ABSTRACT

BUCHOLZ, ETHAN RICHARD. Chemical Biology of Resistant and Susceptible Species of *Abies* to Balsam Woolly Adelgid (*Adelges piceae*). (Under the direction of Dr. John Frampton).

Balsam Woolly Adelgid (*Adelges piceae* Ratz) (Hemiptera: Adelgidae)(BWA), an exotic invasive insect pest, has damaged many natural stands of Fraser fir (*Abies fraseri* [Pursh] Poiret) within the southern Appalachians, and continues to cost hundreds to thousands of dollars per hectare to control on Christmas tree plantations, an important forestry resource for North Carolina. Chemical biology of resistant and susceptible species of the *Abies* genus to BWA were studied via acetone extraction and headspace Solid Phase Microextraction (SPME) to identify differences that could potentially elucidate opportunities to study the underlying resistance mechanisms to BWA. Chemical ID's, specifically Bornyl Acetate (BA) and Isobornyl Acetate (IBA) were first tested to identify if cuttings of Fraser fir could uptake these chemicals and result in differential rates of insertion by BWA crawlers compared to Fraser fir and Veitch fir (*Abies veitchii* Lind.) water controls. Hanging bolt artificial infestations, where BWA-infested logs were suspended over uninfested branch cuttings to allow crawlers to fall and insert on the cuttings, indicated that mean inserted crawlers were not different between BA, IBA and Fraser fir control cuttings, and that BWA inserted on Veitch fir significantly more than the three other treatments, most likely due to increased area to capture falling adelgid crawlers. Concurrently, a study which tested the effects of BA solution (35ppm), pure BA, and Fraser and Veitch fir controls indicated that BWA egg eclosion was significantly inhibited by pure BA, and was slightly, though not significantly, affected by the Veitch fir control compared to the BA solution and Fraser fir controls. However, analysis of the effects of Fraser fir volatiles and Veitch fir volatiles, with

a known number of BWA eggs placed on top in an open environment did not result in a negative effect on BWA egg eclosion.

Looking for a potential concentration effect of BA on fecundity of BWA required the use of headspace-SPME to attempt to replicate signals of BA from Fraser fir and Veitch fir in the headspace of a closed container. Using a proxy insect, the Green Peach Aphid (*Myzus persicae* Sulzer)(Hempitera: Aphididae)(GPA) served as a test for potential concentration effects by placing two reproductive aphids in closed jars with 5 different concentrations of BA. The result showed that as BA concentration increases, GPA fecundity decreases, marked by finding fewer individuals within the higher concentrated environments, showing a negative effect of BA on GPA fecundity. When this study was replicated using BWA eggs instead of GPA, results showed that BA alone did not result in decreased eclosion success of BWA eggs. However, two treatments included in the BWA test that were not included in the GPA test, a Fraser fir and Veitch fir defoliated branch, indicate that within a closed space, Veitch fir volatiles result in significant egg eclosion failure, while Fraser fir volatiles have a slight negative effect on egg eclosion success. The results of this study, coupled with the previous studies of egg eclosion in an open air environment suggest that direct contact with chemical constituents, rather than the interaction in the air, results in a negative effect on eclosion of BWA eggs.

Headspace SPME on a variety of *Abies* species with a known resistance or susceptibility to BWA was then conducted. Three arboreta provided branch samples, and each was defoliated and run twice through GC/MS to obtain qualitative chromatographic information on all peaks. This peak information was used in a partial least squares regression, which identified peaks of importance to resistance and susceptibility. The peaks

identified as beta-myrcene and alpha-pinene are most associated with resistance, but further work is needed to evaluate those chemicals and determine what kinds of synergistic effects occur with multiple chemicals on BWA biology.

Chemical Biology of Resistant and Susceptible Species of *Abies* to Balsam Woolly Adelgid (*Adelges piceae*)

by
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DEDICATION

To Catherine, without you, I would be lost

BIOGRAPHY

Ethan Bucholz was born in Saint Louis, Missouri to Dr. Richard Bucholz (MD) and Dr. Kathleen Bucholz (PhD) on a wonderful spring day in 1989. Education has always been something emphasized heavily in the Bucholz household. It was a main goal of the Bucholz parents to provide their children with a wonderful education with the hopes that it ignited their passions and helped them find their way in this world, whatever that path might be. Each of the Bucholz children (Elizabeth, Eleanor and Ethan) have completed some form of graduate school: Elizabeth completed her PhD in biomedical engineering at Duke University and Eleanor completed her MD at Loyola University of Chicago. Ethan started his undergraduate education at Sewanee: The University of the South, as a history major. Upon needing to fulfill a requirement for lab science, he decided to take an introduction to geology course with the Forestry and Geology Department at Sewanee. From there, he discovered a passion that had lain dormant for many years. The outdoors was something Ethan had been quite fond of from an early age. Although he grew up a "city boy" raised in metropolitan St. Louis, he had the fortunate opportunity to go to summer camp in the middle of the Rocky Mountains in Colorado during the summer, and skied in the Wasatch Mountains of Utah during the winter. It was there that his passions for exploring areas and climbing mountains were first formed. Once he discovered geology at Sewanee, another requirement led him to Forestry, where he found himself enthralled with managing the natural world. Through his 4 years at Sewanee, Ethan spent much of his time exploring the Cumberland Plateau, learning about the hundreds of millions of years represented in the sedimentary rocks as well as the vast species diversity present in the current forests. It was through the forestry and geology

department that Ethan would meet a woman named Catherine Thomas from South Texas who would later become his wife.

It was his passion, ignited for Forestry, that led him to the Colorado Rockies once again, but this time to work for the US Forest Service. Carrying out silvicultural prescriptions, researching sudden aspen decline, cruising and preparing timber sales, wildland firefighting, setting prescribed fires, cone collection in Engelmann spruce-all were activities and pursuits the Forest Service introduced to Ethan. When he moved to San Antonio with Catherine, he found it hard to find something there that suited his interests and began the process of applying to graduate school, where he would later choose NC State as his destination. He hopes to receive his masters and move on to a PhD, hoping it can be somewhere in the western US where he can explore the backwoods, shred the gnar, and raise a family all while trying to research and understand the litany of problems that face this nation's vast forested expanses.

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I would like to acknowledge members of my committee, specifically Dr. John Frampton, for giving me this opportunity, as well as many others, to explore the forestry research world, as well as providing me insight into the disappointing and uplifting nature that research can be, while helping me analyze and put this thesis together, and Dr. Robert Jetton, who despises getting called Dr. but I will do it anyway, who helped me on the track towards Entomology. Finally, I would like to acknowledge Dr. David Tilotta, who never seemed to shut his door when I would come by, usually seeking help and guidance, and who showed me the truly frustrating nature of chemical analysis.

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I would like to acknowledge the Christmas Tree Genetics group, who, even though my study diverged from their primary interests, was there to help me and listen to my research, providing me with insight and encouragement along the way. Specifically I extend thanks to Anne Margaret Braham, Will Kohlway M.Sc, Dr. Lilian Matallana and Dr. Ross Whetten. Also, to my colleagues in the Forest Service, Arthur Haines, Matt Etzenhouser, Dr. Jim Worrall, Suzanne Marchetti and Tim Leishman, thank you all for helping inspire my desire to work public lands and for encouraging me to attend graduate school.

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Chapter 1: Literature Review

Balsam Woolly Adelgid Life Cycle

Balsam Woolly Adelgid (BWA) (*Adelges piceae* Ratz.)(Homoptera: Adelgidae) is an exotic insect introduced into the United States around 1908 in Maine (Kotinsky 1916), and detected for the first time nearly 50 years later in the Southern Appalachians in 1955 on Mt. Mitchell (Speers 1958). Since reaching the southern Appalachians in 1955 and interacting with natural Fraser fir (*Abies fraseri* [Pursh] Poiret), BWA has had a profound effect on the spruce-fir ecotype that is limited to the higher elevation Appalachian forests. Studies from Mt. LeConte, Tennessee, analyzing changes in stand structure between 1979 and 2001 note a significant decline in basal area of overstory living fir, and a significant increase in dead overstory fir, evidence of the longevity and lasting impact BWA has played on southern Appalachian spruce-fir forests (Jenkins 2003). From 1958 to 1960, aerial photography revealed an increase in the number of dead trees on Mt. Mitchell from 11,000 to 200,000 (Amman and Speers 1965). Spread occurred to nearby Roan Mountain in 1962, and Great Smoky Mountain National Park in 1963, with further spread hypothesized to be aided by the completion of the Blue Ridge Parkway (Amman and Speers 1965). In spruce/fir, fir, and spruce/fir/hardwood, ecotypes in which Fraser fir is a major vegetative component, researchers measured 98, 82 and 95 percent mortality respectively of Fraser fir, determined from recurring measurements taken on ecosystem studies established in 1966 (Witter and Ragenovich, 1986). While the decline seen in these forests may also be attributed to a number of factors, including air pollution that stresses trees to the point that innocuous insects become mortality-causing agents, BWA remains a primary agent in the dieback and

mortality of Fraser fir seen in the Southern Appalachian red spruce/Fraser fir ecosystems (Hain 1987). The loss of Fraser fir from these high elevation ecosystems has resulted in changes to the successional trajectory of these forests, species composition changes, and potential adverse effects on water quality (Witter and Ragenovich, 1986).

BWA, like most adelgids, are endemic to Northern Hemisphere boreal and temperate environments (Havill and Foottit, 2007). Since its introduction to North America, different population groups representing different subspecies of *A. piceae* have been identified (Foottit and Mackauer, 1983). One subspecies, *A. piceae* var. *piceae*, is found typically in British Columbia and the Southern Appalachians. *A. piceae* var. *canadensis* is found in maritime Canada and the Northeastern United States. *A. piceae* var, *occidentalis* is another subspecies found mostly in British Columbia (Foottit and Mackauer, 1983). It should be noted that these subspecies are identified mostly by morphologic differences, not genetic differences. Within its native range in Northern Europe, BWA infests European silver fir (*Abies alba* Mill.). Closely related to BWA is *Adelges nordmannianae* (Eckstein) which is holocyclic, meaning is has two hosts, a primary and secondary, within the Caucasus Mountains (Havill et al. 2007). The taxonomy has yet to be fully resolved, but with *A. nordmannianae*, *Picea* spp. are always the primary hosts, while *Abies* spp., constitute their secondary host and the only hosts for BWA (Havill et al. 2007; Hain et al. 1991). Secondary host selection depends on the adelgid species in general, but is always another variety of conifer (*Larix, Pinus, Pseudotsuga, Tsuga* and *Abies*) (Havill et al. 2007). Many species of adelgid are holocyclic, but BWA in Europe and North America is anholocyclic, which means it only has one host. Reproduction occurs via parthenogenesis alone; there is no sexual generation as is found in

holocyclic adelgids (Hain et al. 1991). The life cycle of BWA in North America consists of an egg, three larval instars and the adult. The first instar, a crawler about 0.4mm in length, represents the only mobile phase of BWA (Hain et al. 1991). The crawler is relatively passive and incapable of spreading great distances alone. Abiotic forces, mainly wind, likely govern the manner in which infestations expand into new areas (Amman, 1966). Biotic forces such as birds and other animals can aid in transmission of BWA infestations (Hain et al. 1991). Once a suitable feeding site is found, the crawler phase will insert its stylet where it will remain permanently attached and sessile (Bryant 1976). It then transforms, without molting, into a flat, waxy resting stage (neosistentes) and later molts to the second instar, where the second instar through adult are referred to as the *sistentes* (Hain et al. 1991). It secretes dense woolly material, its namesake, which will provide shelter for all phases except the crawler phase (Balch, 1952). The number of generations produced each year can vary, with higher elevations producing two and lower elevation Appalachian stands producing three (Arthur and Hain, 1984).

The mortality caused by successive infestations of BWA on Fraser fir results from the wound response of the tree itself. BWA insert their stylets into the intercellular space, penetrating the phellum, and feed on the parenchyma (Balch 1952). Sometimes the phloem is accessed by the stylets, but that is normally seen in younger shoots only. Infestations are generally classified as either crown or stem infestations; the two have differing effects on the damages seen to the trees. Crown infestations generally result in gout issues, as a result of feeding on parenchyma cells that cause swelling and abnormal growth. The tendency for BWA to settle at the base of buds has damaging repercussions and inhibits growth, leading to

the development of twisted and swollen branchlets (Balch 1952). This results in growth deficiencies, losses of leader shoots and ultimately, after years of infestation, mortality (Arthur and Hain, 1986). Stem infestations, which cause the production of irregular abnormal heartwood, referred to as 'rotholz', is widely viewed as caused by some interaction between the tree defenses and saliva from BWA (Balch 1952). Balch 1952 also notes that rotholz was similar in appearance to compression wood, although different morphologically, and was formed in greater amounts in those trees with more vigorous growth rates. The formation of this irregular sapwood compromises the ability of the tree to conduct water through the xylem. Indeed, it has been shown that the formation of rotholz and other irregular heartwood does impede the water conductive abilities of fir based on dye movement experiments (Mitchell 1967). Mortality and growth loss due to BWA infestation within *Abies* results from the altered carbohydrate metabolism within an infested tree, most likely due to the interruption of water and mineral transport within the xylem, or the state of physiological drought within an individual tree (Puritch and Talmon-de l'Armee, 1971). Considering multiple species of *Abies* those that are more tolerant to water stress in terms of net photosynthesis loss are also those more resistant to BWA associated mortality (Puritch 1973). These studies give support to both the damage rotholz formation causes within *Abies* in addition to supporting the idea that those trees more intolerant of drought are also more susceptible to BWA.

Mullick 1975, hypothesized that there was an inhibition of the formation of NIT (non-suberized impervious tissue) in *Abies* attacked by BWA, a precursor to the formation of necrophylactic periderm, a non-specialized tree response to wounding by both biotic and

abiotic factors. This has been one proposed explanation for the susceptibility seen to BWA in *Abies* spp. It has been hypothesized that the delay in formation of this periderm is a possible reason for the continuation of the wound response into the xylem of the tree (Mullick, 1975). There is a large correlation of adelgid infestation and rotholz formation, evidenced by studies with *Abies grandis* (Puritch 1977).

Fraser Fir Ecology

Fraser fir is an important species, both economically and ecologically. Ecologically, Fraser fir represents a glacial remnant from the last ice age of the Pleistocene (Buell 1945). Fraser fir today represents the shift in the boreal forest that covered much of the southeastern US during the Wisconsin glacial maximum, and as the climate changed, over time, migration of the species northward restricted Fraser fir to the high elevation areas of the Southern Appalachians (Potter et al. 2005, Delcourt and Delcourt 1984). As Wisconsin glaciation (85,000-11,000 yr before present) destroyed most of the vegetation in Canada and the Northern US, it created favorable conditions for the formation of boreal forests as far south as present day South Carolina (Zavarin and Snajberk, 1972). It was during the warming following the end of the Wisconsin glaciation that *Abies balsamea* (L.) Mill. underwent a drastic reduction in habitat, resulting in the loss of this fir species from many Southern Appalachian peaks, restricting it to an altitude 91-305 m greater than its present lower elevation limit, and forming the modern Fraser fir of the Southern Appalachians (Mark, 1958). Fraser fir occurs in six distinct areas of high elevation Southern Appalachian forests, and is the only Southern Appalachian fir species (Figure 1). The geologic history represented

by Fraser fir's presence in the Southern Appalachains as well as its status as the only *Abies* spp. within this geographic region, the protection and preservation of Fraser fir in its natural stands is justified and important (Hollingsworth and Hain, 1991). In addition to being unique, these high elevation ecosystems are home to a variety of rare endemic species of animals and other plants. Within the high elevation spruce/fir ecotype, two species are federally listed as endangered and ten species are of federal concern (Southern Appalachian Man and the Biosphere 1996). Economically, this tree is an important source of revenue for the state of North Carolina; Fraser fir Christmas trees are an important commodity. In 2012, Christmas trees generated $75 million in total sales- in previous years it generated $100 million in total sales (Potter et al. 2005), and accounted for around 20% of the United States' total Christmas trees (NC Department of Agriculture and Consumer Services). Fraser fir's important ecological and economic value warrants preservation, in both *in situ* and *ex situ* situations. A main cause for concern with BWA infestations is the loss of genetic diversity within the Southern Appalachian Fraser fir populations from which more effective selection of particular traits can be made (Potter et al. 2005). However, recent studies of the effects BWA has had on the Southern Appalachians present a more hopeful picture of this situation. The regeneration-mortality hypothesis (Smith and Nicholas, 2000) proposed that in the face of continued BWA presence, as well as competition with other invasive species and climatic factors, Fraser fir populations would decrease with successive generations. However, high elevation Southern Appalachian spruce/fir stands are currently in a period of recovery, evidenced by a decrease in overstory mortality and recovery of regeneration in the understory, even in the continued presence of BWA (McManamay et al. 2011). Although

potential future climatic changes add uncertainty to the continuation of this trend, McManamay et al. 2011 concluded that BWA would continue to be a disturbance factor, however, with emphasis placed on more patchy disturbance rather than the wide-scale destruction inflicted by the initial invasion. In other areas of the United States, BWA attack has persisted 40 years after initial infestations, and continues to cause mortality in important pioneer species, like subalpine fir (*Abies lasiocarpa*), which has successional ecological implications within the mountainous western United States (Mitchell and Buffam, 2001), indicating that its relevance as a pest continues across a wide variety of *Abies* spp. throughout North America.

Coniferous Defenses

Generally, when viewing resistance to insects, there are a few terms one must understand in order to attempt to categorize that resistance. The terms tolerance, antixenosis, and antibiosis all are types of resistance, but their effects on the insects or pests themselves are quite different. Tolerance generally means that the plant can handle levels of infestation that would ordinarily kill susceptible plants. Antixenosis means the insects do not actively seek out certain plant species for oviposition, feeding or shelter as a result of certain qualities of the plant. Antibiosis is the biological interaction between the plant and the insect attacking it that adversely affects the biology of the pest (Painter 1958). Within these broad categories lies the idea of constitutive and induced defenses. Constitutive defenses are those defenses that the plant has already prepared should an insect attack occur, where induced defenses are those defenses the plant prepares upon attack, or things that are not readily

prepared (Larsson, 2002). The difference between what is considered a constitutive and induced defense is very vague, however, and worth noting. Constitutive defenses are largely viewed as the "first line of defense" for any plant, however, inoculation with a pathogen or insect attack can serve to induce systemic induced resistance (SIR), or basically a form of immunization of a tree or other plant from further attacks (Bonello et al. 2006).

Chemical biology of plant species in general is an important field in understanding the ability of plants to self-regulate. Understanding chemical biology also helps identify differences within species, as it does between species. Investigations of the chemical biology of various provenances of Scots pine (*Pinus sylvestris* L.) showed that pines from certain provenances in the north and south of Finland had the largest total monoterpene content, while simultaneously giving researchers the ability to assess correlations between provenance chemical biology and known growth characteristics with trees from those provenances (Maninnen et al. 2002). As plants age, changes occur in their biochemistry. One foliar chemical, 13-keto-8(14)-podocarpen-18-oic acid, determined 63.5% of the feeding deterrence of multiple species of sawfly larvae (*Neodiprion* spp., Midd.) (Hymenoptera: Diprionidae), but this chemical decreased as the foliage matured, triggering oviposition by adults (Ikeda et al. 1977). Addressing the differences in Norway spruce [*Picea abies* (L.) Karst] seedlings by age enabled researchers to focus on selecting certain features of a tree that may make it less favorable to feeding from certain pests, specifically selecting for higher amounts of green leaf volatiles (GLVs) that could potentially delay or prohibit selection for feeding by certain species within the *Hylobius* genus (Kannaste et al. 2013).

Within the framework mentioned above, resistance relates heavily to the biochemistry of the tree and the secondary metabolites it produces naturally. Coniferous trees have well-developed defense systems, useful for defense against insect herbivory and pathogens (Phillips and Croteau, 1999). However, these developed defenses can play a role in host selection as well. Different species of bark beetles are able to manipulate conifer defenses in many instances to aid in pheromone production that aids in signaling suitable host selection for mass attack or for sexual attractants, as well as cessation of attack to prevent competitive mortality amongst a population (Borden 1989). Looking more in depth at the kairomone/synamone/allomone complex between insects and plants, the potential for new management strategies increases. Kairomones are those chemicals that are harmful to the sender and beneficial to the receiver, allomones are those that harm the receiver and benefit the sender, and synamones are those that benefit both the sender and receiver. Success stories, such as the boll weevil (*Anthonomus grandis* Boheman)(Coleoptera: Curculionidae) eradication program, that involved isolation of the sexual pheromones as attractants to females (Tumlinson et al. 1969), and the ensuing use of substances developed from that, such as grandlure, enabled land managers to accurately trap down populations as well as provided more detailed survey and accurate deployment of insecticides to combat this invasive pest (Benedict et al.1985: Dickerson et al. 2001). Using pheromone complexes has also been transferred into the forestry world more recently. Isolating the aggregation pheromone used by many different species within the genera *Dendroctonus* and *Ips*, and combining it with certain host-tree monoterpenes (α-pinene and myrcene), researchers found the combinations differed in their ability to both attract members of their own species as well as certain

combinations serving to attract predatory species (Hofstetter et al. 2012). Certain predatory species seemed to cue in more on the tree terpenes than others. Similarly, (E)-β-farnesene has been shown to be a volatile released by potato plants infested with *Myzus persicae* (Homoptera:Aphididae) (Harmel et al. 2007), and served as a cue for oviposition by a common predator of *M. persicae*, *Episyrphus balteatus* (Diptera: Syrphidae), when compared with non-infested potato plants (Verheggen et al, 2008). Phillips and Croteau 1999 noted the potential genetic role of secondary metabolite formation and its potential in breeding resistance in plants.

Volatile organic compounds (VOCs) have shown their ability to act as antifeedants. Estragole and bornyl acetate, VOC's found infrequently in spruce seedlings, have a demonstrated antifeedant capability against pine weevil (*Hylobius abietis* L.) (Coleoptera: Curculionidae)(Klepzig and Schlyter, 1999), and thus, their infrequent appearance in VOC analyses has led researchers to hypothesize about them being a potential marker of resistance to certain pests, like the pine weevil (Kannaste et al. 2012). However, the antifeedant behavior elicited depends on both the plant, and the feeding preferences of the insect. The difference in plant resistance to polyphagous insects, or more generalist feeders, compared with oligophagous and monophagous plant feeders (more specific in their plant host species choice), is that the more specific the feeder, the more plant-borne stimuli are likely to affect that feeding (Beck, 1965). The role of plant chemistry and VOCs are crucial towards understanding potential resistance mechanisms among closely related plant species, as well as understanding chemical cues insects are attracted to, and that influences activities from oviposition to feeding cessation (Beck, 1965).

Looking at hemlock woolly adelgid (*Adelges tsugae* Ratz.) (Homoptera: Adelgidae) (HWA), studies comparing within and among *Tsuga* species populations, potential constitutive terpene defenses for resistance work were identified. Total monoterpene amounts were found to be different between resistant and susceptible varieties of *Tsuga canadensis* (L.) Carr. although the exact extent that all the terpenes identified play in resistance has yet to be addressed, with regards to their actual biological activity on HWA (McKenzie et al. 2014). When assessing species and susceptibility differences, Lagalanate and Montgomery, 2003, identified potential candidate chemicals that are potentially linked to susceptibility and resistance that await future laboratory evaluation. Identification of potential targets within constitutive defenses of trees, and differentiating test subjects by resistance ranking enables researchers to target the potential chemicals that confer resistance to a specific pest within a population.

With respect to the *Abies* genus specifically, few insights on how differences in VOC content may prove useful for understanding potential chemical changes that could confer resistance to BWA on a species or variety are available. Studies have shown age-related differences in foliar nitrogen: tannin levels and terpene contents of *Abies balsamea* influence feeding rates of spruce budworm larvae (*Choristoneura fumiferana* Clemens)(Lepidoptera: Tortricidae) (Bauce et al, 1994), but regarding BWA, studies identifying differences within *Abies* and their effect on BWA are relatively few. However, a tremendous amount of intra- and interspecies variation exists, even within the same genus, with respect to VOCs and monoterpenes, as well as tree responses to infestation with BWA. Initially, it was thought that the Mount Rogers Fraser fir population was more tolerant to *A. piceae* injury than other

populations of Fraser fir, based on the low relative morality experienced by fir at Mount Rogers while other populations suffered heavily mortality (Hollingsworth and Hain, 1992; Arthur and Hain, 1987; Sutton et al. 1997, Arthur and Hain, 1986). Proposed ideas for this intraspecific variation included bark thickness and increased outer bark production (Hollingsworth and Hain, 1992), total monoterpenes content (Sutton et al.1997; Arthur and Hain, 1987), and greater concentrations of juvabione, considered a potential resistance marker, at Mount Rogers locations (Fowler et al. 2001). However, significant mortality has occurred within Mt. Rogers fir stands, as Sutton et al., 1997 noted in the conclusion of their study, bringing to question the validity of this view. A more thorough understanding of the *Abies* genus and the chemical differences among species would be useful in order to understand potential avenues to search for, and understand chemical differences within and between *Abies* spp.

BWA Management Strategies

The use of plant-originated substances to control insects is well documented within the history of insecticides. Synthetics within the class of insecticides known as neo-nicotinoids are synthetic mimics of nicotine, derived from cultivated tobacco (*Nicotiana tabacum* L.), just as pyrethroids are synthetic mimics of pyrethrum, a well known insecticide derived from chemicals produced in chrysanthemum (*Chrysanthemum* spp. L.). However, the largely unregulated use of substances similar to those mentioned above, as exposed by the landmark publication of Rachel Carson, Silent Spring, in 1962, led to a change in how the United States views insecticide use, and gradually brought about the idea of Integrated Pest

Management (IPM), where many different management options are utilized to both prevent insect and pathogenic attack, rather than just one. The discovery of insect growth regulators (IGR) was made when hemipterans, reared with balsam fir-derived paper in chambers, continued molting rather than maturing into adults (Sláma and Williams, 1965). Juvenile hormone, for the insect *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae), was later identified as the methyl ester of todomatuic acid, or the 'paper factor' of the previously unknown source of juvenile hormone to the original failed colonies. This was determined from analysis, extraction and testing of extracts of balsam fir material on *P. apterus* (Sláma and Williams, 1965; Bowers et al. 1966). More recently, derivatives like azadirachtin from the neem tree (*Azadirachta indica* A. Juss.) (Meliaceae) have been shown to be quite useful as an antifeedant, an IGR and a reproductive inhibitor (Hoffmann and Lorenz, 1998). IGRs have become an important alternative to pesticide use, because they disrupt the endocrine systems of insects, interfering with development, reproduction and metamorphosis, and they provide enhanced specificity compared to commonly used synthetic pesticides, but are typically slower acting (Hoffmann and Lorenz, 1998).

With BWA, pesticide chemical controls such as, lindane, esfenvalerate, endosulfan and horticultural oils are the most widely used in plantation settings, although they are quite costly (upwards of $741-1235 per ha) (Potter et al. 2005). It has also been shown that top dipping Pacific silver fir (*Abies amabilis* Dougl.), a highly susceptible fir species, with overwintering adelgids in 1% propoxur suspension, 2% carbaryl suspension, 2% insecticidal soap solution and 0.5% permethrin emulsion up to their roots all provided sufficient protection of Pacific silver fir in nursery settings (Puritch et al. 1980).

Biological control of BWA in the form of predatory insects introduced from other parts of the world has proven insufficient at control. Fifteen species of predators introduced into North Carolina between 1961-1965 were not able to establish themselves in the Southern Appalachians, likely because of climatic differences between native habitat and NC, as well as poor prey acceptance (Amman and Speers, 1971). Similar results have been reported in biological control situations in the Pacific Northwest, as introductions of predatory species from seven countries resulted in a failure to establish, despite populations of BWA (Mitchell and Wright, 1967). There has been some research in potential biocontrol involving HWA. The predator *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) has shown some success at being reared on diets of both BWA and HWA, and could potentially feed on BWA while HWA populations are in diapause, as those HWA stages in nature usually do not provide enough sustenance to maintain large populations (Jetton et al. 2011). While biocontrol is only one of a few of the scenarios that can be employed using IPM, their lack of great success at management of BWA populations highlight the need to further understand the mechanisms that govern BWA resistance in *Abies* spp.

Resistance to BWA has been studied within the *Abies* genus. Table 1 shows a variety of *Abies* species and their relative susceptibility/damage rating for BWA infestation (Mitchell, 1966). Newton, 2007 assessed methods of artificial infestation, determining that hanging bolts of infested logs over seedlings was the most efficient method of artificial infestation for future susceptibility/resistance studies, while also confirming some of Mitchell's 1966 earlier susceptibility studies. Utilizing efficient methods of artificial infestation is integral towards studying potential resistance mechanisms, offering the ability

to ensure adequate infestation while altering certain variables and trying to understand the outcome to elucidate potential management action in the field.

Silvicultural management strategies have not shown any more promise at reducing infestation severity than biological control of BWA. The best management strategies from a silvicultural standpoint are removal of the infested trees. But this removal is best practiced during the winter month as the insect diapauses. The transport of infested wood must be handled carefully, and steps taken to ensure that equipment used in infested tree removal are sanitized to prevent spread to new areas devoid of infestation (Balch and Carroll 1956). Other strategies, such as pre-commercial thinning of balsam fir stands have shown lighter infestations, or at least less damage than non-pre-commercially thinned stands (Milne, 1990). But, overall silvicultural management has not had a significant impact on BWA populations.

Gas Chromatography and Mass Spectrometry

At a very basic level, the tandem use of Gas Chromatography and Mass Spectrometry (GC/MS) is employed to determine what chemicals comprise an unknown substance and their relative amounts. Generally, the identification of chemicals in a substance is referred to as qualitative analysis, and the determination of the amounts of certain substances is considered quantitative. Gas chromatography and mass spectrometry are incredibly useful tools for researchers in identifying and quantifying the biochemical profile of a plant, which has numerous applications to our understanding of the natural world.

Gas chromatography takes a gaseous solute, and transports it through a column with a stationary phase (Harris, 2000). While one can directly sample volatile components through

the use of a gas-tight syringe, many times the samples placed in the GC are not in gaseous form. The purpose of the injector on a GC is to instantly vaporize a sample and convert it to gaseous form, where it is then transported by the mobile phase through the column. Injectors are normally kept at very high temperatures to ensure vaporization of the sample. The stationary phase is normally a poly(dimethylsiloxane) of some variety, and is necessary to partition the injected chemicals between it and the mobile phase, which is normally a gas like helium, hydrogen or nitrogen. The overall process of gas chromatography is to separate chemical components of a sample by passing them through the column, with the only stipulation being the solvent moving the solute through the column is a gas, as opposed to liquid chromatography where the mobile phase and chemical components are liquids. Separation generally occurs through adsorption qualities of the solute that is being tested (Harris, 2000). Retention times are based on how long it takes the solute to elute through the column. Those that are adsorbed more within the column, have longer retention times than those that do not and move more freely through the column. Coupling gas chromatography with mass spectrometry enables users to begin to identify compounds eluted from the gas chromatograph based on their mass fragmentation patterns (Harris, 2000). Ionization of gaseous compounds within the mass spectrometer will separate them based on their relative mass and produce a spectrum, which can be used to directly identify what the compounds are, or to compare the spectrum of unknown compounds to a vast library of known compounds.

GC/MS analysis yields a detailed record of volatized substances contained within a sample, even when they are present in trace amounts. These techniques have been developed

to enable researchers to accurately identify chemical components within their respective professional arenas. The usefulness of GC/MS extends to many different disciplines. For example, GC/MS enables food science researchers to better understand flavor profiles for better production of popular food items (Dong et al. 2013). In forestry, it allows researchers to identify the metabolite differences between BWA infested trees and uninfested trees or to identify how BWA changes tree chemistry (Arthur and Hain, 1987). Identification of volatiles, which are those chemicals that have high vapor pressures and volatize into the atmosphere easily, is important within the natural resource community. Understanding the differences in volatile contents can help identify differences in species of tree, as well as understand the usefulness of certain metabolites to the interaction pathways between insects and plants (Wajs et al. 2007). Using GC/MS enables researchers to understand seasonal foliage changes, identifying those trees with certain terpene increases useful for defense from herbivory associated with spruce budworm (Zou and Cates, 1995). Its usefulness in forestry and agriculture has enabled researchers to identify volatile organic compounds that are of importance to resistance to insect attack (Klepzig and Schlyter, 1999; Kannaste et al. 2012; Hofstetter et al. 2012).

Using traditional extraction methods for identification purposes can be costly, in both sample material amounts and solvent amounts necessary (Wajs et al. 2007). Utilizing techniques for studies aiming to uncover volatiles rather than determining their relative amounts is much more attractive. Liquid sampling is one popular way of analyzing solutes of interest to research and industry. It entails the extraction of analytes into a medium that they can solubilize within, normally non-polar substances in GC/MS, as water tends to be

damaging to the instruments. Basically, the idea behind liquid sampling is to dissolve, extract or convert one substance into liquid and then insert that liquid into the GC/MS where it is volatized and transported into the column for analysis by the mobile phase. There are many ways of getting compounds into a liquid for analysis; it often depends on what type of study (qualitative or quantitative) is being conducted. A very simple method of extraction is solid-liquid extraction, which is very similar to how one makes coffee, placement of a solid in a liquid that its components can solubilize within, filtering that liquid to remove any remaining solids and injection of the liquid into the GC/MS for analyzing components. The key for any analysis is to determine what medium will be best for capturing the substances of interest. Often, very small amounts of the solution is necessary for analysis in a GC/MS, mostly less than 2 microliters, as high volumes of liquids require more detailed processes to enable their analysis. Liquid sampling is a very useful method of analyzing chemical components of a sample.

The process of collecting volatized substances from samples can be done through sampling of the headspace above a sample or the direct sampling of a volume of gas collected directly from the headspace above a sample. Solid-Phase Micro-Extraction (SPME) is another tool that has been developed for more quickly analyzing volatile contents of various substances without using a gas-tight syringe. Gas-tight syringes take contents of the headspace and inject them directly into the GC/MS where they are carried into the column by the mobile phase. In contrast, SPME utilizes a coated fiber, which is heated upon insertion into the GC/MS and the contents transported to the column. While traditional gas chromatography is time consuming and expensive, due to costs of solvents necessary to

conduct quantitative studies, SPME is a much simpler and more cost-effective method to determine the volatile composition of samples (Todisco et al. 2014). SPME requires closed containers, and is very susceptible to corruption by environmental contaminants, and the condition of the fiber (Wajs et al. 2007). SPME is a sampling of those volatile components that will fill the volume of the headspace, or the area above and surrounding the sample in question. The volatiles that fill the headspace are then adsorbed and concentrated onto a thin filament surrounded with polymer (Vereen et al. 2000). Due to the fragile nature of the filament, it is normally sheathed when piercing any rubber septum, both those of sample vials and the GC/MS injector. Upon placement of the syringe in the injector, the plunger pushes the filament out of the syringe where the heat of the injector thermally desorbs the analytes into the GC/MS. Because the fiber is reaching equilibrium with the volatile chemicals in the container it is placed into, extraction times vary by chemical. This requires the determination of exactly how much time is necessary for the accurate sampling of the volatile contents of the headspace. In GC/MS analyses, SPME fibers are placed in the injector where the compounds that have adsorbed to the filament are desorbed within the injector and analyzed by the GC/MS (Vereen et al. 2000). The main advantages of SPME as a technique are the speed, sensitivity and ability to determine volatiles without the use of solvents (Vereen et al. 2000). SPME is an incredibly useful technique for qualitative identification purposes, but is difficult to use when conducting quantitative studies (Wajs et al. 2007). It has been shown to be reliable at determining volatile contents of the stemwood of selected conifers, and is much easier than other methods, like hydrodistillation, which finds similar amounts of VOCs within the stemwood, but requires a much longer extraction time, and a larger amount of

sample to yield results similar to SPME (Wajs et al. 2006). Using techniques like SPME, the analysis of compounds within coniferous species is now much more accessible (Cvilickova and Kuban, 2004). Understanding the volatile makeup of plants enables researchers to understand the interactions that enable bark beetles to locate suitable hosts (Hofstetter et al. 2012), determine the preferences for certain species of mites (Xugen and Luqin, 2006), and determine potential target chemicals for resistance to hemlock woolly adelgid (*Adelges tsugae* (Lagalante and Montgomery, 2003), among many other potential interactions. SPME is a very useful tool for researchers in identifying differences and similarities between groups based on volatile content.

Research Goals

Research goals for this project are multidisciplinary. I will attempt to chemically differentiate species of fir with varying levels of BWA resistance using multiple methods of GC/MS analysis. The goal of identifying chemical differences will elucidate possible avenues for research on the respective chemicals and their effect on reproduction, survival and insertion on BWA. I will test both susceptible and resistant species of fir for comparison, and identify differences in chemical composition, or relative (qualitative) differences between chemical amounts within each species.

In Chapter 2, using GC/MS of acetone extractions, bornyl acetate was identified as an obvious chemical difference between BWA-resistant Veitch fir and BWA-susceptible Fraser fir. With this in mind, a study attempting to assess whether bornyl acetate and isobornyl acetate present in increased amounts of a Fraser fir cutting would result in lower insertion

success by crawlers compared to controls of Veitch fir and Fraser fir in water was carried out. Concurrently, the same bornyl acetate solution was compared to pure bornyl acetate with regards to their impact on eclosion success of BWA eggs.

Studies presented in Chapter 3 will attempt to influence bornyl acetate concentration differences within the headspace of a closed jar to elucidate how altered concentrations of this terpenoid influence eclosion success of BWA eggs as well as effect the reproductive abilities of a proxy insect, the green peach aphid (*Myzus persicae* Sulzer) (Hempitera: Aphididae). Chapter 4 will be a large qualitative study on the constitutive chemical biology of those *Abies* species with known damage ratings, from nil to severe, to BWA. Three arboreta from across the United States will provide *Abies* branch samples. SPME will be used to assess biochemical profiles of these species and chromatographic information will be analyzed to identify those chemicals most associated with resistance and susceptibility to damage from BWA. These target chemicals identified will be useful for future testing to identify biological impacts of these chemicals, both individually and synergistically, on BWA.

Ultimately, these differences that are identified through various methods of GC/MS testing we hope will reveal part of the resistance mechanisms employed by different species of fir, to better understand how trees are able to naturally defend themselves from this pest's effects on growth and survival.

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Table 1: Damage rankings of various *Abies* species and their native geographic areas

Scientific Name	Common Name	Origin	Damage Rating[1]
Abies lasiocarpa	Subalpine fir	Western North America	Severe
A. fraseri	Fraser fir	Eastern U.S.	Severe
A. balsamea	Balsam fir	Northeastern North America	Severe
A. amabilis	Pacific silver fir	Northwestern North America	Severe
A. grandis	Grand fir	Western North America	Moderate
A. lasiocarpa var. *arizonica*	Corkbark fir	Southwestern	Moderate
A. magnifica var. *shastensis*	Shasta red fir	Western U.S.	Moderate
A. koreana	Korean fir	Korea	Moderate
A. sachalinensis	Sakhalin fir	Northeastern Asia	Moderate
A. religiosa	Sacred fir	Southern Mexico	Slight
A. procera	Noble fir	Western U.S.	Slight
A. concolor	White fir	Western U.S.	Slight
A. alba	European silver fir	Western Europe	Nil
A. cephalonica	Grecian fir	Greece	Nil
A. pinsapo	Spanish fir	Spain	Nil
A. sibirica	Siberian fir	Northern Asia	Nil
A. firma	Momi fir	Japan	Nil
A. veitchii	Veitch's silver fir	Japan	Nil

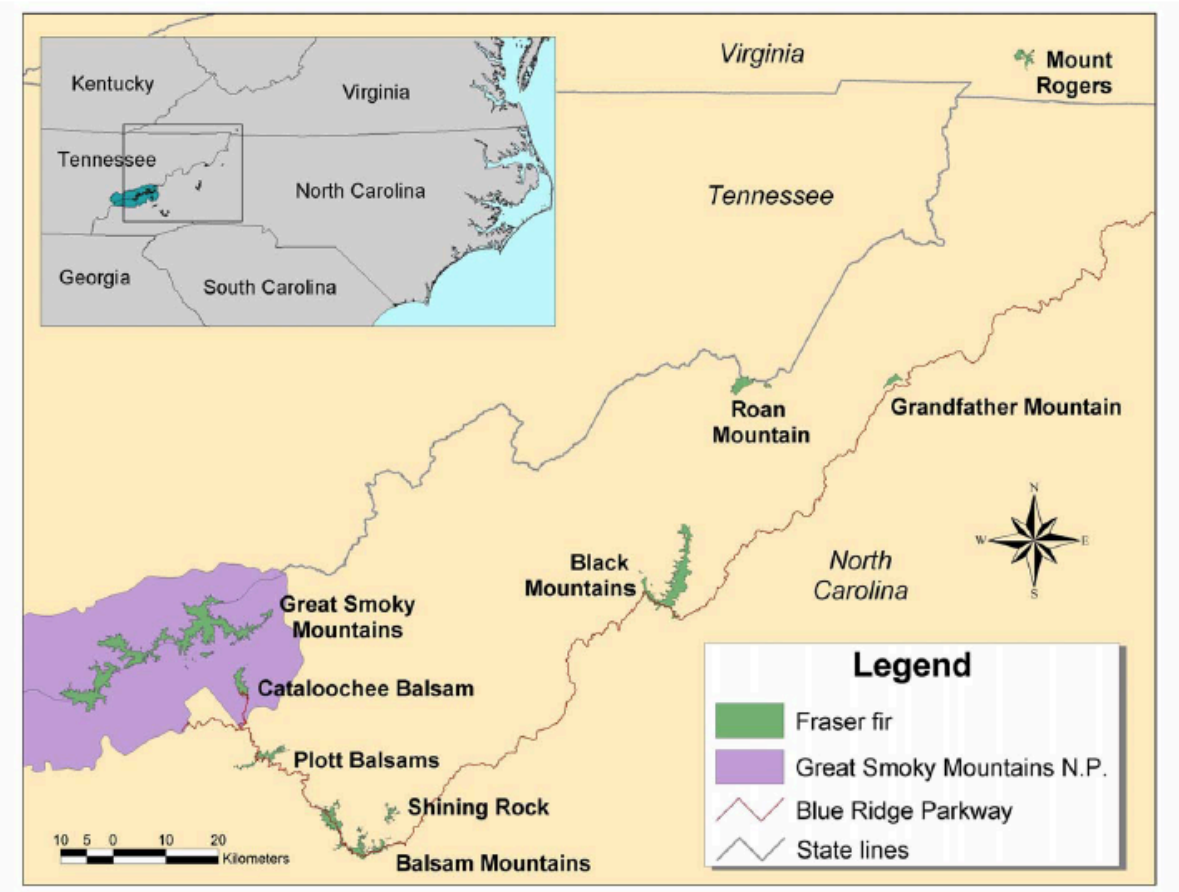

Figure 1: Natural Fraser fir distribution in the Southern Appalachians (Potter et al. 2005)

Chapter 2: Identification of Chemical Differences Between Fraser and Veitch Fir and their Effect on Balsam Woolly Adelgid Insertion and Egg Eclosion

Introduction

Balsam Woolly Adelgid (*Adelges piceae* Ratz.) (Hemiptera: Adelgidae) (BWA) is an exotic invasive insect pest within the United States and native to Northern Europe. Introduced in the early 1900s to upper New England, BWA worked its way south along the Appalachian spine, eventually reaching the Southern Appalachians by 1955 (Speers, 1958). Upon reaching the Southern Appalachians, it wrought serious damage on the only native fir species in the Southern Appalachians Fraser fir (*Abies fraseri* [Pursh] Poiret). Mortality in 1966 in all three main fir-ecotypes (fir, spruce/fir, and spruce-fir-hardwood) was measured at 82, 98 and 95% respectively, with significant reductions in Fraser fir seedlings/hectare between 1966 and 1978 (Witter and Ragenovich, 1986). Initial rates of spread, studied with aerial photography, placed an estimated 11,000 trees killed by 1958, with surveys in 1960 indicating over 200,000 trees had been killed by this pest (Amman and Speers, 1965). Fraser fir has a very narrow window in which it occurs in the Southern Appalachians. It is a dominant tree present around 1371 meters and above in elevation with species composition of stands varying due to topography and aspect, among other things (Whittaker, 1956). Fraser fir holds most of its economic value as a Christmas tree species, bringing in around $75-100 million, depending on the year, in revenue to North Carolina annually (Potter et al. 2005). It is also an important forest ecotype that supports many different endangered species of flora and fauna to the Southern Appalachians, and holds significant recreational value for tourists and hikers (Hollingsworth and Hain, 1991) (Whitaker, 1956).

Resistance to pestiferous insects is a large field, with many projects aimed at either determining the mechanisms responsible or testing varieties of plants that are deemed resistant. But resistance to a certain insect manifests itself several ways. Traditionally, it is viewed from the concepts of non-preference (antixenosis), defined by some quality of the plant preventing selection by the insect for oviposition, feeding and shelter or antibiosis which is defined as a quality of the plant that has a negative effect on the biological processes of the insect (usually resulting in death). Tolerance is a plant that can survive infestation levels that would cause other susceptible plants to succumb and die (Painter 1958). The interaction of plant metabolites and insects is widely used when searching for resistance, as these metabolites are viewed as evolutionary traits. Plants evolved suites of secondary metabolites that can both confer resistance and be utilized by insect species as chemical cues for host acceptance (Beck, 1965). Ikeda et al. 1977 identified one foliar chemical, 13-keto-8(14)-podocarpen-18-oic acid, responsible for feeding deterrence of sawfly larvae (*Neodiprion rugifrons* and *N. swanei* Midd.) (Diptera:Diprionidae) and adult oviposition. Western Pine Beetle (*Dendroctonus brevicomis* LeConte) (Coleoptera: Curculionidae) (WPB) is a common pest of *Pinus ponderosa* Douglas. in the Rocky Mountain West. Verbenone has been identified as an anti-aggregation pheromone used by WPB to identify when a host species is sufficiently infested, reducing competition-caused mortality between individuals within the same host tree. While studies that have tested the efficacy of pouches of verbenone have found that it is ineffective as a treatment in an open-air environment, they do suggest that use with other semiochemical clues from the host species could prove effective at management of WPB (Fettig et al. 2009). Other studies have used volatile

emissions from host plants and have studied the behavior of predatory species as a result. (E)-β-Farnesene (EBF) is a volatile that is released by potato plants when a certain species of aphid attacks it (Harmel et al. 2007). Verheggen et al. 2008 studied how oviposition behavior of *Episyrphus balteatus* DeGreer (Diptera: Syrphidae), an aphidophagous hoverfly, was affected by this particular volatile and found that predatory behavior was induced by this particular volatile, a relationship that has developed to aid predators finding their prey. Understanding chemical cues of insects searching for adequate hosts, as well as those of plants defending themselves, are important from a management perspective, but are also useful in uncovering resistance mechanisms associated with them.

Mitchell, 1966 evaluated the interaction between BWA and many different species of *Abies*. He developed a preliminary rubric for these eighteen species of fir and how they are affected by BWA infestations. Those that were the most susceptible were given a "severe" rating, followed by moderate, slight and nil. The *Abies* species of main interest to the following studies are Veitch fir (*Abies veitchii* Lindley) and *A. fraseri*. Although they are native to areas outside the endemic habitat of BWA, they are on opposite ends of the susceptibility spectrum. Even the BWA host European silver fir (*Abies alba* L.) is listed as tolerant, which means infestations can survive without damage to the host organism. The tolerance of *A. alba* is most likely due to its coevolution with BWA along with host alternation. Within its native habitat, *A. piceae* is closely related to a holocyclic adelgid (asexual and sexual generations), with host alternation (Havill et al. 2007). Host alternation has been proposed as one potential resistance mechanism. While the insect population expands on the primary host (a member of *Picea*), the secondary host (*A. alba*) may recover,

and vice-versa (Havill and Foottit, 2007). However, BWA is anholocyclic, which means it does not have any sexual generations and reproduces via parthenogenesis (Hain et al. 1991). With *A. veitchii* and *A. fraseri* that relationship does not occur. *A. veitchii* is found entirely in Japan, and *A. fraseri* is restricted to the Southern Appalachians. The one difference is in their susceptibility levels. Veitch fir is wholly resistant while Fraser fir is completely susceptible (Mitchell, 1966). Understanding differences between these two species could potentially illuminate resistance mechanisms and/or susceptibility characteristics thus highlighting avenues for tree breeding and genetics research.

From qualitative GC/MS analyses, discussed later, a starting point for highlighting the differences between Veitch and Fraser fir, the terpenoid bornyl acetate was identified as being present in higher quantities in Veitch fir compared to Fraser fir. Bornyl acetate stood out as a potential avenue for resistance research based on the stark discrepancy from GC/MS acetone-soluble compound analysis, as well as mixed research reports regarding its role in plant defense. With regards to the latter, bornyl acetate fed at high concentrations to western spruce budworm (*Choristoneura occidentalis*) (Lepidoptera: Tortricidae), significantly reduced survival of larvae into the adult stage, and when combined with high levels of nitrogen significantly reduced larval growth rates (Cates et al. 1987). Bornyl acetate, among other Douglas-fir (*Pseudotsuga menziesii* Mirb.) foliar terpenes, was indicated as one of the more important determinant factors of female dry-weight for spruce budworm (Cates et al. 1983). It was also shown to be important across two populations of spruce budworm in reducing larval weight and growth (Zou and Cates, 1997). Bornyl acetate was also identified as the acaricidal component of the oil derived from *Juniperus chinensis* var. *globosa,* and has

been indicated as a possible management tool for house dust and stored food mites (Lee et al. 2009). Studying variation in Douglas-fir terpene content throughout the growing season, increases in bornyl acetate concentration have been identified for potential management strategies acting as deterrents to feeding activity from the spruce budworm (Zou and Cates, 1995). Bornyl acetate has also been suggested to not result in any effect on larval growth or survival of 4 selected species of tussock moth (Lepidoptera: Lymantriidae) (Raffa and Powell, 2004). Bornyl acetate has been shown to be a mild antifeedant when administered to the pine weevil (*Hylobius abietis*) (Klepzig and Schlyter, 1999). It has also been found to be a large component in the essential oils of *Eupatorium adenophorum,* which have a studied and profound antibacterial, antifungal and phytotoxic effect, potentially a synergistic effect of all the components that comprise these essential oils (Ahluwalia et al. 2013). When considering carrot fly larva, *Psila rosae* Fab. , bornyl acetate is a feeding attractant (Ryan and Guerin, 1982). Isobornyl acetate, an isomer of bornyl acetate, has been shown to be present in higher concentrations in species of *Tsuga* that are more susceptible to hemlock woolly adelgid (*Adelges tsugae*) (Lagalante and Montgomery, 2003). In addition, although it reduces larval weight of spruce budworm when fed at higher levels than those found in balsam fir needles, its efficacy may be increased with other chemical constituents and terpenes present in the plant structures of note (Kumbasli and Bauce, 2013).

Due to the specific nature of the literature surrounding bornyl acetate and its biological activity on insects, investigating the effects it has on BWA may prove useful to determine whether or not the differences seen in relative abundance between susceptible and resistant species of *Abies* to BWA is a contributing factor towards general BWA resistance.

Testing insertion choices by BWA is one measurement of antixenosis-type resistance. Testing effects of terpenes on BWA egg eclosion is one test of antibiosis resistance. Understanding secondary metabolites and their biological activity on insects is important to resistance studies. Further comprehension of the biologic impact of chemical defenses produced by resistant varieties of plants on pest species enables a fuller understanding of the natural world around us, as well as increases our abilities to understand their genetic foundations. Resistance to insects is likely not governed by one metabolite alone (Painter, 1958), but seeing if a particular terpenoid, or other chemical, has an effect on BWA will enable us to look for potential synergistic effects it has when the whole suite of secondary metabolites are investigated, enabling researchers, tree breeders and land managers to begin implementing changes to better protect the natural environment. Therefore, I will attempt to alter the concentration of both bornyl acetate and isobornyl acetate within cuttings of Fraser fir, and identify if any differences in insertion success. If bornyl acetate acts as a repellant to insertion by BWA crawler, then I will expect to see fewer adelgids inserted on Fraser fir cuttings with increased bornyl acetate concentrations compared to controls and isobornyl acetate. I will also attempt to elucidate whether bornyl acetate has an effect on fecundity of BWA. I predict that eggs in the presence of bornyl acetate will eclose at lower rates than those eggs separated from bornyl acetate sources as well as those eggs around Veitch fir will eclose at a lower rate compared to those near Fraser fir volatiles.

Methods

Gas Chromatography/Mass Spectrometry Analysis

Branch samples of Fraser fir and Veitch fir were obtained from Avery County, North Carolina in December 2013 (35.952117, -81.890290). Branches were removed from three Fraser fir and three Veitch fir individuals, stored in plastic bags and placed in a cooler with ice for transport back to Raleigh. They were then kept in a walk-in cooler at 5°C until ready to be used for extraction. Extraction of the chemical components of the woody material was carried out in 99.5% pure acetone (EMD chemicals). Samples from Veitch and Fraser fir were defoliated, cut into smaller stem material and weighed before being placed in glass vials filled with 10 mL acetone for a week. After a week's time, the liquids were removed from their jars with fir material, filtered and placed into smaller sampling jars labeled accordingly. 1 microliter of this material was then injected into a GC/MS for analysis.

All GC/MS sampling was conducted on a Thermo-Finnegan Trace GC with coupled Polaris Q Mass Spectrometer. The column was an Agilent Technologies DB-1701 60 m x .0250 mm x 0.25 micron length. All MS analysis and identification were done using the NIST library database. Oven temperatures were held for 4 minutes at 45°C and ramped from that point onward at a rate of 5°C per minute until oven temperatures reached their peak temperature at 280°C, where it was held for 5 minutes. Run times were 56 minutes.

Uptake Experiment

Infested Fraser fir logs were obtained from an abandoned Christmas tree plantation on the border between Allegheny and Ashe Counties, North Carolina (36.356972, -81.232002) in mid-May of 2014. The trees with more woolly masses were selected, cut, pruned and

placed in a truck for transport to Raleigh, where they were suspended from wire racks. Standard size (7.62cm x 12.7cm) note cards were placed underneath with sticky tack spray to assess whether crawlers were present. Once crawlers were detected, the experiment was set-up. Three cuttings of Fraser fir and one Veitch fir cutting made up a complete rep. Treatments were 35 ppm bornyl acetate solution, 35 ppm isobornyl acetate solution, and 2 distilled water vials. 50 mL plastic vials were filled to 45 mL and had the cuttings placed in solution for uptake. The placement of each treatment within its row was randomized. Three reps were placed on metal vial holders placed under the suspended infested logs for crawlers to fall onto the cuttings and establish infestations. There were 10 holders, for a total of 30 repetitions for each individual treatment. There were 2 shelves with 4 metal racks (each containing 3 reps) and one shelf that had 2 metal racks. Figure 1 shows a picture of the setup. Density counts on sticky cards were conducted every other day to ensure crawlers were continuing to fall and metal racks were rotated each day (i.e., 1 moved to 10 spot, 10 moved to 9 etc). Each day, solution levels were assessed to make sure that the cutting ends were always submerged. If one cutting needed solution, all the cuttings were given their respective solution. Before refilling each plastic vial, solution uptake was noted, and recorded. At the end of the 7-day period, all cuttings were taken out of solution, placed in well-marked bags and frozen at -20°C to await microscopic inspection which was conducted a few days after freezing to ensure that all biological activity of any adelgids present had ceased. All adelgid counts were done underneath a dissecting microscope. Any adelgids found inserted on the cuttings were counted and tabulated for data analysis.

In addition to the reps done with infested bolts hanging overhead, an additional metal rack with three reps was kept surrounded by fabric impenetrable by BWA and placed away from the experiment, so as to not allow infestation. These cuttings were kept in solution and then removed, weighed, and placed in acetone to sit for a week. They were then run through the GC/MS to analyze whether or not significant or noticeable changes in chemical concentrations within the cuttings had been achieved.

Petri Dish Experiment

Run concurrently with the above uptake experiment, a vented Petri dish experiment was conducted to ascertain any effects on egg eclosion of BWA. 20 egg masses were counted for egg numbers and averaged to determine the amount of eggs that would be placed on the cuttings. After determining the average, 4 egg masses were checked to ensure presence of BWA eggs before placement of the masses on cuttings. Moistened filter paper was placed at the bottom of each Petri dish to help maintain humidity. Figure 2 shows a picture of the setup of this experiment. Before placement of the egg masses on the cuttings, 3 Fraser fir samples were treated with distilled water, the bornyl acetate solution (35 ppm) from the uptake experiment and pure bornyl acetate (Sigma Aldrich). A Veitch fir cutting was also treated with distilled water. Treatments were applied by plastic pipette. 10 drops of an individual treatment were placed on each of the cuttings along its entire length. Egg masses were then placed on the treated cuttings, randomized on their placement on the racks and left for a six day period to allow eclosion of the eggs. After that period, reps were bound together with bands and placed in a -20°C freezer for future counting. Similar to the adelgid

counting conducted with the uptake experiment, all Petri dishes analyzed by microscopy for crawlers as well as the bark discs for uneclosed eggs.

Eclosion Experiment

In late August, more BWA infested logs were collected, along with Fraser and Veitch fir cuttings from the same abandoned Christmas tree plantation in Ashe County, NC. Egg masses were removed from the infested logs in the lab, and all eggs counted before placing them in their respective Petri dishes. In each Petri dish, a cutting (either Veitch or Fraser fir) was placed on top of the moist filter paper. Between 10-15 eggs (in their egg mass) were placed on each cutting. Figure 3 showa a picture of the placement of the Petri dishes in this experiment. The exact number of eggs placed in each numbered Petri dish was recorded at the beginning of the experiment. There were only two treatments, a 5.1-7.6 cm cutting of Veitch fir and a 5.1-7.6 cm cutting of a Fraser fir. Treatments were randomized and placed on a wire rack. Every other day, some deionized water was added to the filter paper in each treatment to keep the samples moist. Samples were kept on the wire rack for a week. After that time, they were grouped by treatment rep and frozen to await counting. Each egg mass was inspected for any non-eclosed eggs, along with any crawlers still present on the egg masses. Cuttings were then inspected, and each crawler found was recorded. Each piece of filter paper was also examined to determine if any crawlers were present.

Statistical Analysis

All statistical processing of the results were conducted using analysis of variance via Proc GLM (SAS version 9.3, SAS Institute 2014). Both the uptake and Petri dish experiments had rep and treatment as the sources of variation. The sources of variation for

the eclosion experiment were the rep and the species of tree. Tukey-Kramer multiple comparison tests were used to identify significant difference among treatment means. Data is presented as least squares means (and standard error).

Results

Gas Chromatography/Mass Spectrometry Experiment

Using the GC/MS to analyze acetone-soluble chemical components present in Veitch and Fraser fir identified potential areas for resistance research. Seen in Figure 4, which is a chromatogram of the subtraction of Fraser fir from Veitch fir, where anything that appears above the line as a substance present in higher quantities in Veitch fir as opposed to Fraser fir, bornyl acetate stands out, among other potential chemical differences. Qualitative analyses of acetone soluble compounds routinely indicated a stronger BA signal present in Veitch fir compared to Fraser fir. Peak areas, qualitative measures of relative abundance, routinely ranged between 1.9-5.2 million counts, as seen in outputs of peak information, with BA, whereas the peak areas ranged between 200,000-500,000 counts in Fraser fir chromatogram peak outputs, a difference of ten times. This difference guided our choices for selecting BA in the following experiments. Also, delta-carene was found to be consistently higher in abundance in Fraser fir compared to Veitch fir, but was not considered within this experiment. Delta-carene will be discussed in Chapter 4 as it was significantly correlated with susceptibility to damage within *Abies* agreeing with our findings in this chapter.

Uptake/Petri Dish Experiments

The overall ANOVA of all treatment mean differences was significant ($p<0.001$). Looking at the sources of variation within our model, rep is not statistically significant, but treatment is significant ($p<0.0001$) (Table 1). Using Tukey-Kramer pairwise comparisons, the Veitch fir control treatment mean number of inserted adelgids is significantly different than the Fraser fir control, isobornyl acetate and bornyl acetate treatment means (Table 2). The three other treatments were not significantly different from one another.

Figure 5 shows a graphical representation of this data. Analysis of the uptake of solution was required to identify if uptake amount played a significant role in adelgid insertion. A regression analysis of the adelgids inserted on the total BA solution uptake over the week of the experiment was performed. The R-squared value was 0.000146 which indicates no relationship between solution uptake and insertion by the adelgids.

The Petri dish experiment showed some different results than the above uptake experiment. The overall ANOVA model is significant (p-value <.0040). Rep was not significant (p-value < 0.7976), but treatment was (p-value <.0001) (Table 3). The mean numbers of inserted adelgids present on each of the treatments (water Fraser fir, water Veitch fir, pure BA, BA solution) are 19.7, 10.7, 1.1 and 18.8 respectively (Table 4). Conducting a pairwise comparison of means revealed that the pure BA treatment was significantly different than the water Fraser fir and BA solution treatments but not from the water Veitch fir treatment (Table 4).

The eclosion experiments did not yield any statistically significant results. ANOVA results (JMP Pro) of both uneclosed eggs present as well as total eclosed eggs (based on

crawler counts) indicated that there were no significant differences among treatments (p-values <0.4099 and <0.3418). Analysis of means revealed that the mean number of eclosed eggs (each started with 10-15 eggs) was not different between egg masses placed on top of a Fraser fir cutting and egg masses placed on Veitch fir cuttings. The mean number of eclosed eggs (attained from counting crawlers) on Veitch fir cuttings was 10.7 (std. error 0.6337). The mean number of eclosed eggs on Fraser fir cuttings was 9.9 (std. error 0.6337). The mean number of uneclosed eggs found on the egg masses placed on Veitch fir cuttings was 1.00 (std. error 0.2817). The mean number of uneclosed eggs found on the egg masses placed on Fraser fir cuttings was 0.667 (std. error 0.2817). BWA egg eclosion on Fraser fir vs. Veitch fir was not statistically significantly impacted by either species of fir.

Discussion

Uptake Experiment

Overall, the results seen in these sets of experiments did not suggest anything significant perhaps due to the low bornyl acetate concentration within the cuttings. Bornyl acetate is extremely water insoluble, and the only quantifiable information I was able to uncover indicated an aqueous solubility of 23 ppm (Weidenhamer et al. 1993). Any aqueous solution made with BA would be very low in concentration; the solution used in this experiment was intended around 35 ppm to try to make a solution as concentrated as possible. The uptake experiment, conducted with BA in an aqueous solution, was dependent on the Fraser fir cuttings being able to uptake a fair amount of the solution. If adequate uptake were to occur, there was no guarantee that the uptake would result in a concentration

change of BA in the overall cutting, or that the placement of the BA would enable it to come into contact with the BWA crawler as they inserted their stylets for feeding. GC/MS analysis of uninfested controls indicated that uptake did not occur in significant amounts, at least not enough to result in differences between uninfested water controls and uninfested BA controls in relative abundance of BA. There were many confounding variables present for the uptake experiment, but one result was very interesting: the number of inserted crawlers present on the Veitch fir cuttings. Veitch fir has been shown to be a highly resistant species to BWA (Newton, 2013 and Mitchell, 1966). This experiment took place over the course of a week. Crawlers are not active searchers for their particular niche because they are generally passive dispersers. The spread of BWA south along the Appalachian spine or even locally between trees was not due to crawler mobility, but wind and human transport (Amman, 1966). The fact that they inserted in higher numbers on the Veitch fir is most likely related to the numbers that were captured by the Veitch fir branches. At the time they were cut, most of the Fraser fir terminal buds had not broken from winter dormancy. The Veitch fir buds, however, were further along in breaking out of winter dormancy. The Veitch fir cuttings hung and spread out, compared to the almost conical shapes of the Fraser fir branches. Due to this, it is most likely that more crawlers were intercepted by the Veitch fir branches and inserted on them. Their insertion shows that they will actively attempt to feed on the Veitch fir, whether because they perceive it to be a viable host or because they have no other options. This implies that something about their nutrition in their plant host, whether already constitutively present or an induced response to their feeding activity, will most likely result in their death or inability to produce offspring. Their willingness to insert on Veitch fir

indicates that the crawlers are most likely not affected negatively by fir volatiles in the air, but that the item that likely prevents infestations from spreading or becoming more severe is likely the direct contact with either a prepared or induced defensive chemical within the tree. Indeed, Newton et al. 2013 observed that any life phases found 4 weeks after artificial infestation were not present on Veitch fir after 7 weeks, indicating that inserted adelgids perished and were unable to sustain infestation.

Crawlers inserted in similar levels in all of the Fraser fir treatments, indicating that, if BA has detrimental effects to BWA insertion, its concentration was likely not increased within the cuttings due to the lack of statistical support from the analysis. As discussed earlier, GC/MS analysis of samples kept un-infested did not show any differences in BA content between the treatments for Fraser fir. It was likely that uptake did not result in the concentration change we were hoping for, most likely based on the fact that the solution was low in concentration of BA as well as the lack of adequate uptake of solution. Average uptake was 12.85 mL, averaged across the entire experiment, for the week (range: 6-20.5 mL). Some of that uptake is likely evaporation, but overall, the cuttings were not taking up much solution. Coupled with the low concentration, this is likely the reason no changes were engendered by the treatments. Even if a higher concentration had been achieved, perhaps the crawlers would have inserted as they did on the Veitch fir branches but not survived. This indicates that antixenosis, or non-preference is likely not the general resistance mechanism governing resistance to BWA as crawlers inserted on Veitch fir at high rates, and there was not statistically significant difference between the three other treatments.

Petri Dish Experiments

There were statistically significant differences among the treatments within the first BWA Petri dish experiment. Being that one of the main concerns from the uptake experiment centered around the solution being taken up by the cutting and making it available for contact with the adelgids themselves, the Petri dish experiment aimed at making sure the substances would come into contact with the adelgids. While the egg masses were not doused with the solutions, they were placed on top of cuttings that were covered in the treatment solutions, such that crawlers would come into contact with them, and eggs would be in direct presence of the target volatiles. Eclosion on the BA solution (35 ppm), water control on Fraser fir, and water Veitch fir control cuttings were not significantly different from one another (Table 4). The pure BA treatments were significantly different from the water Fraser fir and BA solution treatments, but not the water Veitch fir control (Table 4). It would appear that the pure BA treatment inhibited eclosion of nearly all eggs already laid by the adelgids, based on the low mean number of crawlers recovered from the Petri dishes. No crawlers were found inserted on any of the pure BA treatment cuttings, indicating a negative biological effect by the pure BA. All crawlers found within these Petri dishes were either still on their bark disc or on the filter paper. While this is not surprising, the chemical, a known monoterpene indicated in studies as having a negative effect on the biology of different pestiferous insects, inhibited eclosion when applied in pure form and in a high concentration. Being that the egg masses were not doused in BA, just the cutting, it indicates that the volatility of BA most likely was how it interacted with the uneclosed adelgid eggs. It also had a slight phytotoxic effect on the cuttings, indicating its potency.

While it was intriguing to witness the negative effects of pure BA on the life cycle of the adelgids, the item of most interest was that of the Veitch fir control results. In an "open-air" environment, Veitch fir had a slightly negative impact on BWA egg eclosion and crawler presence when compared to the Fraser fir water control and the Fraser fir BA solution treatment while not being statistically significant. This brings into question a confounding variable of this experiment. The exact number of eggs placed on each cutting was unknown. All egg masses were looked at for the presence of uneclosed eggs, but not counted, before being placed on top of the treated cuttings to begin the experiment. While not statistically significant, the fewer number of crawlers found in the Veitch fir control dishes indicated that an experiment with more control over the exact number of eggs was necessary to determine if there is a true negative effect of Veitch fir on egg eclosion for BWA.

The Petri dish eclosion experiment was the follow-up in this case. It was a very simple design to see if Veitch fir volatiles in an "open environment" had an effect on eclosion of BWA eggs. Based on our findings, Veitch fir was not different from Fraser fir in effect on BWA egg eclosion. Both variables had a very similar effect, in that they did not inhibit or hinder BWA egg eclosion and insertion success. The findings from the first Petri dish experiments were therefore either due to fewer eggs being placed on the Veitch fir cuttings, as when an equal number were placed on Fraser and Veitch fir there was not a significant difference, or seasonal variation differences in Veitch fir volatiles. Veitch fir collected in the spring for the Petri Dish Experiment had just broken winter dormancy and had several centimeters of new growth. Veitch fir collected for the Eclosion Experiment in late August had already begun to prepare for winter dormancy, and the new growth bark had

since hardened considerably compared to the early spring new growth. It is possible that a change in volatile profiles could be responsible for the different results, but more analysis of Veitch fir seasonal volatile changes would be needed to elucidate this alternative explanation for the differences seen between the Petri Dish Experiment and the Eclosion Experiment.

From these experiments I gleaned a few pieces of data: one of the more important findings we learned was that BWA would insert quite readily on Veitch fir. Whether they were then going to be able to subsist while inserted on the Veitch fir is unknown from this experiment, but, due to Veitch fir's resistant status, it is likely that they would not have survived a longer investigation. The increased surface area, provided by new seasonal growth already present on Veitch fir accounts for the higher number of crawlers, but whether those crawlers would have survived to reproduce is unlikely.

I was unable to affect a concentration change within Fraser fir to address whether or not bornyl acetate or isobornyl acetate will engender a disruption in host selection in BWA. I did find that crawlers will insert at high rates on Veitch fir, a resistant species, leading me to believe that the volatiles present in the air column will not inhibit BWA from inserting on a particular host, which means Veitch fir resistance is more than likely characterized as antibiosis, as antixenosis would traditionally mean that crawlers will not insert as Veitch fir is not a proper host. Veitch fir volatiles do not significantly inhibit BWA egg eclosion in an "open-air" environment. Influencing a change in concentration within Fraser fir and the air column to see if BA has an effect on egg eclosion remains to be seen. Pure BA was inhibitory on egg eclosion success, but the weakly concentrated BA solution (35 ppm) was not, indicating that altering the concentration could potentially produce a negative biological

response in the adelgids. Cates et al. 1987 indicated that both high and low concentrations of BA, when fed to spruce budworm, significantly reduced fecundity of reproductive adults. This study indicates that pure BA, when not in direct contact with eggs has a negative effect, but low quantities of BA in an aqueous solution (35 ppm) do not have that same effect. This could mean there is some threshold for tolerance within BWA, but further research is needed to elucidate that potential.

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Table 1: Analysis of variance results for mean number of adelgids inserted in the uptake experiment. Treatments included bornyl acetate solution with Fraser fir, isobornyl acetate solution with Fraser fir, water Fraser fir control and water Veitch fir control.

Source of Variation	D.F.	F-Ratio	Prob>F
Rep	29	1.53	0.0679
Treatment	3	15.91	<0.0001***

***=Significant at the alpha=0.0001 level

Table 2: Pairwise comparisons of mean aphids present (least squares mean) of all treatments and associated standard errors for the aphid bornyl acetate concentration experiment

Treatment	Significance*	Mean Inserted Adelgids	Standard Error
Veitch Fir- Water	A	16.03	1.13
Fraser Fir-Water	B	7.83	1.13
Fraser Fir-Isobornyl Acetate	B	6.87	1.13
Fraser Fir-Bornyl Acetate	B	6.57	1.13

*=Levels not connected by the same letter are significantly different

Table 3: Analysis of variance results for mean (least squares mean) crawlers present in each Petri dish for the Petri dish experiment. Treatments included Fraser fir with pure bornyl acetate, Fraser fir with water, Fraser fir with bornyl acetate solution (solution from uptake experiment) and Veitch fir with water.

Source of Variation	D.F.	F-Ratio	Prob>F
Treatment	3	11.84	<0.0001***
Rep	9	0.59	0.7976

***=Significant at the alpha=0.0001 level

Table 4: Pairwise comparisons of the mean (least squares mean) crawlers present within each Petri dish at the conclusion of the Petri Dish Experiment

Treatment	Significance Level*	Mean Crawlers Present	Standard Error
Bornyl Acetate Solution	A	18.8	2.51
Water Fraser Fir Control	A	19.7	2.51
Water Veitch Fir Control	A B	10.7	2.51
Pure Bornyl Acetate	B	1.1	2.51

*= Levels not connected by the same letter are significantly different

Figure 1: Uptake Experiment setup

Figure 2: Petri Dish Experiment setup

Figure 3: Veitch and Fraser fir cuttings at time of placement in Eclosion Experiment.

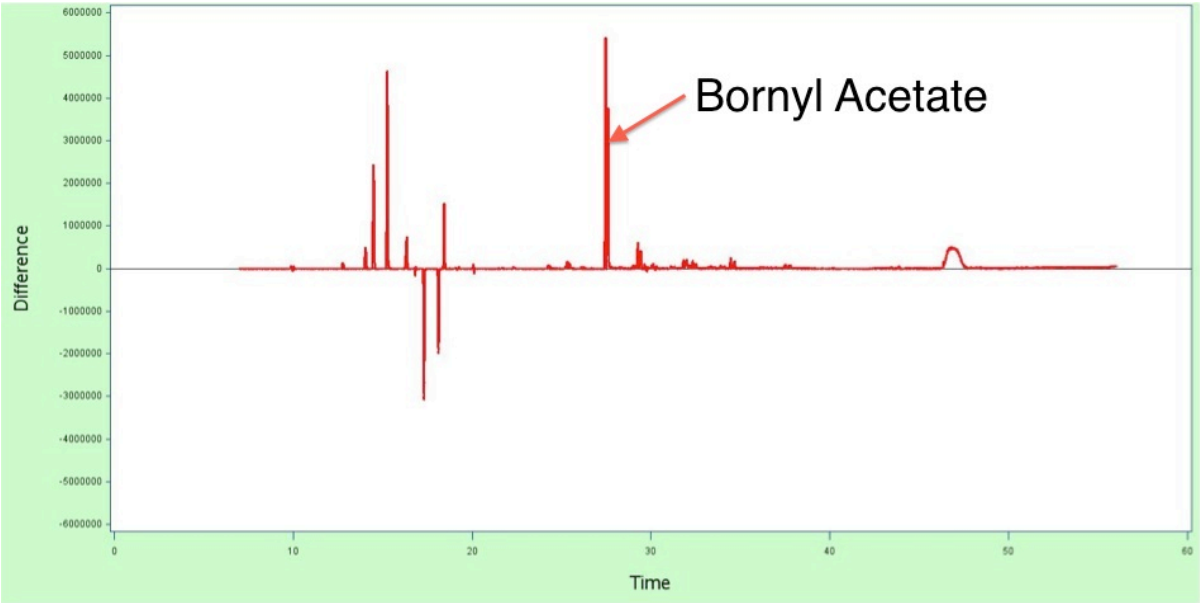

Figure 4: Subtraction chromatogram comparison Veitch fir vs. Fraser fir with bornyl acetate peak identified. Any peaks above 0 line indicate a higher signal from Veitch fir GC/MS analyses, any peaks below the 0 line indicate higher signal from Fraser fir GC/MS analyses.

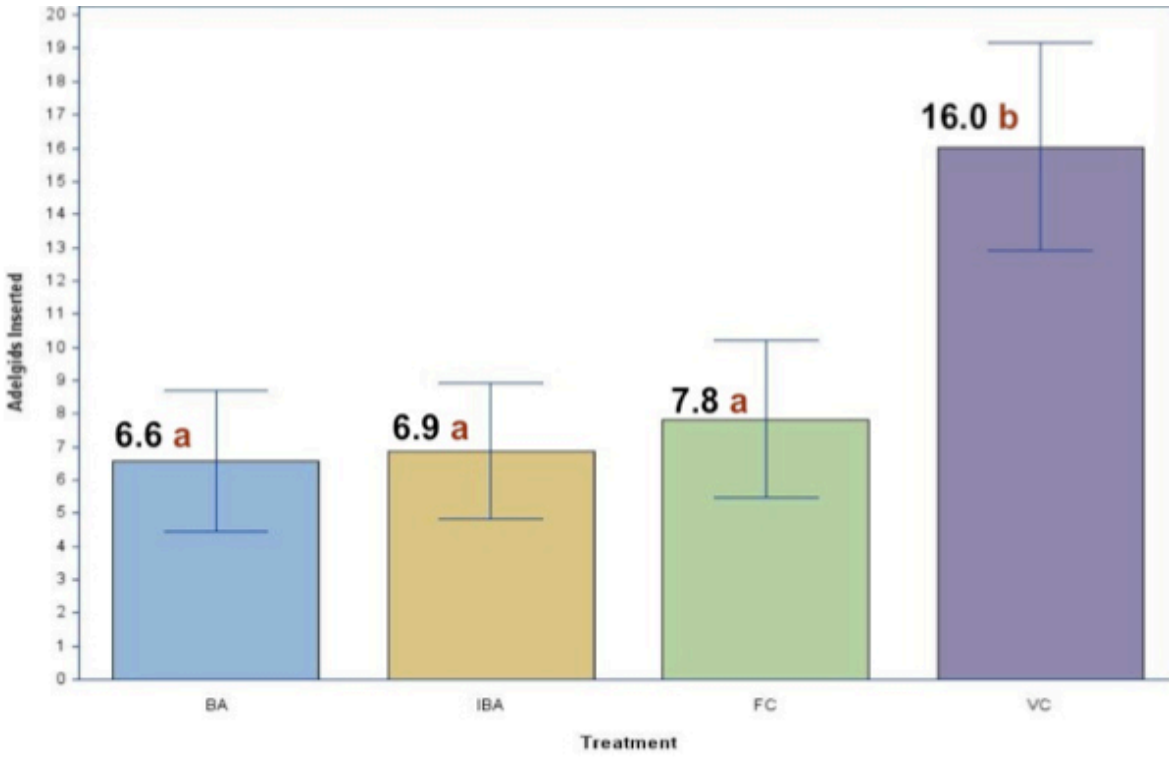

Figure 5: Mean number of inserted adelgids and standard error of each measurement by treatment and statistical significance.

Chapter 3: Effect of Different Headspace Concentrations of Bornyl Acetate on Fecundity of Green Peach Aphid and Balsam Woolly Adelgid

Introduction

Balsam Woolly Adelgid (*Adelges piceae* Ratz.) (Hempitera: Homoptera: Adelgidae) (BWA) is an exotic invasive insect pest, introduced to the Eastern United States in the early 1900's, and reached the Southern Appalachians by 1955 (Kotinsky 1916)(Speers 1958). It is a pest of many different species within the genus *Abies* worldwide (Mitchell 1966). Within its native range of Northern Europe, it infests European silver fir (*Abies alba* Miller), but does not significantly damage or have a major affect on the life cycle of that species. Within the Southern Appalachians of the United States, it readily infests Fraser fir (*Abies fraseri* (Pursh) Poir) and has a significant effect on growth and survival of this endemic species (Hain et al. 1991). Within the United States, the BWA life cycle is anholocyclic, where it does not change hosts and reproduces via parthenogenesis only. It is closely related to a species of holocyclic adelgid, *A. nordmannianae* (Eckstein), which is cyclically parthenogenetic, with sexual generations and asexual generations (holocyclic) as well as host alternation (Havill et al. 2007).

Resistance screening for BWA across a wide range of members of the *Abies* genus shows that Fraser fir is among the most susceptible species of fir to BWA, while Veitch fir (*Abies veitchii* Lindley), a Japanese species of fir, is among the least susceptible to damage from BWA (Mitchell 1966). Resistance to pests in plants is generally defined within three broad categories: non-preference, tolerance, and antibiosis (Painter, 1958). Resistance to BWA can be placed in multiple categories depending on the species. Host tree species is the

most important factor determining adelgid populations (Amman, 1970). Others have suggested that juvabione, a compound that mimics juvenile hormones in insects, increased with infestation by BWA within Fraser fir, but were ultimately unsuccessful in finding significant differences in concentrations (Fowler et al. 2001). The formation of secondary periderm around infestation sites may also be a resistance mechanism, protecting the underlying bark, as well as keeping the wound response of the tree from spreading (Mullick 1975). Others suggested resistance mechanisms to BWA include those species better adapted to handle drought stress (Mitchell 1967), as well as bark thickness depending on genetic variability within fir (Hollingsworth and Hain, 1992).

While these potential mechanisms have taken place mostly by assessing Fraser fir and susceptibility based on intraspecies variation, few have attempted to address differences of an interspecific nature. Based on GC/MS analyses performed on the highly resistant Veitch fir and susceptible Fraser fir, one highlighted difference discovered was the abundance of bornyl acetate, a terpenoid, present in Veitch fir, compared to lower levels in Fraser fir (Chapter 2). Bornyl acetate, a terpenoid, found commonly in gymnosperms and angiosperms, is a monoterpene especially present in coniferous trees (Raffa and Powell, 2004). Studies have shown a difference in its interactions with various insect species. Certain studies have found that bornyl acetate reduced larval growth and survival to adult stage of the western spruce budworm (*Choristoneura occidentalis* Freeman) (Lepidoptera: Tortricidae) when in combination with both high and low levels of nitrogen, administered in an agar diet (Cates et al. 1987). Others have found it to be one of the few monoterpenes isolated from analysis of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) needles that reduced survival and growth

rate of multiple, geographically different, populations of western spruce budworm (Zou and Cates, 1997). Similarly, bornyl acetate fed to spruce budworm (*Choristoneura fumiferana* Clemens) at levels higher than what is found in balsam fir (*Abies balsamea* (L.) Mill.) needles reduced larval growth (Kumbasli and Bauce, 2013). Bornyl acetate has been implicated as a potential management technique for control of stored food and house dust mites (Lee et al. 2009), but has also been implicated as a feeding attractant for certain fly larvae species on carrot root (Ryan and Guerin, 1982). Bornyl acetate, when fed to 4 separate species of tussock moths (Lepidoptera: Lymantriidae), did not yield any changes in larval growth rate, consumption rate and development times (Raffa and Powell, 2004). The reproductive success of two scale insect species (Hemiptera: Dispididae) was evaluated on *Tsuga canadensis* (L.) Carrière and *Tsuga sieboldii* Carrière. On *T.canadensis* the number of eggs was significantly less than that laid by the species on *T.sieboldii*, and one of the significant differences between terpene content between the two species was bornyl acetate, among other chemical variations (McClure and Hare, 1984). These studies do not show definitive proof of the effect bornyl acetate has on various insect species, but they do suggest that there is an effect on certain insect species, and not on others, and whether it alone has potential negative/positive effects on various insect species remains in question (Kumbasli and Bauce, 2013). With that in mind, bornyl acetate became a target for my investigations into possible resistance mechanisms for *Abies* spp. to BWA.

Being that BWA is difficult to rear in a laboratory setting, and most successful uses of BWA come from naturally collected field populations, in order to ascertain if there is an effect on BWA during the winter, while it is still in diapause, we used a proxy species of

insect, the green peach aphid (*Myzus persicae* Sulzer) (Homoptera: Aphididae) (GPA) to simulate BWA for beginning our investigations into varying the concentration of bornyl acetate in the headspace of a sealed container. GPA was chosen as a proxy due to its ease in rearing in a laboratory situation (Gavkare and Gupta, 2013) as well as similarities between BWA and GPA in parthenogenetic reproduction, meaning there is no sexual recombination. GPA is a common agricultural pest, capable of vectoring many different plant pathogens (van Emden and Harrington, 2007). Similar to BWA within its natural habitat, GPA, like many aphid species, exhibits cyclical parthenogenesis, enabling it to alternate between sexual generations and asexual generations, an evolutionary advantage that enables GPA to both produce large populations while also enabling it to maintain genetic diversity (Poupoulidou et al. 2006) and beacause of the similarity in reproductive histories, while BWA exhibits an anholocyclic reproductive cycle in the Eastern United States, and the easy rearing capacity of GPA, it was selected as a proxy for experiments.

Based on my previous results from the Uptake Experiment in Chapter 2, affecting a concentration change within Fraser fir cuttings by placing them in BA solutions proved to be ineffective. I also showed in Chapter 2 that pure BA seemed to have a negative impact on egg survival, but a fair amount of confounding factors surrounded that finding. Coming into contact with a substance of that concentration would likely have a detrimental affect on a wide variety of insect species. Chapter 2 also showed that between Veitch and Fraser fir cuttings, there wasn't much of an effect on egg eclosion success when placed on each cutting within a Petri dish. What I really wanted to attempt was altering the concentration of BA within a closed environment, and seeing if that altered concentration would have a

corresponding decrease in reproductive ability of the insect in question. Altering the concentration of BA in the headspace, to prevent direct contact with BA, should reduce the number of offspring produced by GPA and BWA confirming the ability for this terpenoid to result in fecundity differences.

Methods

Aphid Experiment:

BWA can be very difficult to rear in a lab setting. Being that most BWA, when used for experiments, is collected in the field and brought back to the lab, rather than raising the BWA in the lab itself, and it is only available from late Spring to Fall, *Myzus persicae* (Hemiptera (Homoptera): Aphididae), the Green Peach Aphid (hereafter GPA), served as a proxy for BWA in this experiment. GPA reproduces in a viviparous manner, but reproduces parthenogenetically, similar to BWA. Both are part of the order Hemiptera and suborder Homoptera, and are phytophagous plant feeders. Given their similar clonal reproduction methods, assessing the affects that differing concentrations of BA had on their reproductive abilities would enable me to determine whether to similarly test BWA with the same environmental parameters.

GPA were collected in March from a PhD. Entomology student at NC State, from a lab assessing the effects of GPA on tobacco and various *Brassica* species. Before placement of GPA-infested plant material into a rearing chamber, a colony housing unit was built (Figure 1). Twelve *Brassica oleracea* L. plants were placed in 6 plastic pots (2 per pot) and covered by the top of the housing. Once the newly transferred plants were allowed to grow

for a week, GPA-infested plant material was placed on top of the plants, and given 2 weeks to allow for the infestation of the new plant material, and to allow for populations of the colony to build.

Determination of the experimental conditions required the GC/MS analysis of many different samples. Samples of Veitch and Fraser fir were collected from Avery County, NC in mid-March, 2015. Defoliated samples were placed in 60 mL SPME jars from Cole-Parmer and allowed 2 days to equilibrate. SPME fibers were exposed to the headspace for 2 hours before being run through the GC/MS. Being a qualitative look at BA, peak areas were matched to determine concentrations that roughly simulated what is found in Fraser and Veitch fir. Silicone oil was used as the diluting agent for the BA, because BA is highly water insoluble. To determine peak area similarities to Veitch and Fraser fir, concentrations were developed, analyzed through SPME analysis and peak areas offered insight into whether the concentration was too high or too low. Once approximate concentrations had been analyzed, leaves from the host plants within the aphid colony were added to the SPME jars. The leaves absorbed some of the BA, and thus weakened the signal, as opposed to having no leaf material in the jars, wherein the BA signal was unhindered. Small changes were made based on those findings, and 5 concentrations were developed. Concentration 2 and concentration 4 represented Fraser and Veitch fir concentrations respectively.

The 5 concentrations of BA were dilutions. Concentration 1 was 1µ BA in 10 mL oil. Concentration 2 was 1µL BA in 2.5 mL oil. Concentration 3 was 1µl BA in 1mL oil. Concentration 4 was 10µl BA in 1 mL oil and concentration 5 was 25µl BA in 1 mL oil. One-hundred µl of each solution was added to each SPME jar. The jars themselves had a

small 3 mL vial glued to the bottom, as a place for the BA solutions to be added such that GPA would not come into contact with the solution, or at least provide a physical barrier to contact with the solution itself. There were 5 reps, 7 treatments: concentrations 1-5, as well as 2 controls, a water control and an oil control. Two late-instar/adult aphids were placed in each jar, along with one leaf, taken from the uninfested colony, and cut in half to fit inside the 60mL SPME jars.

Once all the aphids, solutions and leaves had been placed in SPME jars, they were randomized within each rep as to their position within the growth chamber and kept at 23°C on a 16-8 hour photoperiod. After 1 in the growth chamber, one rep was randomly selected and tested using the SPME fiber to ensure that concentrations had indeed been altered within the headspace. Extraction times were 2 hours based on analyses conducted analyzing differing times and the signal change accompanying those different extraction lengths. After testing was complete, all samples were frozen and then analyzed. Counts on the number of individuals in the jars were conducted, along with the development of a rudimentary 5-point scale assessing leaf health. A rating of 1 meant both halves of the leaf were completely degraded or yellowed, whereas 5 represented both halves still green and turgid. Figure 2 shows a picture of the set up of all jars within the growth chamber.

The GC/MS component of these investigations was performed on an Agilent Model 7280A GC system with an attached Agilent Model 5977E Mass Spectrometer. Oven temperatures started at 40°C for 2 minutes, and increased at a rate of 8°C per minute up to 224°C final at 25 minutes. Sample separation was accomplished using an Agilent Column HP-5MS-UI (30 meters, 0.25mm i.d, 0.25 micron thickness). Injector temperatures were

kept at a constant 250°C. The SPME fiber was inserted into the injector for all 25 minutes of the run, before removal for other runs.

BWA Experiment

This experiment, based on the results from the previous GPA experiment, was carried out at the beginning of June 2015. BWA infested logs were collected from two different Christmas tree plantations in Allegheny County, NC (36.356972, -81.232002) and brought back to Raleigh. Veitch and Fraser fir branch clippings were also collected from Allegheny County on the same trip.

Bark discs with BWA egg masses were placed in each 60 mL SPME jar, equipped with the same 3 mL vial for the 100 µl of BA solution. The same concentrations were used as in the above GPA concentration experiment. Before placing the bark disks with egg masses on them inside the SPME jars, each disk was analyzed. Any unenclosed eggs within the egg mass were counted, as well as any live crawlers already present on the disks. In total, there were 13 treatments. Nine of those treatments had the bark disks stuck to transparent adhesive tape, to prevent crawlers from too much mobility. Concerns about the chemicals, specifically the plasticizer chemicals used in the making of the tape, led to the addition of 4 more treatments. The treatments with tape included 5 concentrations (the same as from the aphid experiment), a water control, an oil control, a treatment that had a defoliated Fraser fir clipping placed in the SPME jar and one that had a defoliated Veitch fir clipping added to the jar. Weights for the Fraser fir and Veitch fir clippings varied from 1.085-1.477 g. The treatments without tape were Concentrations 1, 3 and 5, plus an oil control. Because certain

plasticizers are used in pesticides, the addition of 4 more treatments without the tape was to assess if there was a negative effect of the tape on the eclosion of the eggs. Figure 3 shows the inside of the growth chamber with all treatments and reps.

The GC/MS component of these investigations was performed as previously described.

Once all treatments were added and all BWA egg information was tallied, the jars were randomized and placed in a growth chamber kept at a constant 20°C. They sat for a week, before being removed from the chamber and frozen. Each jar was then inspected, and all remaining live eggs were counted as well as any crawlers that could be found, using a dissecting microscope. Those treatments which had tape included within had their tape removed and counted for crawlers that had become stuck throughout the course of the experiment. The bottom and sides of each jar were also inspected for crawlers.

To compile the data for analysis several changes needed to be made: in many cases, there were more individuals present at the end of the experiment than there were at the beginning, meaning more were produced as the experiment continued. To account for this in the data collection process, adjustments to the number of uneclosed eggs needed to be made. For example, if there were 18 individuals (crawlers + eggs) at the beginning of the experiment, and there were 16 crawlers and 4 uneclosed eggs at the end, the adjusted number of eggs to be counted in the data analysis was 2, because there were 20 individuals present at the end of the experiment, so clearly more eggs had been laid and eclosed during the experiment. Also tabulated was percent difference in uneclosed eggs, from start to finish as

well as percent difference in individuals from start to finish. All of this was tabulated before the data were analyzed.

Statistical Analysis

For both experiments, JMP Pro software (version 11.2.0) was used to analyze the data collected at the end of the experiment. For the aphid experiment, an analysis of covariance, with leaf health serving as the covariate, allowed the normalizing of the aphids present to a common mean of leaf health. The sources of variation were the treatment (concentration of BA), leaf health rating (covariable, 1-5 scale) and rep. Tukey-Kramer honestly significant differences were also used to make all pairwise comparisons between the treatments.

The BWA experiment required several layers of statistical analysis. The first was to determine whether or not there was a tape effect. The tape used in this experiment, based on SPME analyses, contained chemical plasticizers that are sometimes used in insecticides. A multi-way ANOVA was used to conduct this analysis being that there were multiple independent variables that I wanted to take a look at to see if they had an effect on the number of eggs eclosed during the experiment. F tests for the sources of variation were done on rep, treatment, tape and all interactions. Upon determining whether or not those treatments with tape included were significantly different from those same treatments where tape was excluded, a complete analysis could be done. For this analysis, a multi-way ANOVA was used with rep, treatment, and all interactions as the sources of variation, along with a Tukey-Kramer HSD to make all pairwise mean comparisons.

Results

Aphid Experiment

Numbers presented are measurements of individuals of all life stages of GPA. The analysis of variance for the entire model (p-value <0.0001) was significant. Looking at the F test for the sources of variation, both treatment and leaf health were significant (Table 1) at the p<0.01level. LSmeans, using leaf health as a covariate, and significance level for all treatment means, along with standard errors, are shown in Table 2. A general decreasing trend can be seen as concentration rises. Concentration 1, control 1 and control 2 are all significantly different from concentration 3, 4 and 5 (Table 2). Figure 4 shows the graphical representation of the LSMeans along with standard error associated with each treatment. Figure 5 shows a comparison of the peak signals of BA between treatments C1, C3 and C5, tested by randomly selecting the 4^{th} rep. The concentration of BA was significantly altered between the treatments.

BWA Experiment

Altering the concentration of bornyl acetate did not have a significant effect on BWA as it did on GPA. As mentioned previously, the first statistical determination to be made was whether or not tape presence in the SPME jar played a significant role in determining egg eclosion success. A subset of the data that included those concentrations and controls that had tape and no tape included within the treatment (Concentrations 1, 3 and 5 as well as the oil control). The overall ANOVA model was significant at p-value <0.0603. Inspecting the effects and sources of variation, there was a significant rep effect, but tape and their

interaction were not significant (Table 3). Tape mean uneclosed eggs were 1.8 (std. error 1.19) and no tape mean adelgids were 4.2 (std. error 1.19) which are not significantly different from one another at an alpha level=0.05. There was not a significant tape effect on the eclosion success of BWA eggs.

After looking for significant tape effects, the rest of the data could be analyzed. The overall ANOVA p-value < 0.0035 indicating statistical significance. Breaking it down by sources of variation, treatment was significant and rep was not (Table 4). LSmeans for the treatments ranged from 1.2 for concentration 2 to 12.6 for the Veitch fir control. Tukey-Kramer HSD tests showed only one statistically significant difference was found (Table 5). The Veitch fir control differed significantly from all other treatments in the experiment when accounting for different response variables as it was significantly different when the response variable was adjusted uneclosed eggs. Mean uneclosed eggs per treatment within the BA concentration treatments ranged, non-uniformly from 1.2-4.0. Figure 6 shows a graphical representation of the BWA concentration experimental data and standard errors associated with each treatment value. The four control treatments mean uneclosed eggs ranged from 2.8-12.6, with Veitch fir, as previously mentioned the only significantly different mean at 12.6 (std. error 1.14). Figure 7 shows a picture of uneclosed eggs found on one 9 mm bark disk from the 4th rep of the Veitch fir control treatment at 75x magnification. Worth noting was the Fraser fir control treatment that had a mean uneclosed egg count of 6.2 (std. error 1.14). Though not significantly different than the other treatments (outside of Veitch fir), the Fraser fir control was the second highest uneclosed egg mean (Table 5). The Veitch fir control is the only statistically significantly different treatment.

Looking at percent difference in eggs, a similar trend was seen in the data. The overall ANOVA p-value was significant (p-value < 0.035). Treatment was significant, and rep and rep*treatment interaction were not (Table 6). Table 7 shows the results of the Tukey-Kramer HSD, comparing the mean percent difference in eggs from start to finish for the BWA concentration experiment. The mean percent difference amongst the 5 concentrations varied non-uniformly from -74.06% to -92.68%. For the 4 control treatments, mean percent difference changed from -24.82% to -82.54% with the only significant difference being Veitch fir at -24.82% (std. error 6.84) difference in uneclosed eggs (Table 7). Worth noting was the Fraser fir control mean percent difference at -62.90 with a standard error of 6.84. Just as seen with mean uneclosed eggs, Fraser fir was the next lowest percent difference in uneclosed eggs, but not statistically significantly different from all the other treatments (outside the Veitch fir control). The Tukey HSD, and all pairwise comparisons of means of both uneclosed eggs and percent difference in eggs all indicate the only statistically significant difference being the Veitch fir control treatment.

The 2nd rep was randomly chosen for SPME testing to ensure that the concentration of BA was altered. Comparing the signals shows that the concentrations between the environments were different increased from treatment to treatment as anticipated (Figure 8).

Discussion

The above results indicate a significant concentration effect on the reproductive successes of GPA with regards to bornyl acetate concentration. Altering the concentration to roughly match the volatile signal of BA, based on SPME analyses done on defoliated

Veitch and Fraser fir cuttings, was sufficient enough to have a significant effect on the production of GPA offspring. It should be noted that the approximation for the signal in Fraser fir, represented by concentration 2, was not significantly different from concentration 4, the approximation of bornyl acetate's signal in Veitch fir. This suggests that while bornyl acetate is a terpenoid that can negatively impact the ability for certain insect species like GPA to reproduce; it does not mean that the difference between Veitch and Fraser fir in GC/MS relative abundance of this particular terpenoid is responsible for the studied difference in resistance to BWA. It does suggest the potential biologic effects of BA are more geared towards fecundity rather than outright mortality, at least when present at different concentrations in the headspace and not in direct contact with the insect, because the insects were still reproducing even at the highest concentration tested.

Returning to the idea that the concentrations shown in Veitch and Fraser fir (reflected in Concentrations 2 and 4) were not statistically significant in their differing effects on the fecundity of GPA reveals that perhaps it is the direct contact with the substance that will produce the most drastic results. Altering the concentration in the headspace was enough to see a difference in fecundity, but this experiment did not measure the effect BA could have on GPA coming in direct contact with BA through feeding. GPA is also not accustomed to interacting with this particular terpenoid. While aphids are generally polyphagous feeders, studies analyzing the chemical components of the more popular food sources for GPA did not show the presence of bornyl acetate in the volatile make-up (Fernandes et al. 2009). GPA has not been conditioned to interact with this coniferous terpenoid. It is not unknown for insects to come into contact with terpenoids that have a profound effect on their biology.

The discovery of juvenile hormone was noted when certain insect species, after coming in contact with the paper products used within the rearing chamber were kept in a state of perpetual juvenility. It was found that their paper bedding, made from balsam fir, had a certain chemical that kept them in that state of immaturity (Bowers et al. 1966). While the aphid data yielded a useful concentration gradient, and showed the ability for bornyl acetate to have an impact on the biology of GPA, the results from testing the same concentrations of bornyl acetate on BWA in a similar manner did not yield the same result.

BWA did not show a concentration effect in fecundity associated with increasing the concentration of bornyl acetate in the headspace of a septum jar. Kumbasli and Bauce, 2013 showed that while bornyl acetate at higher concentrations than those found in leaves in natural settings reduced larval growth of spruce budworm, it alone was not enough to have toxic effects on the larval instars studied, indicating that a synergistic effect of other monoterpenes or nutrients in addition to these may be needed. In agreement with this is the Cates et al. 1987 study which saw the effectiveness of bornyl acetate at high and low levels of nitrogen.

The effect on BWA fecundity of all concentration treatments within our study were not significantly different than the controls, outside of the Veitch fir control. Being that Veitch fir was the only significantly different treatment, it notes a difference from what was seen in the Chapter 2 experiments. While in Chapter 2, the eclosion experiments were conducted in an "open environment", in these experiments the environment was sealed. While the sealed environment does not reflect a natural setting, the fact that the build up of volatiles within the septum jars with Veitch fir, and, to a limited extent, the Fraser fir control

jars, indicates that the trees contain volatiles, whether working singularly or in concert, and that they do have an antibiotic effect on BWA survival. While bornyl acetate on its own does not result in a decrease in fecundity or egg survival and eclosion within BWA, even at varied concentrations, the cumulative effect of many different volatiles, produced by Veitch fir, does result in a decreased egg eclosion success. It suggests that the volatiles alone in the headspace may have a biological effect on BWA, without coming into direct contact with the insects themselves. Fraser fir is highly susceptible to BWA, but when placed in a closed space, Fraser fir volatiles have some negative effect on fecundity, suggesting that the volatiles present even in susceptible species may have an impact on the survival or reproductive ability of an insect pest. When considering resistant species, the effect is more pronounced. This could potentially signal overall concentration of volatiles within the tree has an effect. Based on previous GC/MS analyses, Fraser and Veitch fir produce many of the same or very similar suites of chemicals for defense purposes. The relative amounts of those produced remain unknown. Relative abundance of chemical components from previous analyses tends to show Veitch fir with a greater qualitative abundance, which may contribute to the differences seen in this eclosion experiment. Those terpenes that are important to the negative biologic effect of both Veitch and Fraser fir volatiles in a closed headspace may be more abundant in Veitch fir, accounting for the statistical differences between these two treatments. That these secondary metabolites present in Fraser fir, within this contained environment, have a similar effect as Veitch fir, but not as statistically significant, offers insight that a similar suite of volatiles, without direct contact, results in decreased fecundity or at least fewer eclosion successes within BWA. However, it must be noted that those same

volatiles do not have that kind of effect when in an open environment, which suggests that the direct contact, perhaps through feeding activity, is important towards interrupting the life cycle of BWA, rather than indirect contact with volatiles in the headspace. However, these terpenes and other secondary metabolites generally identified through GC/MS analysis have a wide range of usefulness, from growth regulation to defense (Kačík et al. 2012). When thinking about my results and resistance to BWA, Veitch fir may produce these secondary metabolites that have a negative impact on the biology of BWA at higher levels, which is why identifying and addressing the quantitative and qualitative differences between Veitch and Fraser fir is important to determining which substances and at what quantities they act in a negative biological manner on BWA. Fraser fir does produce similar volatiles, but perhaps the lower relative amounts produced by Fraser fir are the reason for Fraser fir's susceptibility and the resistance seen in Veitch fir. It also signals that constitutive defenses may be those compounds producing this negative biological activity. These cuttings were placed on ice in a cooler for transport and kept in a cooler at temperatures just above freezing. Biological activity and production of defenses would have been limited. This suggests that those chemical defenses already prepared were enough to effect this change in fecundity for BWA. A closer look at constitutive defenses within *Abies* could yield some target volatiles for similar analysis to ascertain biological outcomes for BWA. Constitutive defenses may be one potential resistance mechanism within the *Abies* genus as it related to BWA, highlighted by the difference in reaction of egg eclosion between open and closed environments.

Lower amounts are inferred from the concentration effect seen in GPA: as the quantity of BA in the headspace was lowered, the number of offspring produced increased.

Veitch and Fraser fir produce a similar suite of metabolites that have a detrimental effect on BWA egg eclosion in a closed space. Relative concentrations and chemicals that account for the differences seen in this instance are unknown, but based on the concentration effect seen that damaged the reproductive abilities of GPA when concentrations were raised, the same thing with the potentially synergistic effects of these metabolites is seen in this experiment with BWA. BA could potentially play a role, but by itself, does not appear to effect BWA egg eclosion success, at least not at the concentrations tested. Instead, our findings seem to support the notion that bornyl acetate potentially works in conjunction with other features of tree chemistry to bring about its negative biological activity or not (Kumbasli and Bauce, 2013).

Looking specifically at bornyl acetate, as mentioned previously, it alone is not enough to engender any real results for resistance efforts. Pure BA has a negative effect on BWA survival, as seen in Chapter 2, but not at varied concentrations that approximate more natural conditions. It does not mean that it does not have an effect on insect fecundity, as shown with the aphid experiment, but it alone does not affect BWA at the concentrations tested. It is not unprecedented to see mixed results, especially when taking into account the results of the BWA experiment that followed. BA has been shown as effective at reducing both larval growth and survival to the adult stage of the spruce budworm when administered into their diets (Cates et al. 1987; Zou and Cates, 1997). It has also been shown to have no effect on the life cycle or survival of other insect species, such as many varieties of Tussock moth (Raffa and Powell, 2004). It has also been correlated to be a significant part of *T. canadensis* terpenoid acetate leaf volatile profile, on which significantly fewer eggs/female were

produced by two species of scale insect (McClure and Hare, 1984). It, along with many terpenes and terpenoids, increases in concentration in *T. canadensis* between branches infested with HWA and those that remain free of infestation, although this does not indicate a negative biologic effect on HWA as *T. canadensis* is highly susceptible (Broeckling and Salom, 2003). It appears that, in our case, bornyl acetate is a compound that, at varying concentrations, can reduce the number of individuals produced by GPA over a week, and alone, at varied concentrations, does not have a significant effect on egg eclosion success of BWA.

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Table 1: Analysis of variance of mean aphids present with all sources of variation and associated F-ratios and p-values within the aphid bornyl acetate concentration experiment.

Source of Variation	D.F.	F-Ratio	Prob>F
Treatment	6	8.33	<.0001***
Leaf Health Rating	1	18.37	.0003**
Rep	4	1.90	0.1446

***=Significant at the alpha=0.0001 level

**=Significant at the alpha=0.01 level

Table 2: Pairwise comparisons of mean aphids present (least squares mean) of all treatments and associated standard errors for the aphid bornyl acetate concentration experiment.

Treatment	Significance*	Mean Aphids Present	Standard Error
Control 1 (Water)	A	17.08	1.48
Control 2 (Oil)	A	17.01	1.40
Concentration 1[1]	A	16.24	1.44
Concentration 2[2]	A B	12.48	1.48
Concentration 3[3]	B	9.55	1.40
Concentration 4[4]	B	8.30	1.42
Concentration 5[5]	B	8.15	1.40

*=Levels not connected by the same letter are significantly different (alpha=0.05)

[1]= 100 µl of 1 µl BA in 10 ml oil solution

[2] =100µl of 1 µl BA in 2.5ml oil solution

[3] =100µl of 1 µL BA in 1 ml oil solution

[4] =100µl of 10 µL BA in 1 ml oil solution

[5] =100µl of 25 µL BA in 1 ml oil solution

Table 3: Analysis of variance for BWA concentration experiment comparing treatments with tape to those without tape. Sources of variance and associated F-ratio and p-values are presented.

Source of Variation	D.F.	F-Ratio	Prob>F
Rep	4	4.39	0.0071**
Tape	1	2.02	0.1664
Treatment	3	0.41	0.7437
Treatment*Tape	3	0.83	0.8299

**=Significant at the alpha=0.01 level

Table 4: Analysis of variance for mean BWA uneclosed eggs with all sources of variation and associated F-ratios and p-values presented.

Source of Variation	D.F.	F-Ratio	Prob>F
Rep	8	9.21	0.2678
Treatment	4	1.41	<0.0001***
Rep*Treatment	32	1.63	0.1274

***=Significant at the alpha=0.001 level

Table 5: Tukey-Kramer honestly significant differences between BWA uneclosed eggs mean (least squares mean) with standard errors reported for the BWA bornyl acetate concentration experiment.

Treatment	Significance*	Mean Uneclosed Eggs	Standard Error
Veitch Fir Control	A	12.6	1.15
Fraser Fir Control	B	6.2	1.15
Water	B	4.2	1.15
Concentration 4[4]	B	4.0	1.15
Concentration 3[3]	B	3.7	0.81
Concentration 1[1]	B	3.0	0.81
Oil	B	2.8	0.81
Concentration 5[5]	B	2.4	0.81
Concentration 2[2]	B	1.2	1.15

*=Treatments not connected by the same letter are significantly different (alpha=0.05)

[1]= 100 µl of 1 µl BA in 10 ml oil solution

[2] =100µl of 1 µl BA in 2.5ml oil solution

[3] =100µl of 1 µL BA in 1 ml oil solution

[4] =100µl of 10 µL BA in 1 ml oil solution

[5] =100µl of 25 µL BA in 1 ml oil solution

Table 6: Analysis of variance with sources of variation and associated p-values of significance when response variable is percent difference in eggs from start to finish during the BWA concentration experiment.

Source of Variation	D.F.	F-Ratio	Prob>F
Rep	4	1.34	0.2911
Treatment	8	9.17	<0.0001***
Treatment*Rep	32	1.68	0.1132

***=Significant at the alpha=0.0001 level

Table 7: Tukey-Kramer HSD pairwise comparisons of all mean percent difference in uneclosed eggs start to finish for BWA concentration experiment.

Treatment	Significance*	Mean % Change Eggs	Standard Error
Veitch fir Branch	A	-24.82	6.84
Fraser fir Branch	B	-62.90	6.84
Concentration 4[4]	B	-74.06	6.84
Water	B	-76.70	6.84
Concentration 3[3]	B	-78.06	4.84
Concentration 1[1]	B	-82.34	4.84
Oil	B	-82.54	4.84
Concentration 5[5]	B	-85.66	4.84
Concentration 2[2]	B	-92.68	6.84

*=Levels not connected by the same letter are significantly different (alpha=0.05)

[1]= 100 µl of 1 µl BA in 10 ml oil solution

[2] =100µl of 1 µl BA in 2.5ml oil solution

[3] =100µl of 1 µL BA in 1 ml oil solution

[4] =100µl of 10 µL BA in 1 ml oil solution

[5] =100µl of 25 µL BA in 1 ml oil solution

Figure 1: Green peach aphid housing unit with *Brassica* plant food-source.

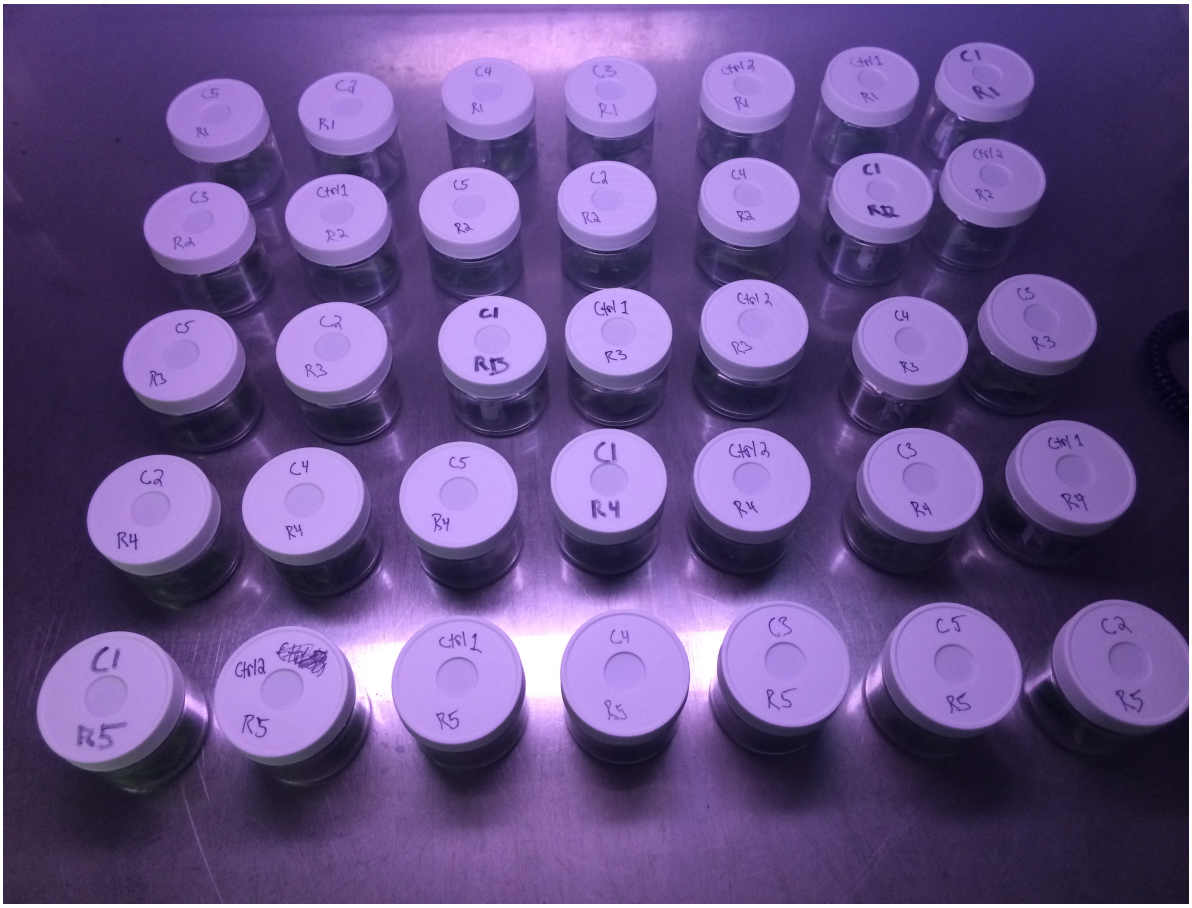

Figure 2: All treatments and repetitions from the Aphid experiment in their 60 mL SPME jars.

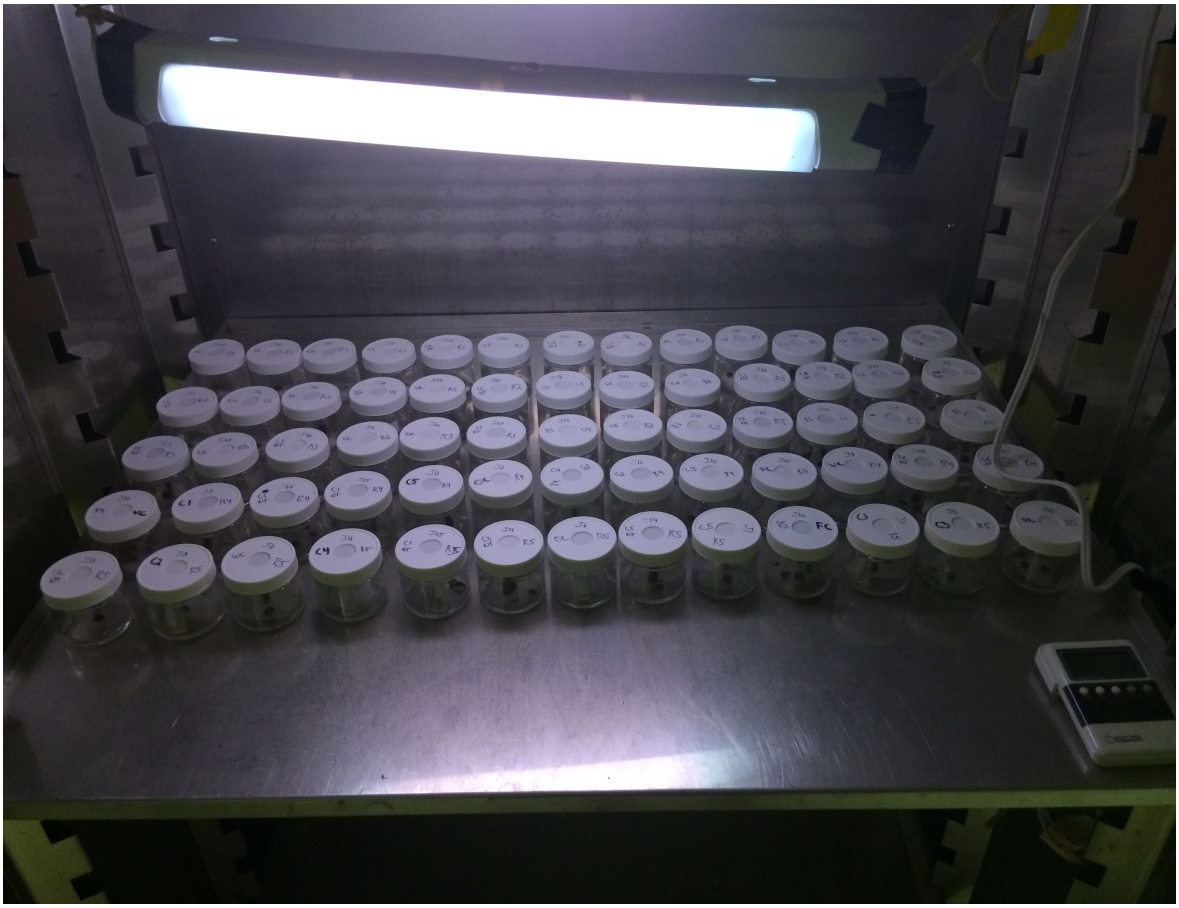

Figure 3: treatments and repetitions for the BWA Experiment in their 60 mL SPME jars**.**

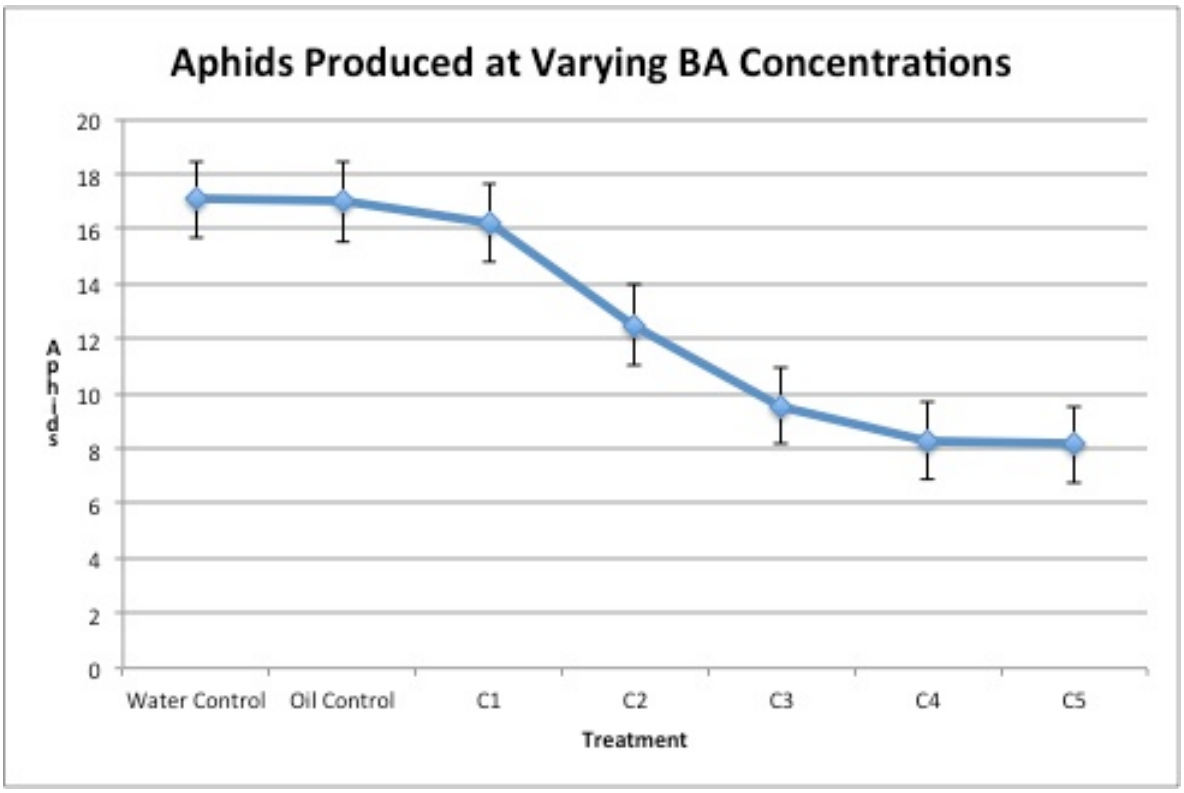

Figure 4: LSMeans of aphids produced within each treatment and standard errors associated with those measurements**.**

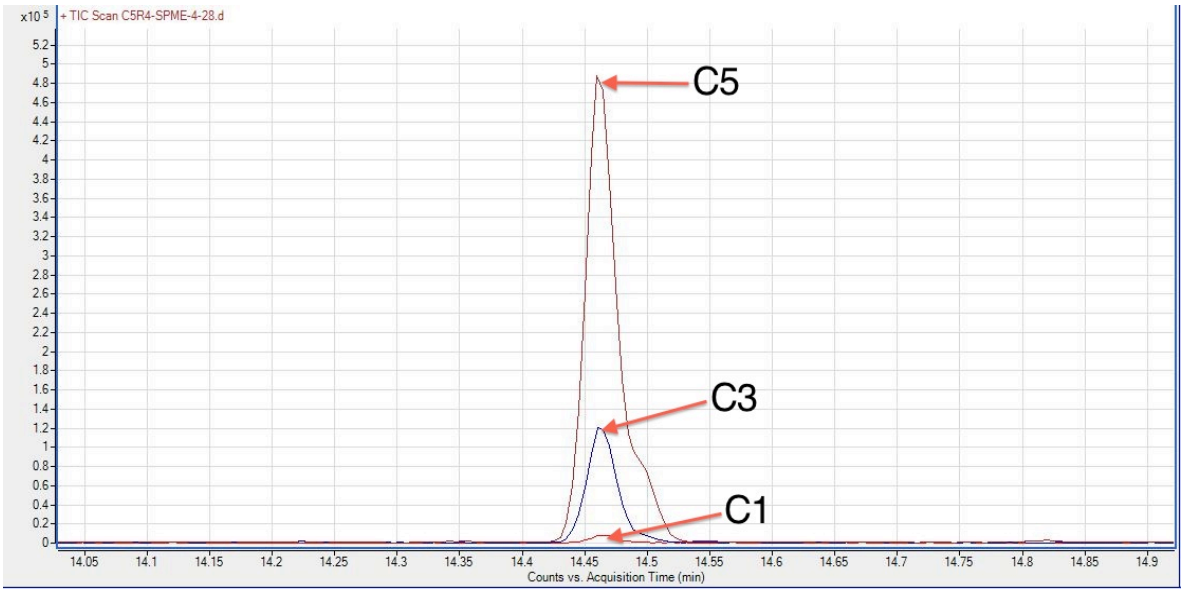

Figure 5: Comparison of bornyl acetate peaks between concentration 1, 3 and 5 during the Aphid Experiment.

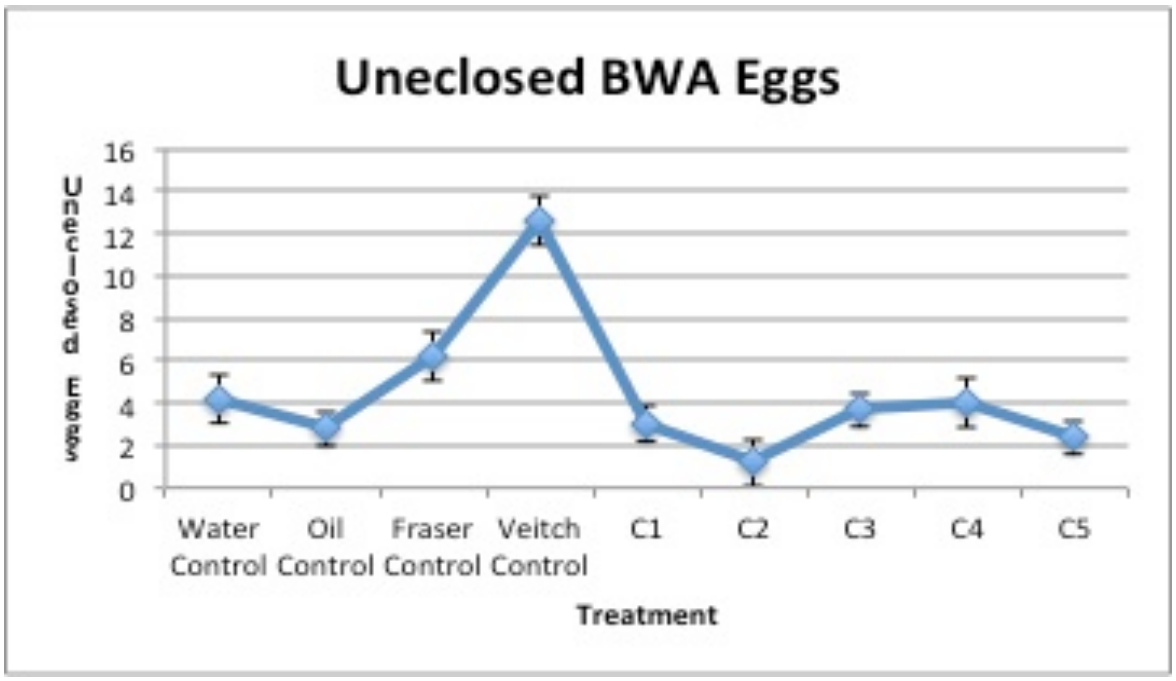

Figure 6: Uneclosed eggs mean (least squares mean) for each treatment and associated standard error**.**

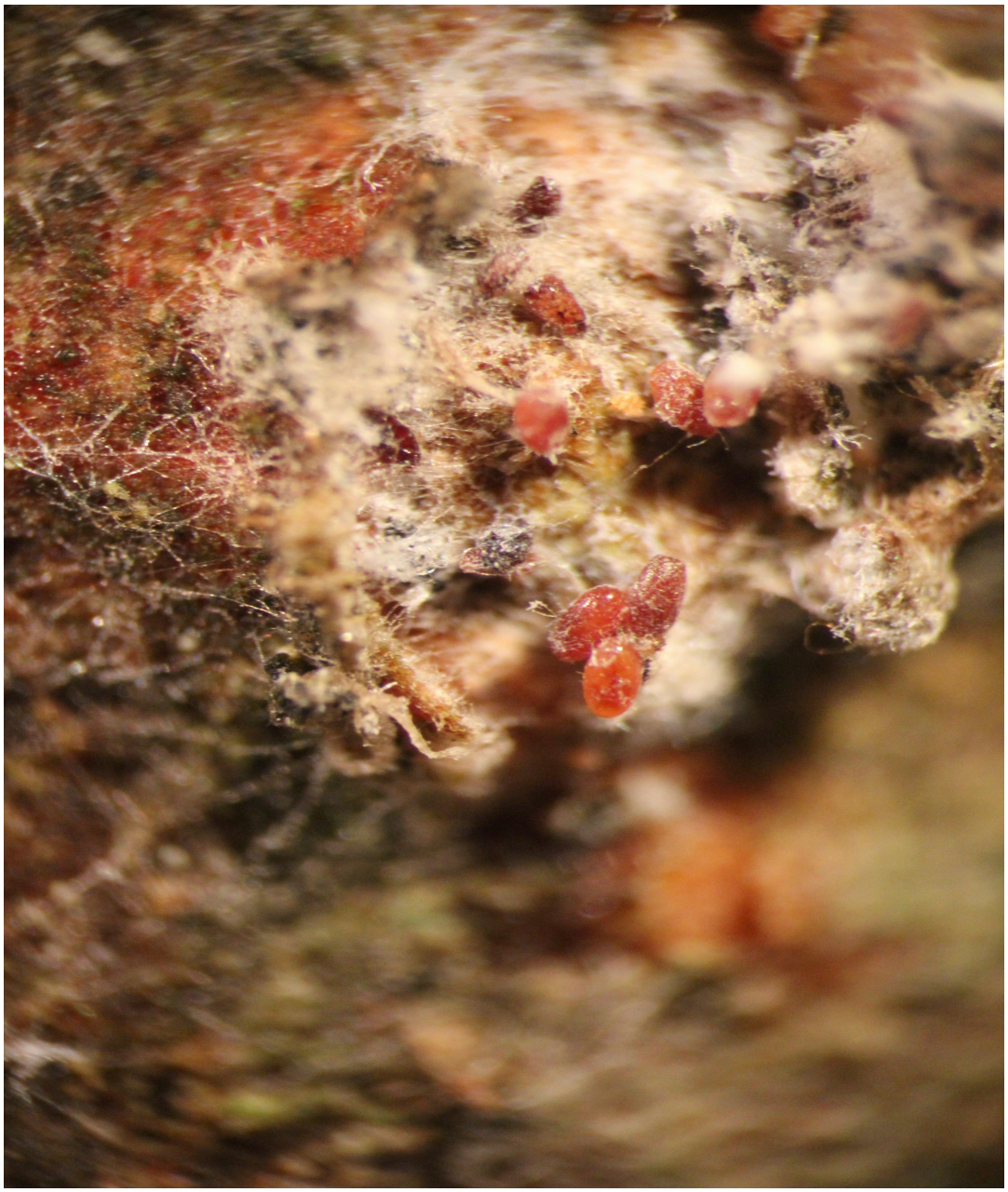

Figure 7: Uneclosed eggs on 9 mm bark disk from Veitch fir control rep 4

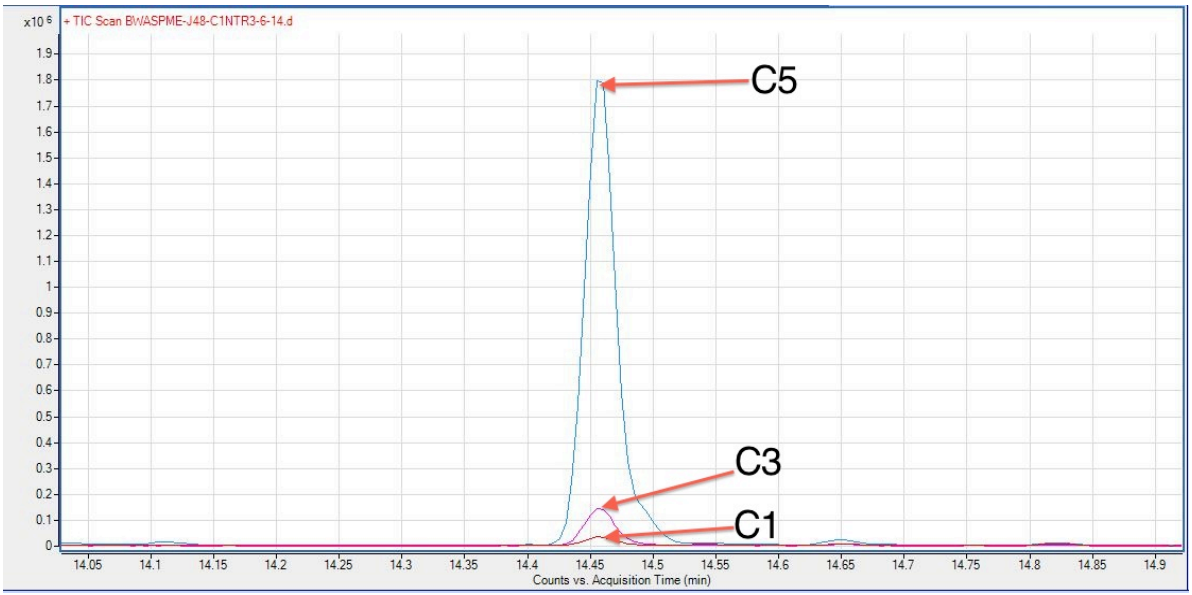

Figure 8: Comparisons between bornyl acetate signal in concentration 1, concentration 3 and concentration 5 during the BWA Experiment

Chapter 4: Qualitative Solid-Phase Microextraction Gas Chromatographic and Mass Spectrometric Analysis of Seventeen *Abies* Species and Identification of Volatiles Associated with Resistance and Susceptibility to Balsam Woolly Adelgid

Introduction

Balsam Woolly Adelgid (BWA) (*Adelges piceae* Ratz.) (Hemiptera: Homoptera: Adelgidae) is an exotic invasive phytophagous pest in the Southern Appalachians of the United States. Introduced in the early 1900s to the upper New England coast, BWA has since spread to most areas of North America, and was carried south along the ridge of the Appalachian Mountains, making it to the Southern Appalachians by 1955 (Kotinsky 1916) (Speers 1958). Within the Southern Appalachains, BWA has been a significant mortality agent within high elevation Fraser fir (*Abies fraseri*) forests endemic to this region. Fraser fir is the only member of the *Abies* genus found in this part of the Appalachian region, a remnant from the latest period of glaciation, which enabled more boreal species to push farther south as the ice sheet advanced, and to move upward in latitude and elevation as it retreated about 10,000 years ago (Potter et al. 2005). As such, Fraser fir is only endemic to the highest elevation peaks in the Southern Appalachians. Fraser fir holds little timber value economically (Amman and Speers 1965) but is an important part of the North Carolina forest economy due to its popularity as a Christmas tree. Annually, Christmas trees generate between $75-100 million in revenue for the state (NC Department of Agriculture and Consumer Services) (Potter et al. 2005).

BWA has caused a significant amount of mortality in Fraser fir since its introduction to the Southern Appalachians in 1956. BWA infests trees via both crown and stem infestations, but stem infestations tend to be more devastating to overall tree health than

crown infestations (Arthur and Hain, 1987). The feeding of BWA is generally confined to outside the phloem, where BWA crawler stylets are able to penetrate the phellum and feed on parenchyma (Balch, 1952). Rarely do stylets access the phloem, and those cases are normally found on younger shoots rather than mature trees. The wound response of Fraser fir to this infestation is what causes the mortality.

The formation of 'rotholz' or irregular heartwood similar in composition to compression wood is highly associated with the mortality attributed to BWA infestation (Balch 1952). Purtich, 1977 found the formation of this irregular heartwood to be highly associated with adelgid infestation on *Abies grandis* (Dougl. ex. D.Don) Lindl. Puritch, 1973 also hypothesized that those members of *Abies* more tolerant to water stress, are likely more resistant to BWA. The effect this has on the translocation abilities within *Abies* has been noted. Studies assessing the translocation of dye between infested and uninfested controls found that significantly less dye was absorbed by infested trees and dyes were not taken up as high in the column in infested trees as in uninfested trees (Mitchell, 1967). This indicates that the wound response attributed to BWA infestation does significantly limit the trees ability to transport nutrients and water up the stem. Over successive years of infestations, especially bole infestations, mortality results (Amman and Speers, 1965). In the Southern Appalachians, on Mt. Mitchell specifically, 82, 98 and 95 percent of Fraser fir trees over 244 cm in height located in fir, spruce/fir and spruce/fir/hardwood ecotypes respectively were killed by 1966, just 10 years after initial detection of BWA within the Southern Appalachians (Witter and Ragenovich 1986).

Not all members of the *Abies* genus are susceptible to mortality following infestation by BWA. Table 1 shows 17 species, all members within the *Abies* genus with their relative susceptibilities, based on findings from infestation trials (Mitchell 1966). Resistance is typically talked about in reference to one of three things: antibiosis, antixenosis and tolerance. Antibiosis is a resistance mechanism of the plant being fed upon that has a negative impact on the biology of the feeder, usually resulting in death. Antixenosis is typically a non-preference to a type of plant based on some characteristic of the plant. Tolerance is defined as being able to withstand infestations or attacks that would cause more susceptible plants to succumb (Painter, 1958).

Chemical biology often plays a large role in the understanding of resistance mechanisms. Differences between constitutive defenses and induced defenses make the assessment of resistance difficult. Constitutive defenses are those prepared before the attack, whereas induced defenses are those prepared during or following attack (Larsson, 2002). Looking at the leaf volatile composition changes between young and old balsam fir (*Abies balsamea* L.) Mill. trees, target terpenes have been identified that reduced larval growth and survival when fed upon by spruce budworm, indicating the role of both terpene chemistry and age-related susceptibility to pest species (Zou and Cates, 1995). Studies of the chemical biology of Norway spruce (*Picea abies* L.) Karst. trees showed that the prepared (constitutive) secondary metabolites are not what determine resistance to *Ips topographus* (L.) beetles, but that those secondary metabolites produced as a result of infestation (induced) are more indicatory of strength in defending against bark beetles (Schiebe et al. 2012). However, bark beetle behavior and ecology is quite different from that of BWA. Bark

beetles are more evolved towards being sensitive to chemical cues of their hosts as well as environmental cues. It seems that beetle species have evolved to avoid competition with one another based on tree availability as a limiting resource (Amezega and Rodriguez, 1998). In North America, the BWA dispersal stage, crawlers, do not actively search for viable hosts as stringently as bark beetles, who have cued in on various aspects of tree chemical biology. Therefore, assessing constitutive defenses of various fir trees may offer insight into potential resistance to BWA, more so than those of more developed species of Coleopterans (Larsson, 2002).

The identification of those terpene and chemical constituents implicated in tree defense is very important for many fields. From a management perspective, identification of tree terpenes and those that are associated with negative biological activity on target pest species has enabled researchers to pose more management suggestions for stored food mite pests (Lee et al. 2009). Analysis and identification of foliar terpenes of target species of trees have enabled researchers to test the efficacy of different chemicals on biological activity of western spruce budworm (*Choristoneura occidentalis* Freeman) (Cates et al. 1987). Bauce et. al, 1994 identified differences in balsam fir needles based on age, and identified target terpenes that, in conjunction with certain levels of nitrogen and tannins, resulted in decreased feeding activity by the spruce budworm (*Choristoneura fumiferana* Clemens). Using that identification, Kumbasli and Bauce (2013) evaluated the effect of those terpenes within an artificial diet on growth and survival of spruce budworm. Evaluation of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) needles has identified variation in terpenes associated with both age and location, resulting in differences in efficacy in effecting

negative biological activity on different populations of spruce budworm (Zou and Cates, 1995, Zou and Cates, 1997). Identification is always the first step in these studies, to establish target chemicals potentially linked to the desired end.

Studies that have taken a look at Fraser fir volatiles, especially when looking for resistance to BWA, have not found much of note. A study looking to compare bark artificially wounded and treated with chemicals suspected to be associated with BWA stylet insertion yielded no differences in monoterpene content in the bark after wounding (Arthur and Hain 1987). Similarly, monoterpene comparisons amongst Fraser fir trees suspected of being more resistant only yielded slight differences in levels of delta-carene (higher in susceptible trees) and alpha-pinene (lower in susceptible trees) (Arthur and Hain, 1987). Similar studies comparing Mount Rogers and Roan Mountain sources of Fraser fir found that delta-carene, beta-phellandrene and total monoterpene content were statistically significantly different, although the premise that Mount Rogers Fraser fir is more resistant did not pan out as they underwent severe infestation during the investigation (Sutton et al. 1997). As far as testing multiple species of fir and identifying qualitative differences, there are only studies on *Tsuga* species and their susceptibility to Hemlock Woolly Adelgid (*Adelges tsugae* Annand) (HWA) that make comparisons based on known susceptibility to this similar pest. Through qualitative analysis of seven species of *Tsuga* with differing susceptibilities, a list of target chemicals for investigation as attractants for HWA and deterrents for HWA were identified (Lagalante and Montgomery, 2003).

The goal of this study is to similarly identify target chemicals that may be more associated with resistance and susceptibility to BWA. Using the resistance rankings

determined for different *Abies* species, I will qualitatively, using SPME and GC/MS, analyze chemical components across the susceptibility spectrum in *Abies* utilizing materials from several arboreta. I will then use information produced in those analyses to perform statistical analysis to identify chemicals most predictive of resistance to BWA for future testing.

Methods

All investigations of volatile contents were done in the summer of 2015, between late May and early August. Species of *Abies* featured in Mitchell 1966 were those targeted for investigation (Table 1). Susceptibility levels, as defined by Mitchell, were Nil, Slight, Moderate and Severe. Those with a rating of nil are considered resistant, those with severe are the most susceptible. SPME jars used were those used in previous investigations: 60mL wide-mouth septum SPME jars from Cole-Parmer. Samples were kept cool during shipping with dry ice and placed in a -20°C freezer for storage before use. The arboreta which provided samples for investigation were requested to include at least one individual per species for each of those listed in Table 1, and if multiple individuals were present, up to 3 individuals from a single species of *Abies*. A 5-8 cm branch cutting was taken from each individual and placed in a bag for shipping with appropriate labels. Before placement into a SPME jar with an appropriate label, samples were defoliated and cut multiple times to ensure volatile release from the stem and bark material. Samples were then placed in a refrigerator (40°C) and sat for 2 days before SPME investigations were conducted.

All samples used for this experiment were provided from arboreta throughout the country. Three arboreta, in total, provided samples of different *Abies* species for testing.

Hoyt Arboretum in Portland, Oregon shipped samples in late May 2015. Morton Arboretum in Lisle, Illinois also shipped samples overnight in late May 2015. Longwood Gardens in Kennett Square, Pennsylvania, shipped overnight samples in late July 2015. Between the arboreta, a total of 55 samples across 17 *Abies* species listed in Table 1 were examined at least twice.

SPME investigations entailed allowing the samples to reach room temperature before placement of SPME needle in the headspace of the jar. A Supelco (Subsidiary of Sigma Aldrich, Bellefonte, PA) field sampling SPME fiber was used to conduct these investigations. Samples and needle would then sit for 2 hours of extraction before being run on a 25-minute GC/MS round. Two hour extraction times were determined from investigations of differing extraction time on chromatographic signals. Each individual sample was run at least twice through the GC/MS to provide a better estimate of the sample values.

The GC/MS component of these investigations was performed on an Agilent Model 7280A GC system with an attached Agilent Model 5977E mass spectrometer. Oven temperatures started at 40°C for 2 minutes, and an increase at a rate of 8°C per minute up to 224°C final at 25 minutes. Sample separation was accomplished using an Agilent Column HP-5MS-UI (30 meters, 0.25mm i.d, 0.25 thickness). Injector temperatures were kept at a constant 250°C. The SPME fiber was inserted for all 25 minutes of the run, before removal for other runs. Each individual chromatogram was integrated using the same program and same specifications by peak area 1% of total chromatogram area. Anything less than 1% of the total chromatogram area was not considered in our analysis because of noise. All

chromatographic peak information for each individual sample was somewhat different. Total peak numbers between samples were unequal, and as such, needed to be moved. Grouping of peaks was done by matching retention times. Retention times vary run to run, and therefore they were grouped by +/- 0.05 minutes in order to have a complete data set where all peaks were represented in each chromatogram. If a chromatogram did not have a certain peak, it was given a height and area of 0 to signify that these compounds were not found in high enough relative abundance to be considered. Once a complete data set was compiled, statistical analysis could begin. Mass hunter workspace software qualitative analysis (Version B.06.00) was used to integrate and determine peak area and peak height. Selected peak spectra were analyzed and identified using NIST (National Institute of Standards and Technology) libraries, and peak spectra were compared to existing spectral information for identified compounds to best match information present in chromatograms. Any identification of compounds is therefore tentative as substances were not compared to the actual chemical, and should be considered as potential targets for future investigations.

Statistical Analysis

All qualitative analysis of peak information was analyzed using the PROC PLS procedure with SAS Software (Version 9.3). Partial least squares (PLS) analysis is a type of multivariate analysis, that differs from operations like principle component analysis (PCA) because it is a supervised analysis, meaning comparisons of a wide variety of factors within the model can be contrasted with a dependent variable, unlike PCA which does not compare a set of independent variables to a dependent one (Maitra and Yan, 2008). PLS enables variable reduction while also allowing predictions to be made based on the data set. In the

analysis of this data, the dependent variable was the BWA susceptibility ranking (1-4) with 1=Nil, 2=Slight, 3=Moderate and 4=Severe, derived from Mitchell's 1966 classification of *Abies* spp.

Data were analyzed using PLS in two separate ways. Individual tree mean data were compiled by averaging chromatographic information on peak height and peak area over the two runs conducted per sample (n=55 tree means). Species mean data were compiled by averaging all chromatographic peak and height data for each species, across all three arboreta (n=17 species means). For each analysis, 4 subsets of the data were tested: peak area and percent peak area, peak height and percent peak height. Percent peak area was calculated as the ratio of individual peak area to the total area of the individual chromatogram. Percent peak height was calculated in a sim4ilar fashion, utilizing the height data for each peak contained on an individual chromatogram. The data were not centered or scaled prior to the PLS analyses. The data reported are the percent height and percent area information for both the individual tree data and species mean data.

One-at-a-time cross-validation was conducted in order to determine the number of factors to extract; the selected models minimized the predicted residual sum of squares (PRESS). For each model, peaks with a variable importance projection (VIP) (Wold, 1994) exceeding 0.75 were selected and putatively identified. For each peak identified, correlations were calculated, comparing chromatographic peak information with resistance to determine relationship of the peak variable with resistance ranking. Those with a negative correlation indicate a role in resistance while those with a positive sign are more indicative of

susceptibility. P-values were also recorded to determine the relative significance of the correlations.

Results

Table 2 shows the top identified peaks from the percent height analysis of the individual tree data. The VIP numbers and their importance is also featured in Table 2, along with correlations with resistance, the sign of the estimate suggests it is more indicative of a resistance trait (negative sign), or a susceptibility trait (positive sign). Correlation size and associated p-values are also reported. Table 3 is a list of the top most predictive peaks from the partial least squares regression analysis on the percent area of the individual tree data. Table 4 shows the tentatively identified compounds from the analysis of the percent height of the species mean data, and Table 5 shows those top predictive peaks associated with the PLS analysis of the percent area of the species mean data.

Overlapping compounds identified in all the analyses were readily apparent. Those that appeared in the top predictive peaks, across all 4 data sets were: beta-phellandrene, beta-pinene, alpha-pinene, beta-myrcene, camphene, delta-carene, d-limonene, toluene, and a terpinolene (cyclohexene, 1-methyl-4-(1-methylethylidene)-)isomer. Other compounds of interest that were not featured across all four data sets were: caryophyllene, bornyl acetate and y-muurolene.

Tables 2-5 also show the R-square value of our models. Percent area data from the species mean analysis had the highest r-square value at 0.95, whereas the other three analyses had r-square values of 0.91, indicating that these models explain much of the variation in the

data. Also, these models predicted susceptibility ratings (1-4) with 1 representing resistance (nil damage) and 4 indicating susceptibility (severe damage). Table 6 shows the predicted damage intensities, our measure of relative resistance levels, made by these models and their actual damage intensity level for the percent area and percent height models using the species mean data. Based on the table, the model was able to predict resistance levels more accurately using percent area data rather than percent height data (Table 6).

Discussion

Our qualitative analysis of different species of *Abies* identified candidate compounds that need further study in order to elucidate more clearly their impact on BWA survival, fecundity, and other potentially inhibitory biological functions. Although we have some guidance and potential avenues, the impact monoterpenes have on insects is not limited to one biological function or even one biological outcome. Monoterpenes and those secondary metabolites produced by trees and plant species highlight the intricacies of the kairomone, synamone and allomone complex. Because this study is attempting to draw statistical correlations with various compounds found within resistant and susceptible species of *Abies* the following will discuss studies that include the top identified compounds and their potential use in the pest management field.

These results seem in line with those observations made by Lagalante and Montgomery, 2003, a study conducted in a very similar manner to this one. We identified many of the same terpenes, and highlighted their potential importance to resistance. Lagalante and Montgomery, 2003 indicated that alpha-pinene and beta-caryophyllene were

potential indicators of resistance to HWA infestation. Similarly, identification of monoterpene changes between HWA-infested and healthy eastern hemlock branches showed that alpha pinene, beta pinene, beta phellandrene, terpinolene, bornyl acetate, limonene and camphene, among other monoterpenes, increased as a result of infestation (Broeckling and Salom, 2003). Consistent with this finding, is the finding that comparing resistant eastern hemlock populations with susceptible populations, general terpene concentration increases between susceptible and resistant trees, rather than a few specific terpenoids (McKenzie et al. 2014). While these terpenes represent a change in induced defense in response to HWA attack or constitutive defense prepared already, their effects on HWA survival are unknown. General studies of volatile composition of Fraser fir indicate that those terpenes, save for toluene, identified are important constituents of fir foliage and that beta-phellandrene may be emitted in response to foliar injury (Vereen et al. 2000). When comparing terpene content to BWA resistance in Fraser fir, although resistance with Fraser fir has yet to be truly identified, the monoterpenes alpha- and beta-pinene as well as delta-carene were identified in lower concentrations in those trees tentatively viewed as resistant to BWA, or at least less susceptible (Arthur and Hain, 1987). Delta-carene and beta-phellandrene were two monoterpenes that showed significant differences between resistant and susceptible populations of Fraser fir, alluding to potential involvement in resistance to BWA (Sutton et al. 1997).

Other tree species and their secondary metabolite constituents have been analyzed for their potential impact on pests. Studies of the seasonal changes to foliar terpene chemistry identified bornyl acetate, camphene and alpha-pinene as potential deterrents to the spruce

budworm, based on studies of Douglas-fir foliage (Zou and Cates, 1995). Based on analyses of balsam fir needles, lower consumption rates by the sixth-instar larvae of the spruce budworm were highly correlated to lower nitrogen and tannin ratio, as well as the increased presence of bornyl acetate, terpinolene and delta carene (Baucé et al. 1994). Similarly, beta-pinene with high concentrations of nitrogen improved spruce budworm larval growth while bornyl acetate impeded and reduced larval growth and survival (Cates et al. 1987). When comparing species of pine, the Swiss stone pine (*Pinus cembra* L.) was found to have volatile emissions from cones, of which alpha-pinene, beta-phellandrene, limonene and beta-pinene were dominant monoterpenes. When resin extracts were applied to cones from mountain pine (*Pinus unciata* Ram.), whose resins contained markedly lower amounts of alpha pinene, damage by herbivory from a number of different phytophagous insects was significantly reduced (Dormont et al. 1997). Identifying differences between species enables inroads to be made into uncovering supplementary or different control methods. The identification of other tree and woody plant compounds is important to uncovering new methods of pest control.

Understanding chemical properties through identification of compounds enables researchers to look for potential uses on an industrial scale. Plant essential oils are now being more widely viewed as "reduced-risk pesticides" as they have a more desirable environmental fate, compared to many of the chemical control methods still widely used in agriculture (Isman, 2000). Originally viewed as deterrents, their full range of insecticidal activities is now being processed more regularly for pest control (Isman, 2000). Essential oil derived from *Eucalyptus grandis* Hill ex. Maiden shows that both alpha- and beta-pinene are

active larvicidal compounds against mosquitos and could be useful in determination of more naturally derived pesticides (Lucia et al. 2007). Alpha-pinene has also been identified as a major constituent of essential oils within cultivated members of the Cupressaceae family in Vietnam (Dai et al. 2013). One should note that the literature consensus as to the action of certain monoterpenes and compounds of interest is not complete. Alpha-pinene, among other terpenes identified in this experiment, is also highly attractive for certain bark beetle species. The Japanese pine sawyer (*Monochamus alternatus* Hope) had a significantly greater consumption rate than the control at all rates of addition of alpha pinene to its diet (Fan and Sun, 2006). Limonene, on the other hand, had an inhibitory effect on consumption rate of *M. alternatus*. Similarly, limonene had an inhibitory effect of attraction to alpha-pinene and ethanol baited traps, which, without limonene, attracted 5-16 times greater numbers of *Hylobius* spp. Germar beetles (Nordlander, 1990). This attraction has been used against pest species as well. Tree bolts, baited with the same alpha pinene/ ethanol mixture were used to successfully attract antagonistic species of insects to help manage pest populations significantly more than control trees (Schroeder and Weslien, 1994). Traps baited with toluene, found to be present in half-ripe olives, were found to be an attractant to olive fruit flies (*Dacus oleae* Rossi) (Scarpati et al. 1993).

Another aspect of this research that must be accounted for are individual effects and synergistic effects. Nordlander, 1990 notes that alpha-pinene did increase attraction to baited traps, but was even more attractive when ethanol was added to the mixture. Commercial lures for bark beetles include a host-tree terpene component. When the commercial lure, which contains myrcene as the terpene component, was compared to a lure with alpha-

pinene, instead of myrcene, attraction of multiple species of bark beetles to the traps increased (Hofstetter et al. 2008). Individual monoterpenes and their individual LD50 (lethal does to kill 50% of test population) can be calculated, and have indicated that camphene, limonene and myrcene do have contact insecticide action, and their individual toxicity increases when applied to test populations of red rust flour beetle (*Trilobium castaneum* Herbst.) (Coleoptera: Tenebrionidae) and the rice weevil (*Sitophylus oryzae* L.) (Coleoptera: Curculionidae) (Abdelgaleil et. al. 2009). When comparing delta-carene, camphene, terpinolene and bornyl acetate to spruce budworm toxicity, individually, these substances are not as effective in their toxicity (Kumbasli and Baucé, 2013), compared to when these substances were administered with different levels of nitrogen and tannins (Baucé et al. 1994). Caryophyllene emitted from leguminous fodder crops in Australia determined attraction by a pest psyllid *Heteropsylla cubana* Crawford, indicating that for certain species of Homopterans, caryophyllene is emitted by plants more attractive to a certain pest, but results in no difference in attracting insects in baited traps compared to controls (Finlay-Doney and Walter, 2005). Mixtures of cultivated potato plant sesquiterpenes, of which caryophyllene was identified as a major component, were found to be very repellant to green peach aphid (*Myzus persicae* Sulzer) (Avé et al. 1987). Clove bud (*Syzygium armomaticum* (L.)) essential oil, which contains a large amount of caryophyllene, has also been shown to have insecticidal properties against pear psylla (*Cacopsylla chinensis* Yang and Lee) (Hemiptera: Psyllidae) (Tian et al. 2015). These studies suggests that synergism is an important part of understanding chemical biology of tree defenses as the components as a whole indicate desired antibiotic effects rather than their individual parts. Individually, toxic

substances are not as effective at managing a pest species, but in conjunction with synergists, their toxicity increases in its efficacy.

My results suggest that there may be many different terpenes indicated as important to resistance and susceptibility within *Abies*. The terpenes and other secondary metabolites that were identified have widespread effects on different species of insects. They can be attractants, inhibitors, or toxic, depending on the insect species. Further research is needed to uncover just what these secondary metabolites will do, if anything, to BWA and at what stage in their life cycle are BWA affected by these substances. Consistently, alpha-pinene, and beta-myrcene showed weak negative correlations with damage ranking, that were not statistically significant, but still indicated their potential within this system (Tables 2-5). Caryophyllene, although not identified as a top peak in the percent area analysis of the species mean data, had a statistically significant negative correlation (alpha=0.001) with damage intensity in Tables 2-4. D-limonene also had a statistically significant negative correlation (alpha=0.05) with damage intensity in Tables 2 and 3, while delta-carene was significantly positively correlated with damage in Tables 2-5 (alpha=0.05). The other terpenes found in tables 2-5 have positive correlation coefficients indicating their correlation with susceptibility (positive correlation with damage), but that does not mean they should be left out of any future analyses. These correlations help provide targets for future resistance studies, and ascertaining their individual and synergistic effects on BWA fecundity, as well as other life stages, would be useful in potentially determining biochemical resistance mechanisms.

Looking at the prediction success of our model, percent area chromatographic information from the species mean data was the most accurate in terms of correctly placing species in their resistance levels, which indicates that this information is quite useful in predicting resistance levels. Our peak identification needs "truthing" or comparing the identified substances and associated spectral information to synthesized versions to ensure identification accuracy.

Future research concerns with our tentative identifications must focus on a few things. The first is the checking of our identifications. This study highlights some of the limits of qualitative analysis through SPME. The MS can produce the spectral data, but due to the similarity of many different known substances in molecular weight and empirical formula, identifications can only be tentative. Testing known compounds and comparing them to our ID is required in order to be more confident. Being that we were identifying substances with the NIST library as well as based on their spectra and how well they matched with compounds within the NIST library, more analysis of these compounds must be made such that accuracy in our identification can be more accurate. For instance, our identification of y-muurolene or 1,6-cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]- represents the difficulty in using just the spectral data. The closeness and similarity between these two compounds makes concrete identification difficult. The next is to test these substances on BWA. It would be useful to test them on multiple life stages, and it would also be useful to identify potential synergisms to add into testing to make the design a bit more complex. Using the methods developed in Chapter 3, future research can ascertain the effects, if any, of target volatiles individually and in conjunction with other potential targets.

Testing them individually will give a good idea of what substances are more toxic than others, and identifying secondary variables like seasonal nutrient composition, nitrogen content etc. can be useful in identifying potential mechanisms that aid in relaying toxicity to BWA in resistant species. It would also be interesting to apply this approach to bark and stemwood. The volatiles most likely to come into direct contact with BWA feeding activity are those in the bark. Perhaps further differentiation between bark metabolites and stem metabolites can yield more avenues for understanding BWA interaction differences amongst the susceptibility ratings. Also, identifying changes between infested and uninfested trees across a wide susceptibility spectrum could help identify differences, if any, between constitutive defenses and induced defenses. As was shown in Chapter 3, constitutive defenses may be responsible for the negative impact on BWA egg eclosion, Understanding how BWA alters chemical composition of multiple *Abies* species, and how that varies by susceptibility ranking could be useful in determining resistance mechanisms. Being that this study was a broad overview of the chemical biology of *Abies*, the exact nature of the biological activity on BWA cannot yet be determined, but this study does give target substances for future resistance efforts.

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Table 1: Damage rankings of *Abies* species (Mitchell, 1966) provided by three arboreta.

Scientific Name	Common Name	Origin	Damage Rating
Abies lasiocarpa	Subalpine fir	Western North America	Severe
A. fraseri	Fraser fir	Easter U.S.	Severe
A. balsamea	Balsam fir	Northeastern North America	Severe
A. amabilis	Pacific silver fir	Northwestern North America	Severe
A. grandis	Grand fir	Western North America	Moderate
A. lasiocarpa var. *arizonica*	Corkbark fir	Southwestern U.S.	Moderate
A. koreana	Korean fir	Korea	Moderate
A. sachalinensis	Sakhalin fir	Northeastern Asia	Moderate
A. religiosa	Sacred fir	Southern Mexico	Slight
A. procera	Noble fir	Western U.S.	Slight
A. concolor	White fir	Western U.S.	Slight
A. alba	European silver fir	Western Europe	Nil
A. cephalonica	Grecian fir	Greece	Nil
A. pinsapo	Spanish fir	Spain	Nil
A. sibirica	Siberian fir	Northern Asia	Nil
A. firma	Momi fir	Japan	Nil
A. veitchii	Veitch's silver fir	Japan	Nil

Table 2: Top chromatographic peaks by Variable Importance in Projection (VIP), associated MS-ID along with correlation values and predictive success for percent height of individual tree mean data.

Number of Factors	4
R-Square Value	0.91
Correct Predictions	53%
Correct Predictions within 1-Resistance Level	94%

Peak Number	**VIP**	**MS-ID**	**Correlation with Damage and P-value***
40	6.90	Beta-Phellandrene	0.18 0.19
37	5.18	Delta-Carene	0.54 <0.0001***
27	5.16	Alpha-Pinene	-0.13 0.36
41	5.07	D-Limonene	-0.27 0.05**
33	4.39	Beta-Pinene	0.25 0.07
34	3.39	Beta-Myrcene	-0.09 0.52
49	1.38	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.49 0.0001***
28	1.16	Camphene	0.08 0.57
113	1.01	Caryophyllene	-0.61 <0.0001***
15	0.99	Toluene	-0.12 0.39
89	0.91	Bornyl Acetate	0.12 0.39

*= Sign on damage correlation indicates association with resistance or susceptibility (+ =susceptible, - =resistance)
**=Significant at the alpha=0.05 level
***= Significant at the alpha=0.0001 level

Table 3: Top chromatographic peaks by VIP, associated MS-ID along with correlation values and predictive success for percent area of individual tree mean data.

Number of Factors		4	
R-Square		0.91	
Correct Predictions		67%	
Correct Predictions within 1-Resistance Level		96%	
Peak	**VIP**	**MSID**	**Correlation with Damage and P-value***
40	7.90	Beta-Phellandrene	0.17 0.22
41	5.61	D-Limonene	-0.28 0.04**
37	5.36	Delta-Carene	0.52 <0.0001****
27	4.13	Alpha-Pinene	-0.11 0.42
33	3.58	Beta-Pinene	0.19 0.17
34	2.73	Beta-Myrcene	-0.12 0.38
15	1.20	Toluene	-0.12 0.39
49	1.00	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.48 0.0002***
28	0.81	Camphene	0.07 0.61
113	0.76	Caryophyllene	-0.61 <0.0001****

*= Sign on damage correlation indicates association with resistance or susceptibility (+ =susceptible, - =resistance)
**=Significant at the alpha=0.05 level
***=Significant at the alpha=0.01 level
****=Significant at the alpha =0.0001 level

Table 4: Top chromatographic peaks by VIP, with associated MS-ID along with correlation values and predictive success for percent height of species mean data.

Number of Factors	3
R-Square Value	0.91
Correct Predictions	52%
Correct Predictions within 1-Resistance Level	94%

Peak Number	**VIP**	**MS-ID**	**Correlation with Damage and P-value***
40	7.40	Beta-Phellandrene	0.27 0.30
27	5.34	Alpha-Pinene	-0.23 0.37
37	5.09	Delta-Carene	0.56 0.02***
33	4.30	Beta-Pinene	0.30 0.25
41	4.25	D-Limonene	-0.31 0.23
34	3.48	Beta-Myrcene	-0.20 0.45
49	1.40	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.40 0.11
28	1.11	Camphene	-0.08 0.75
113	0.98	Caryophyllene	-0.63 0.0067****
15	0.85	Toluene	0.03 0.91
89	0.82	Bornyl Acetate	-0.09 0.72
134	0.80	Y-muurolene**	-0.40 0.11

*= Sign on damage correlation indicates association with resistance or susceptibility (+ =susceptible, - =resistance)
**= Identification incomplete, also tentatively identified as: 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]- which is another name for Germacrene D.
***=Significant at the alpha=0.05 level
****=Significant at the alpha=0.01 level

Table 5: Top chromatographic peaks by VIP, with associated MS-ID along with correlation values and predictive success for percent area from the species mean data.

Number of Factors	4
R-Square Value	0.95
Correct Predictions	100%
Correct Predictions within 1-Resistance Level	100%

Peak Number	**VIP**	**MS-ID**	**Correlation with Damage and P-value***
40	8.39	Beta-Phellandrene	0.26 0.32
37	5.20	Delta-Carene	0.58 0.02**
41	5.02	D-Limonene	-0.32 0.20
27	4.23	Alpha-Pinene	-0.22 0.39
33	3.39	Beta-Pinene	0.26 0.31
34	2.96	Beta-Myrcene	-0.24 0.35
15	0.99	Toluene	0.01 0.96
49	0.99	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	0.35 0.16
28	0.77	Camphene	-0.11 0.69

*= Sign on damage correlation indicates association with resistance or susceptibility (+ =susceptible, - =resistance)
**=Significant at the alpha=0.05 level

Table 6: Actual damage rankings compared with model predicted damage rankings based on chromatographic information utilizing both percent height and percent area of species mean data.

Species	Damage Ranking*	% Area Prediction	% Height Prediction
A. alba	1	1.06	1.17
A. cephalonica	1	0.98	0.58
A. firma	1	1.14	1.69
A. pinsapo	1	0.93	1.59
A. sibirca	1	0.97	1.51
A. veitchii	1	0.96	0.58
A. concolor	2	1.96	2.85
A. procera	2	1.87	2.59
A. religiosa	2	2.01	2.00
A. grandis	3	3.04	2.41
A. koreana	3	3.09	3.03
A. lasiocarpa v *arizonica*	3	3.09	3.18
A. sachalinensis	3	3.32	3.06
A. amabilis	4	4.10	3.47
A. balsamea	4	3.84	4.50
A. fraseri	4	4.01	2.58
A. lasiocarpa	4	3.64	2.92

*= Damage rankings on a scale of 1-4 where 1=Nil (Resistant), 2=Slight, 3=Moderate and 4=Severe (Susceptible)

Thesis Conclusions

The identification of metabolitic differences between susceptible and resistant species of fir revealed that bornyl acetate could be a potentially important difference amongst the species tested. Bornyl acetate is highly water insoluble and therefore is not effective at being uptaken by tree cuttings when in an aqueous solution. However, the increased number of BWA crawlers that inserted on Veitch fir indicates that resistance is likely governed by direct contact with secondary metabolites within the tree. Studying the effect that low and high doses of bornyl acetate has on egg eclosion within BWA, one will find that as a pure substance, bornyl acetate has both a slightly phytotoxic ability and results in almost no BWA egg eclosion. The lower concentrations do not see that inhibitory effect and are no different than water controls on Fraser fir. Veitch fir volatiles showed a slightly negative effect on egg eclosion, as fewer crawlers were found inserted in early experiments, but in more controlled experiments, where egg counts were made, Veitch fir volatiles did not differ at all from Fraser fir volatiles in having an effect on BWA egg eclosion.

Altering the concentration of bornyl acetate in the headspace was accomplished using silicone oil as the solvent. Using green peach aphid as the proxy insect for BWA, altering the concentration of bornyl acetate resulted in decreased fecundity, as those in the highest concentrated environments produced the fewest number of offspring. Conducting the same experiment with BWA did not result in decreased fecundity success, but other treatments did elucidate findings. The Veitch fir and Fraser fir controls showed that volatiles in a closed space have a negative effect on BWA egg eclosion. Veitch fir was the only treatment that had a significant effect on egg eclosion that was different than that of all other treatments, but

Fraser fir produced the second most pronounced effect on overall egg eclosion. These results, as well as our earlier findings, indicate that Fraser fir and Veitch fir volatiles within a closed environment are able to negatively impact BWA egg eclosion and that it is more pronounced with Veitch fir compared to Fraser fir. Even though within an open environment, the volatiles present did not have any discernable effect on egg eclosion, the fact that they do when in a closed environment signals that perhaps direct contact with these same suites of volatiles, perhaps at a greater amount in Veitch fir compared to Fraser fir, are one potential mechanism of resistance. Chemical biology plays a large role in mediating and facilitating interactions between plants, pests and predators, perhaps in the case of BWA constitutive chemistry plays a role in conferring resistance.

My final study, attempting to qualitatively identify volatiles through SPME analysis of a wide variety of fir species of known susceptibility or resistance to BWA indicated that many similar suites of volatiles, as indicated by other similar studies, are present in the fir genus. Moreover, our studies indicated that certain chromatographic peaks, representing different compounds, may be more important than others. Caryophyllene and d-limonene may be significantly correlated with resistance in our assessment, but further research is necessary to elucidate this idea. Alpha-pinene and beta-myrcene are also consistently weakly correlated with resistance, while delta-carene is significantly correlated with susceptibility in *Abies.*Determining whether any synergistic relationships, as our studies above have shown that bornyl acetate does not produce a decrease in egg eclosion success alone, but perhaps with a synergist, any toxic potential would be enhanced, as has been shown through other similarly aimed studies.