Z)-6,9-17:H needs to be present in relative high amounts compared to (Z,Z,Z)-3,6,9-17:H.

There was no effect of dose when above 10 µg. A trend of decreasing the trap captures were observed when pheromone doses increased to 100 µg (Figure 12), similar effects of pheromone dose on trap catch have been reported for other lepidopteran moths (Baker and Roelofs 1981, Turgeon et al. 1983, Grant et al. 1989). Baker and Roelofs (1981) reported that oriental fruit moth trap catches significantly decreased at abnormally high doses of pheromone due to termination of the upwind flight prior to moths reaching pheromone source.

Itame occiduaria (Packard), moths were also attracted by synthetic blends combination of 3R,4S-epoxy-(Z,Z)-6,9-17:H (225 µg) & (Z,Z,Z)-3,6,9-17:H (50 µg) (Millar et al. 1990), however no analysis of *I. occiduaria* female glands has been done. Closely related *Itame* species may share similar sex pheromone components, although moth species exhibit a high degree of enantiomeric specificity in their sex pheromone behavioral responses (Millar et al. 1990, Martin et al. 2004). Results of the present study confirm that (3R,4S)-epoxy-(Z,Z)-6,9-17:H and (Z,Z,Z)-3,6,9-17:H are sex pheromone components of female *I. argillacearia,* with the epoxide likely occurring at a higher ratio. The high rate of captures with these components makes them excellent pheromone candidates for traps in monitoring, mating disruption or mass trapping of this pest. Further research should home in on the optimization lure dosages and ratios for capturing male *I. argillacearia*.

5.3.2. Evaluation of Trap Type and Height

Trap placement and type had a significant effect on moth captures (Table 2). The greatest number of moths was recovered from Wing traps placed a canopy level (Table 3). These traps captured 2- to 3-fold more moths that the other treatments ($P< 0.05$). Whereas efficiency of the Wing trap was significantly influenced by trap height, this factor had no effect on moth captures with the Delta trap (Table 2; Interaction: F=3.42, df=1,76, P=0.0685). Delta traps placed at ground level had significantly fewer moths than Wing traps at either height placement (Table 3).

Table 2. Analysis of variance results for the effects of trap types, heights, and interaction for capturing of male *I. argillaceria* moths.

Factor	*df*	*F*	*P*
Model	3,76	13.95	0.0001
Trap type	1,76	26.33	0.0001
Trap height	1,76	12.11	0.0008
Block	1,76	0.18	0.6727
Trap type x Trap height	1,76	3.42	0.0685

Table 3. Influence of trap type and height on the number of male *I. argillaceria* moths captured in traps baited with a live female.

Trap type	Height level	Mean number of male mothspertrap±2.543 (SEM)
Delta trap	ground	9.85c
Delta trap	canopy	14.00bc
Contech Wing trap I	ground	18.20b
Contech Wing trap I	canopy	31.75a

* values with different letters are significantly different (LSD, $P< 0.05$)

It is likely that at ground level, blueberry foliage hindered the movement of moths into the trap. The foliage may have also intercepted volatiles coming from

female moths (Figure 11). The influence of trap type may be due to differences in area of the sticky surface in the traps and/or access opening. The surface area of the sticky surface was 288cm^2 of the Delta trap, whereas that of the Wing trap was 637cm^2. Further, moths are able to enter the Wing trap through either of four sides, whereas the Delta trap contains only two relatively small openings on the each end of the trap. Easier access to females combined with more sticky surface may have resulted in the higher trap captures with Wing traps. In addition, it is easier to place and monitor traps when at canopy level.

The cost of Delta and Wing traps can vary with the supplier, but Delta traps are generally cheaper than Wing traps. For example, when purchased for the current experiments, the Contech Wing trap was almost double of cost of the Delta trap.

It is unclear with my experiments how many traps should be placed in a field per unit area. Additional field studies are needed to determine the relationship between moth captures per trap and larval densities obtained through sweep-netting to establish an economic threshold of moths/trap that is congruent to the current threshold based on larvae per 25 sweeps (Crozier 1995). Field releases of marked moths could help to estimate how the probability of capture declines with distance between the traps and the release point, and to estimate population density. As an index of attractive area for a trap releasing semiochemicals, the "effective attraction radius" has been proposed (Wagner et al. 2001). This effective attraction radius yet to be determined for the male *I. argillacearia* moths.

Further research clarifying the influence of different trap colors in capture of moths could be important (McNally and Barnes 1981, Hoyt et al. 1983). Shape and design of the trap also seems to be an important factor affecting the catch of moth species. Testing capture of males with other traps such as the Pherotech® Diamond trap or a non-saturating Unitrap® (Green Cross Canada, Mississauga, Ontario) should be considered.

Chapter Five: Discussions

6.1. Study Rationale

The research presented in this thesis identified the sex pheromone constituents of *I. argillacearia,* and confirmed attraction of identified components in field experiments. My research also examined basic behavior of mating and oviposition. The rationale for this work was the limited number of options for management and monitoring for *I. argillacearia*. This insect is currently monitored in blueberries almost exclusively through use of sweep nets. This method has some disadvantages (Howatt 2005, Drummond et al. 2008), which were discussed in the introduction of this thesis.

Additional tools for monitoring or controlling *I. argillacearia* are desirable. Although, there are many examples of pest management benefits accrued from pheromone research in other agricultural and forestry systems (Seabrook and Dyer 1983), there has been relatively little semio-chemical research done on blueberry pests. In most cases, widespread and successful applications of sex pheromones are being used for insect pest detection and population monitoring. Strategically placed and monitored pheromone traps allow growers to accurately track insect population growth and movement to optimize insecticide application in both time and space while greatly decreasing pesticide inputs (Seabrook and Dyer 1983). For an example, apple growers throughout major growing regions of Canada and the United States who use pheromone traps to monitor codling moth (*Cydia pomonella*), oblique-banded leaf roller (*Choristoneura roseceana*), Oriental fruit moth (*Grapholita molesta*), have often been able to reduce the half of insecticide applications (Cade 1985, Pedigo and Rice 2006).

Pheromone-based technologies also are useful in mass trapping and mating disruption of insect pests (Seabrook and Dyer 1983).

6.2. Commercialization of Synthetic Sex Pheromones

Despite the identification of many insect pest pheromones, progress through practical implementation and commercial exploitation of such technologies has been slow (Witzgall et al. 2010). The practical pheromones application depends up the availability of efficient dispenser materials and on the economic synthesis of the pheromone chemicals. With large-scale synthesis it has been possible to use synthetic pheromones commercially and make their sale financially viable (Yamamoto and Ogawa 1989, Witzgall et al. 2010).

For example, apple growers currently use on 160,000 ha worldwide pheromone traps to monitor and mass trap key lepidopteran pests such as codling moth and oblique-banded leaf roller, have often been able to reduce insecticide applications by 50% (Carde and Minks 1995, Pedigo and Rice 2006, Witzgall et al. 2010). The annual production of codlemone, the main pheromone compound of codling moth (*Cydia pomonella*), is on the order of 23metric tons, and the price of codlemone is currently below 1,000 US$/kg. Orchard treatments with up to 100 g/ha of synthetic codlemone effectively control *C. pomonella* populations over the entire growing season (Yamamoto and Ogawa 1989). Although the price of synthetic codlemone in the early 1990s was too high for commercial area-wide applications, the development of large-scale synthesis made codlemone synthesis commercially viable (Yamamoto and Ogawa 1989,

Witzgall et al. 2010). Similar opportunities may develop for blueberry pests. The market prices of the synthetic pheromones of other Geometridae moths that have very similar molecular structures with *I. argillacearia* are approximately $2.25 per dispenser (Great lakes IPM, Vestaburg, United States and Russell IPM, Flintshire, United Kingdom).

It is estimated that at least 20 million pheromone lures are produced each year for monitoring or mass trapping insect pests, which implies this is an emerging market. There are over 2500 wild blueberry producers in eastern Canada and together with high bush blueberries, Canadian blueberry production in 2011 covered 69,900 ha of land (Statistics Canada 2012). Thus, there may be sufficient market to justify production of blueberry insect pest sex pheromones for commercial applications.

6.3. Research Needs

The following should be considered as areas of future research for *I. argillacearia* management with semiochemicals.

- Field experiments confirmed the biological activity of 3R,4S-epoxy-(Z,Z)-6,9-17:H with tri-ene, (Z,Z,Z)-3,6,9-17:H in trapping male *I. argillacearia* moths. However, further study to confirm the optimal ratio of pheromonal components is needed to ensure maximum captures in the field.
- Insect pest management with semio-chemical complements biological control. The best biological control method might be the conservation of natural enemies of the pest (Drummond and Groden 2000, Pedigo and Rice 2006). I identified one wasp species (*Hyposoter exiguae Viereck* [Hymenoptera; Ichneumonidae])

and one fly species (*Voria ruralis Fallen* [Diptera; Tachinidae]), with other natural enemies, that infested approximately 65% my laboratory colony, suggesting natural enemies may have strong impacts in the field. A complete evaluation of *I. argillacearia* natural enemies has yet to be determined.

- Clarifying the influence of different trap colors in capture of *I. argillacearia* males could be important (McNally and Barnes 1981, Hoyt et al. 1983). Shape and design of the trap also seems to be an important factor affecting the catch of moth species. Additional field experiments with different trap type are needed to determine the importance of these factors.
- Trap captures of moths should be relatable to the abundance of damaging larval stages and economic thresholds. If this can be established, decision making for taking action can be optimized (Witzgall et al. 2010). Therefore, captures of male *I. argillacearia* adults in first year need to be correlated with larval incidence and subsequent crop damage in the field the following year. This will provide a basis for establishment of an economic or action threshold based on moth captures.
- A market analysis would be important to forecast the economic opportunities from the sale of traps containing *I. argillacearia* sex pheromones. At this time the prospective uptake of pheromone-based technologies by blueberry growers is unclear.

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