ABSTRACT

BEDNAR, DAVID MICHAEL. Interactions Between the Invasive Balsam Woolly Adelgid (Adelges piceae (Ratzburg)) and a Native Host, Fraser Fir (Abies fraseri (Pursh)). (Under the direction of Dr. Fred Hain).

Adelges piceae, balsam woolly adelgid (BWA), is an invasive insect in the United States where it kills natural populations of Fraser fir (Abies fraseri) and causes economic damage in Christmas tree plantations. Fir tree defensive reactions to BWA infestations vary between species and include a loss of apical dominance, gouting, rotholz production, and eventually tree death. Fraser fir is highly susceptible to BWA infestations; however, putative resistance to BWA has been suggested for natural populations. Tolerance to BWA exists in Turkish fir (A. bornmuelleriana (Mattf.)), a European fir where BWA are native. In the following studies we examine both Fraser and Turkish fir in a variety of studies.

It has been suggested that the reactions observed in susceptible Abies species, i.e. swelling, gouting, rotholz production, are caused by BWA digestive enzymes introduced into the tree during feeding. Whole body homogenates of balsam woolly adelgid (Adelges piceae) adults, crawlers, and eggs were tested for various types of enzyme activity. All samples showed amylase, protease, peroxidase, and polyphenol oxidase activity. Occurrence of these enzymes in crawlers as well as adults indicate they are not a byproduct of feeding but are produced in the insect and most likely introduced into the tree during feeding. Whole body homogenates of BWA have also been shown to possess pectinase, an enzyme important in the digestion of the middle lamella of plant cell walls. If BWA introduce amylase, pectinase, protease, peroxidase, and polyphenol oxidase into xylem ray parenchyma when feeding, it would explain, in part, the local swelling of cells, disruption of phenylalanine ammonia lyase synthesis, and the abnormal formation of heartwood, termed "rotholz".
observed in susceptible Fraser fir hosts. Here we present the possible mechanism for rotholz production in Fraser fir by the action of digestive enzymes present in BWA.

In relation to physical changes, chemical changes and the production of defense compounds were studied in healthy and infested Fraser fir. Comparisons between bark, mature wood and juvenile wood samples from healthy and BWA infested trees were analyzed using gas chromatography with a flame ionization mass spectrophotometer (GCMS) focusing on a known plant compound, juvabione, along with other defense chemicals in the bark. As expected, juvabione concentrations were higher in infested trees compared to uninfested trees, and it was more concentrated in juvenile wood compared to mature wood. Several important insect and fungal defense compounds were differentially regulated in bark samples. Samples were tested in a bioassay using mealworms and compared to pure juvabione and another known insect growth regulator, Methoprene. The IC$_{50}$ for pure juvabione was lower than that calculated for extracts from juvenile and mature wood, indicating a possible antagonistic effect from unknown compounds.

In addition to chemical defenses, energy relations were studied by examining carbohydrate and phenolics changes in Fraser and Turkish fir 5 year old trees before and after infestation with BWA. Plant health was assessed by examining total soluble carbohydrates, lipids, and phenolics in conjunction with anatomical characterization of xylem ray parenchyma cells in Fraser and Turkish fir. Lipids were significantly different between species and infestation level with higher lipid concentrations in Fraser fir and infested samples. There were positive correlations between TSC and TSP for both species, but only infested Turkish fir maintained this relationship, while infested Fraser fir did not. More XRP cells were present in Turkish fir and positive correlations between XRP cells and lateral pits
between cells existed in Fraser fir. Other correlations between anatomical characteristics for each species are discussed.

Finally, methoprene was tested as a possible alternative to current chemical BWA control methods. Chemical control is currently the only form of management for this invasive insect pest. Broad-spectrum insecticides not only reduce natural predator populations, but eliminate competition for other pests which subsequently also require chemical control. Methoprene has a lower toxicity, and negligible impacts on predatory/beneficial insects, and the environment. This study evaluates methoprene as a control treatment for BWA. Methoprene was shown to be a successful growth regulator and its potential use in combination with augmentative biological control is discussed.

Our overall goal is to reduce or eliminate chemical pesticide inputs on Fraser fir Christmas tree farms. This can be accomplished through breeding resistant Fraser fir, use of biological control, and the use of alternative biopesticides. This study focused on determining the mechanisms by which BWA cause deleterious effects in Fraser fir possibly by the introduction of digestive enzymes. Fraser fir chemistry and comparative physiology are discussed as potential traits for selecting BWA resistant fir. The link between digestive enzymes and reactions observed in Fraser fir are particularly useful in determining mechanisms for potential areas for future resistance research.
Interactions Between the Invasive Balsam Woolly Adelgid \textit{(Adelges piceae)} and a Native Host, Fraser Fir \textit{(Abies fraseri)}

by

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DEDICATION

To my wife and daughter. Maria, for pushing me to always be better, and pursue my passion.

Randi (17 mo.), who can already differentiate between a beetle, an ant, and a fly.
**BIOGRAPHY**

David Michael Bednar was born November 12th, 1984 in Klamath Falls, Oregon to Kevin and April Bednar. His parents encouraged him in every endeavor, while teaching him to enjoy nature at every opportunity. David attended a small high school in Condon, Oregon and graduated from a class of 21 students. He spent his summers working various jobs at game bird farms, wheat farms, and the Gilliam County road department. It was at the road department that David was first introduced to the idea of biological control of weeds using insects. He took advantage of every opportunity the school could offer him, allowing him to graduate as a five-sport varsity athlete.

After graduating from Condon High School, David attended Pacific University in Forest Grove, Oregon. The small private college allowed David to compete on the varsity wrestling, track, and cross country teams while at the same time staying active in club sports like crew and ultimate frisbee. The small class sizes at Pacific created a close relationship between students and professors. Three professors in particular inspired David to pursue entomology. However, it was at Pacific where he met Maria, who, later became his wife and encouraged him to follow his passion.

Ed Alkaslassy, worked with David as his majors advisor, but it was his previous work as a museum curator of the insect collection that intrigued him most. Dr. Brook Swanson was a young new professor who introduced David to insect morphology and physiology. Dr. Stacey Halpern probably influenced David the most offering him an internship in Florida the summer after his Junior year. David studied the oviposition behavior of false Colorado potato beetles (*Leptinotarsa juncta*), and immediately became passionate about studying insects.
David wrote his senior capstone on the invasion biology of argentine ants (*Linepithema humile*). In his research for the project David became interested in working with Dr. Jules Silverman.

David was accepted to North Carolina State University, under the advisement of Dr. Jules Silverman immediately after receiving his Bachelor of Science in May of 2007. He received his Master of Science in 2010 studying the interactions between an invasive ant and the native ant it displaces. His thesis is titled: “Predation by *Pachycondyla (=Brachyponera)* on *Reticulitermes virginicus* and competition with *Aphaenogaster rudis.*”

David continued his work in Entomology that same year. Under the advisement of Dr. Fred Hain, David worked toward a PhD studying the balsam woolly adelgid and learned to play handball. In 2013 David became the proud father of Randilynn Elise, a beautiful little girl. In August of 2013 David accepted a job at the North Carolina Department of Agriculture and Consumer Services working in the beneficial insect lab rearing HWA predators. At the 2014 National Collegiate Handball tournament held at NCSU David earned 2nd place in Men’s division IIA singles cheered on by his one year old daughter and wife. David’s life continues to be full as he finishes his doctorate, works a full time job, raises a wonderful little girl, and plays a little handball.
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I would like to thank everyone on my committee for their invaluable scientific input and mentoring, especially Fred Hain my advisor. Allen Cohen was particularly helpful in pushing my thesis in the right direction and allowing me to make scientific connections I would not have otherwise seen. I appreciate all of the hard work done by John Strider in helping me run experiments. A thank you to all of the other occupants of Grinnells during my tenure there, without you the basement is a dark and lonely place. The most gratitude is owed to my wife, who in the last year and a half of my PhD was responsible for caring for me and Randi, our first child. She has been a great supporter, and I cannot thank her enough.
# TABLE OF CONTENTS

| LIST OF TABLES | vii |
| LIST OF FIGURES | viii |
| CHAPTER I: DISSERTATION INTRODUCTION | 1 |
| FRASER FIR AND BWA HISTORY | 1 |
| PLANT DEFENSE SYSTEMS | 6 |
| BWA FEEDING ENZYMES | 11 |
| CHEMICAL CONTROL OF BWA | 19 |
| GOALS | 23 |
| LITERATURE CITED | 23 |
| CHAPTER II: BALSAM WOOLLY ADELGID FEEDING ENZYMES | 32 |
| ABSTRACT | 32 |
| INTRODUCTION | 32 |
| METHODS | 38 |
| RESULTS | 45 |
| DISCUSSION | 47 |
| LITERATURE CITED | 54 |
| TABLES AND FIGURES | 60 |
| CHAPTER III: FRASER FIR JUVABIONE AND BARK CHEMICAL COMPOSITION WITH RELATION TO BALSAM WOOLLY ADELGID INFESTATION | 69 |
| ABSTRACT | 69 |
| INTRODUCTION | 70 |
| METHODS | 74 |
| RESULTS | 80 |
| DISCUSSION | 83 |
| LITERATURE CITED | 93 |
| TABLES AND FIGURES | 101 |
| CHAPTER IV: DIFFERENCES IN FRASER AND TURKISH FIR TOTAL SOLUBLE CARBOHYDRATES, LIPIDS, PHENOLICS, AND XYLEM RAY PARENCHYMA ANATOMY AFTER BWA INFESTATION | 116 |
| ABSTRACT | 116 |
| INTRODUCTION | 116 |
| METHODS | 119 |
| RESULTS | 124 |
| DISCUSSION | 128 |
| LITERATURE CITED | 132 |
| TABLES AND FIGURES | 138 |
| CHAPTER V: CONCLUSIONS AND FUTURE RESEARCH | 146 |
| FUTURE WORK | 147 |
| LITERATURE CITED | 150 |
| APPENDICES | 169 |
LIST OF TABLES

CHAPTER II:
Table 1. Total protein content and weight of BWA adults, crawlers, and eggs as determined by the Bradford protein assay .................................................................60
Table 2. Amylase activity of BWA adults, crawlers, and eggs as determined by the EnzChek ultra amylase assay kit (E-33651). Relative fluorescent units µg protein¹ min⁻¹ insect¹ (RFU/µg protein/min/insect) and Time to reach max RFU .................................................................................................................................62
Table 3. Total protease activity of BWA adults, crawlers, and eggs as determined by the EnzChek protease assay kit (E-6638) ..........................................................63
Table 4. Peroxidase activity of BWA adults, crawlers, and eggs as determined by the Amplex red hydrogen peroxide/peroxidase assay kit (A-22188) ....................65
Table 5. Polyphenol oxidase (PPO) activity with and without phenylthiourea (PTU) a PPO inhibitor .................................................................................................................67
Table 6. HWA and BWA enzyme activity comparison .................................................................................68

CHAPTER III:
Table 1. Wood Disk sample summary from healthy and infested Fraser fir ......................101
Table 2. Petroleum ether extracts of Fraser fir EC₅₀ for mealworm pupae .........................104
Table 3. Mean percent and µg/g JRCs from petroleum ether extracts calculated per juvenile and mature wood samples compared between infested and healthy trees .................................................................106
Table 4. Defense compounds differentially abundant in the Bark of infested and Healthy Fraser fir ......................................................................................................................107
Table 5. Mean percent mortality of Mealworm pupae in Juvabione Bioassay, compared using a Tukey-Kramer HSD .................................................................................109
Table 6. Summary of findings from studies examining JRCs in Abies ...............................110

CHAPTER IV:
Table 1. Total soluble carbohydrates (TSC), lipids, and Total soluble phenolics(TSP) in Fraser and Turkish fir prior to and after 21 days infestation with BWA ........................................................................................................................................139
Table 2. Xylem Ray parenchyma (XRP) anatomical characteristics in stems and branches of Fraser and Turkish fir prior to and after 21 days infestation with BWA .........................................................................................................................141
Table 3. Correlations between %TSC, %lipids, and %TSP .................................................142
Table 4. Correlations between anatomical characteristics by species .............................143
Table 5. Correlations between anatomical characteristics by species and infestation level ...............................................................................................................................144
Table 6. Correlations between BWA/cm anatomical characteristics, %TSC, %lipids, and %TSP by species .........................................................................................145
LIST OF FIGURES

CHAPTER II:
Figure 1. Enzyme kinetics for BWA amylase activity ........................................61
Figure 2. Enzyme kinetics for peroxidase activity. Adult, Lygus 5, and Lygus 1 follow the primary axis on the left, while the crawler and egg samples follow the secondary axis on the right ..............................................................64
Figure 3. Polyphenol oxidase (PPO) activity with and without phenylthiourea (PTU) a PPO inhibitor ..............................................................66

CHAPTER III:
Figure 1. Juvabione standard curve for samples 3, 5, 6, 11, and 12 with standard deviation indicated by vertical bars ..................................................102
Figure 2. Chromatogram for healthy minus the infested bark sample between 20 and 27 minutes. Values displayed correspond to the top one (black line) and bottom ten percent (red line) of peaks ........................................103
Figure 3. Chromatograms for infested juvenile wood, mature wood, and bark from one of the trees sampled, the gray line indicates the external juvabione standard at 60.04ng/ul ..........................................................105
Figure 4. Chromatogram of Healthy and infested bark between 20-27min where 8 peaks were identified as being different between the two samples ............108
Figure 5. Showing *Tenebrio molitor* that did not complete the final molt (left), underwent an incomplete molt (right), and successfully molted (bottom right) ....................................................................................111
Figure 6. Mevalonate pathway and conversion of FPP to (E)-α-bisabolene in the production of Juvabione (reproduced with permission(Bohlmann et al. 1998b)) ..................................................112
Figure 7. Comparison in structure of compound [1] extracted from the bark of Fraser fir to jacaranone .................................................................113
Figure 8. Comparison in structure of labdanes extracted from Fraser fir ([2],[7]) and Sclareol .................................................................113
Figure 9. Comparison in structure of abietane and those extracted from Fraser fir ([3], [4], [5], [6]) .................................................................114
Figure 10. Structures of [8] salvialane skeleton and oxazolidinedione .............115

CHAPTER IV:
Figure 1. Diagram of how anatomical characteristics were measured in cross (top) and radial (bottom) sections of fir tree branches and stems ..............138
Figure 2. %TSC (a), %Lipids (b), and %TSP (c) for Fraser (green) and Turkish (purple) fir samples at two time points for BWA infested (solid) and control (dotted) trees 140
CHAPTER I

DISSERTATION INTRODUCTION:

Phytophagous insects and angiosperms have been engaged in an arms race for over 100 million years (Stotz et al. 1999). Plants respond to insect attack by producing defensive substances that negatively affect insect growth and development or recruit predatory insects. Insects extract nutrients from plants while plants develop complex systems of defense. In order to successfully feed, insects must extract nutrients (and energy) from plants and convert them to usable forms. They accomplish this through the production of digestive enzymes such as proteases and carbohydrases. Diet is a major determining factor for the types of enzymes found in insect guts. Insects respond to plant defenses by producing more enzymes, which counteract these defense systems. Today the interactions between plants and insects are known to be complex and involve a diverse array of chemicals, enzymes, inhibitors, and signal molecules that interact in various ways, depending on the plant and insect.

FRASER FIR AND BWA HISTORY:

Adelgids (Adelgidae) and their hosts (Abies spp.) have coevolved for over 60 million years (Havill and Footit 2007, Havill et al. 2007). During that time, Adelgids have evolved the appropriate salivary enzymes to extract nutrients and combat host defenses. As different lineages of fir (Abies spp.) diverged and spread across the northern hemisphere, adelgids specialized on different coniferous species. Balsam woolly adelgid (Adelges piceae, BWA) is a native pest to European fir species and is closely related to Adelges nordmannianae, a common pest of Turkish fir (Abies nordmanniana subsp. Bornmuelleriana) (Havill et al.)
Since its introduction into the United States BWA has devastated North American fir species, especially Fraser fir (*Abies fraseri*) (Hain and Berryman 1988).

Fraser fir is a keystone species occurring naturally in the high elevations of the Southern Appalachians. In 1951 Fraser fir comprised 34% of the total basal area in the Great Smokey Mountains, and outnumbered spruce 3-5 times in these stands (Amman and Speers 1965). Trees ranged in age from saplings to 125 years old, with the oldest recorded fir standing at 168 years old (Oosting and Billings 1951). Fraser fir, along with red spruce, is the climax species for elevations above 1500m, providing habitat for several endemic biota, including bird, mammal, invertebrate, and plant species (Potter 2012).

Commercial production of Fraser fir began in the mountains of North Carolina in the 1950’s, and grew from 430,500 being planted between 1953-1964 to 42 million trees in recent years (Sidebottom 2009). This hundred-fold increase in Christmas tree production brought North Carolina to the forefront of the Christmas tree industry, ranking 2nd in the nation. Fraser fir are grown in nurseries and exported all over the world. In the 1950’s a tree would sell for $7; today total Christmas tree sales average $100 million in North Carolina (NCDA 2012). North Carolina produced 18 % of the nation’s Christmas trees, ranks first in dollars made per tree, and 96 % of the trees grown and sold are Fraser fir (Potter et al. 2005).

In 1908 the balsam woolly adelgid was introduced, probably on ornamental planting stock from Europe, to the northern spruce-fir stands of Maine. In 1958, the Southeastern Forest Service Research Station surveyed the Mt. Mitchell area in North Carolina and estimated that 11,000 trees had been killed by BWA. Aerial surveys in 1960 estimated more than 200,000 fir trees were killed. Throughout the 1960’s, thousands of additional trees were
killed and in some stands mortality of Fraser fir reached 75 to 100%. By 1965, over 1.5 million trees were killed by BWA. During this time, Fraser fir was considered ecologically important in protecting watersheds, but had low economic value (Amman and Speers 1965). The loss of Fraser fir in these stands saw a dramatic decline in overall basal area and growth, with drastic changes in sunlight, temperature, moisture, water run-off, and nutrient regimes. These habitat changes were directly linked to several species becoming threatened or endangered (Wagner and Van Driesche 2010, McManamay et al. 2011). In combination with climate change and other anthropogenic effects, animals and plants in North America have continued to decline due to the loss of Fraser fir, with 307 currently listed as threatened, endangered, or of concern (Annkatrin 2012).

BWA feed on all North American fir species including balsam (Abies balsamea), subalpine (A. lasiocarpa), and Fraser fir (A. fraseri) (Havill et al. 2007, Newton et al. 2011), even though these species are distantly related to their native host European silver (A. alba) and Turkish fir (A bornmuelleriana). Fraser fir shares a more recent common ancestor with Veitch fir (A. veitchii), an East Asian species, indicating that they probably split more recently than Fraser and Turkish fir. The Abies sect (IX) probably diverged from the Balsamea sect (II) during the beginning of the Miocene, about 23 million years ago (mya) when the Ağrı Mountains were forming on the eastern border of what is now Turkey (Bagci and Babac 2003, Xiang et al. 2009, Aguirre-Planter et al. 2012). It was most likely at this time that European adelgids (A. piceae, A. nordmanniana) split from Asiatic adelgids (A. tsugae, A. pectinatae) (Havill and Foottit 2007). The Balsamea sect continued to migrate eastward into what is now North America during the late Miocene. Divergence from the
Asian lineage probably occurred at the beginning of the Pliocene, marked by cooler drier temperatures encouraging the expansion of temperate conifers, and a reduction in deciduous forest cover (Aguirre-Planter et al. 2012). During this time period it is likely that North American firs escaped adelgid infestation, while European and Asiatic fir species continued to coevolve with adelgids. The late Miocene and early Pliocene were an important divergence time for all fir species prompted by isolation and adaptation to high elevation areas as mountains rose in Asia and North America (Jaramillo-Correa et al. 2008, Xiang et al. 2009, Liepelt et al. 2010, Carlos Linares 2011, Aguirre-Planter et al. 2012).

Speciation in *Abies* appears to have happened over a relatively short period of time, and without secondary contact among species (Liepelt et al. 2010), indicating that as populations of *Abies* became isolated, pest species like the adelgid became obligate specialists. In fact, *Adelges nordmannianae* is a holocyclic pest of Spruce, its primary host, where it reproduces sexually, and *Abies* are its secondary host where it reproduces asexually. BWA (*A. piceae*) are anholocyclic and only reproduce parthenogenically on *Abies*. *Adelges nordmannianae* only differs from *A. piceae* by a few genetic substitutions, suggesting they are of a monophyletic lineage, and possibly the same species (Havill and Foottit 2007, Havill et al. 2007, Sano and Ozaki 2012).

An examination of adelgid phylogeny indicates that *Adelges nordmannianae* share a recent common ancestor with *A. piceae*, more recently divergent than the split from *A. pectinatae* (Havill and Foottit 2007). This is highly significant in understanding how the species coevolved. However, *A. nordmannianae* is more closely related to *A. piceae* which attacks Fraser fir, but is native host to European firs. These lineages split around 5 mya, and
when Fraser fir became isolated it did not have to combat adelgid pests like Veitch and silver fir species did (Havill et al. 2007). The lack of co-evolutionary time for Fraser fir and BWA has led to devastation of this North American fir species after BWA introduction; however, resistance/tolerance may be present in certain populations, or bred into existing populations through hybridization.

Currently, Fraser fir pests are limited to aphids (*Mindarus abietinus, Cinara sp.*), scales (*Aspidiotus cryptomeriae, Fiorna externa*), a wood borer (*Monochamus marmorator*), mites, and a gall midge (*Dasineura balsamicola*), most of which occur in Fraser fir commercial plantations (Sidebottom 2007). Very few diseases are native to Fraser fir and are mostly associated with decay: *Armillaria mellea, Heterobasidion annosum, Haematostereum sanquino lentum, Oligoporous balsameus, Trichaptum abietinum, Lachnellula agassizii* and occur at very low percent incidences in natural fir stands (<1-39%) (Bruck 1989). These conditions suggest Fraser fir did not co-evolve with bark beetles in Eastern North America, and has little use for defense against pathogenic fungi spread by beetles, as many other fir species have (Bruck 1989, Raffa and Smalley 1995b, Franceschi et al. 1998, Katoh and Croteau 1998, Phillips and Croteau 1999, Franceschi et al. 2000, Huber et al. 2004).

Although Fraser fir did not co-evolve with pests like BWA or bark beetles, the genomic potential for defense response to this kind of attack is most likely still present (Huber et al. 2004) and may be elicited through the indirect or direct plant defense systems. It is likely Fraser fir developed different defense mechanisms against different pest species than European and Asian firs which have all coevolved with an adelgid.
PLANT DEFENSE SYSTEMS:

Different categories of direct plant defense have been described as outlined by Chen (2008). Direct plant defenses are plant traits that interact with the attacking insect to decrease plant susceptibility. Indirect defenses involve the recruitment of natural enemies to the attacking insect by release of volatile organic compounds (VOCs) by the plant. Direct plant defense includes an ‘anti-nutrition’ strategy where prior to ingestion the food supply is limited or nutrient value is reduced after ingestion (Chen 2008). One way that a plant might limit feeding of an insect is cell wall fortification. Many genes are involved in thickening of cell walls and have been shown to be up regulated after insect attack. Cell wall fortification works to hinder feeding by insects with sucking mouthparts by improving mechanical barriers that are already in place (Chen 2008).

Cell wall fortification works in conjunction with hypersensitive response (HR), which also reduces nutrient availability at the feeding site through rapid programmed death of cells in that area (Fernandes 1990, Chen 2008). Hypersensitive response is a specialized defense against pathogenic fungi and stylet-feeding insects allowing the host plant to isolate the infected/infested cells and fungus/stylets to prevent it from spreading and limit nutrient flow to the site of infection/infestation (Fernandes 1990). The HR is especially successful in defending against galling insects (Chen 2008). Initial detection of the insect or pathogen begins signal transduction pathways elicited by reactive oxygen species (ROS) and Ca$^{2+}$ ions. There is a moiety of different ROS that can be produced instantly in multiple locations within a plant cell leading to the induction of a specific plant defense mechanism. Most research has focused on H$_2$O$_2$ as the prominent ROS and it is one of the few characterized with a specific
function in plant cell death associated with HR (Fernandes 1990, Mayar 2006). Hydrogen peroxide can diffuse freely in plants and remains relatively stable, but is also highly reactive, making it an ideal signaling molecule (Maffei et al. 2007).

After an insect ingests plant material, the plant starts to secrete different types of proteases and protease inhibitors at the feeding site to reduce the nutrient value of the material that is subsequently ingested. Insects cannot synthesize some amino acids, e.g. arginine, lysine, methionine, and threonine, so these amino acids must be present in their food, extracted from the diet using proteases (Chen 2008), or synthesized by symbiotic gut microbes. Plants can produce enzymes that remain active once ingested by the insect, such as arginase, asparaginase, and threonine deaminase, that work to degrade certain essential amino acids, such as arginine, asparagine, and threonine, respectively (Chen 2008). Insects use proteases and amylases to remove essential amino acids and starch not glycogen from plant material once it is ingested, and plants have evolved to produce protease and amylase inhibitors to combat this process. Galling insects, as well as BWA, have been shown to change the feeding site making it a more favorable habitat for the insect. Plants may defend against this by not allowing modification of plant tissue at the feeding site, thus making it less favorable than a susceptible host (Miles 1968, Sopow et al. 2003, Chen 2008).

Plants also produce secondary metabolites which may act as inhibitors or reduce the nutritive value of the food. Plant toxic defenses can cause either physical damage or disrupt chemical pathways. One way plants can cause physical damage is by producing a cysteine protease at the feeding site that remains active after ingestion. This 33kDa cysteine protease
found in maize has been shown to break down structural proteins within the gut of the insect including the peritrophic matrix of the fall armyworm (Pechan et al. 2002).

Conifers have coevolved with insects and pathogens since the early Cretaceous (200 mya), leading to one of the greatest defense systems in the plant world. All conifers studied contain polyphenolic parenchyma (PP) cells. Polyphenolic parenchyma cells are considered as a part of the constitutive defensive system for conifers, but can be induced to produce more resin. Traumatic resin ducts (TD) are a part of an induced defensive response. Constitutive resin ducts function to store and produce resin, while traumatic resin forms after an attack and may contain different types of resin than pre-existing constitutive ducts. Both systems employ resin, which contain terpenes, phenolics, and other resin acids. Phenolics are tremendously important chemicals in conifer defense against pests and pathogens, so it is logical that they are also abundant. Phenols bind proteins inactivating them hindering the ability of pests/fungi to use digestive/feeding enzymes. Phenolics and tannins are also highly toxic, reducing ability to feed and nutritive value of the plant. Presence of phenols also aid in lignification of cell walls to help prevent the spread of pathogens, or close off the feeding area for some insects. (Franceschi et al. 2005)

Bark beetle attacks on conifers are the most well documented cases of TD and PP cell formation. Dendrontonus, Ips, and Scolytus bore into the phloem of conifers and introduce pathogenic fungus killing the tree ((Phillips and Croteau 1999). Initially, trees defend themselves with preexisting resin canals that can push beetles out as they sever the ducts while boring into the tree. However, beetles have evolved aggregation behaviors to mass attack the tree and overcome initial resin flow. Healthy trees can either push beetles out, or
stop fungal invasion via inducible changes. The formation of impervious necrotic tissue at
the site of infection is known as the hypersensitive response. Another induced change is the
formation of TD and PP cells that secrete defensive compounds into the wound site. The
necrotic lesion, TD, and PP form a reaction zone around the infection sealing it off from the
rest of the tree, killing the infection (Johnson and Croteau 1987, Franceschi et al. 2000, Nagy
parenchyma cells accumulate polyphenols in response to wounding and fungal inoculation in
Norway spruce (Picea abies) (Nagy et al. 2000). Polyphenolic parenchyma cell proliferation
is also important in formation of wound periderm formation after attack by bark beetles or
fungal inoculations (Franceschi et al. 2000). Resistance to subsequent beetle attacks and
related pathogenic fungi in Pinaceae are determined by the formation of TD and PP cells.
Activation of PP cells in several families of Pinaceae indicates a pivotal defense mechanism
against general pathogens and pests (Hudgins et al. 2004).

Abies and Tsuga lack preformed resin ducts in the phloem and xylem; however they
still form TD and PP in response to methyl jasmonate (Hudgins et al. 2004). Metzier et al
(2012) compared TD formation in Norway spruce and silver fir in response to fungal
infestation following wounding. They found that silver fir was better at preventing the spread
of fungi, and contained fewer types of fungi because it was able to seal off wounds better
than Norway spruce. Lesion development is important in grand fir resistance to pathogenic
fungi introduced by bark beetles (Wong and Berryman 1977). It is likely that TD and PP cell
formation evolved in response to bark beetle and fungal attack. When Abies are attacked by
BWA cortical parenchyma cells swell (Balch et al. 1964b). Both Abies and Tsuga produce
abnormal xylem tissue referred to as rotholz and false rings, respectively (Hollingsworth and Hain 1994a, b, Balakshin et al. 2005, Gonda-King et al. 2012)

It is likely that TD do not play a role in defense against adelgids; however, PP cells may aid in the hypersensitive response and wound formation throughout the tree. The formation of enlarged parenchyma cells was hypothesized as a response to salivary secretions by BWA mimicking IAA or other plant growth hormones (Balch et al. 1964b). One interesting aspect to this system is the down regulation of the gene responsible for phenylalanine-ammonia lyase (PAL1) in Fraser fir in response to BWA and jasmonic acid (Emerson 2012). In Norway spruce PAL is localized in the ray parenchyma cells, PP cells, TD, and accumulates in response to wounding (Franceschi et al. 1998). Moreover, PAL is mostly concentrated in the middle lamellae of the xylem ray parenchyma cell wall (Franceschi et al. 1998) where BWA are known to feed. Phenylalanine-ammonia lyase is an important factor in triggering the phenylpropanoid biosynthetic pathway responsible for polyphenol, flavonoid, and lignin production as well as defense against insect/pathogen attack (Ferrer et al. 2008). The down regulation of PAL1 is discussed later, potentially as a result of the introduction of salivary enzymes.

As conifers produce more toxic phenolic compounds and defense systems, insects respond in kind through the production of feeding enzymes. More specifically, it appears as if enzymes present in BWA may be responsible for causing many of the reactions observed in Fraser fir including the down regulation of the phenylpropanoid biosynthetic pathway, rotholz, swelling, and gouting. As conifers produce more toxic phenolic compounds and
defense systems, insects respond accordingly through the production of feeding enzymes to counter these systems.

**BWA FEEDING ENZYMES:**

BWA feed on xylem ray parenchyma cells (XRP) (Kloft 1957, Balch et al. 1964b) which act as storage cells and facilitate medial transport of materials including starch grains (Nakaba et al. 2006) and proteins (Kloft 1957). Transport occurs between XRP through pits along the cell walls, which range from 2-4 per cross-field (Garcia Esteban et al. 2009). Xylem ray parenchyma maintain organelles and may stay alive for as long as 20 years, even after secondary wall formation and lignification. Moreover, XRP are integral in contributing to the formation of heartwood (Nakaba et al. 2006). (Forbes and Mullick 1970a, Garcia Esteban et al. 2009). When fed upon by BWA parenchyma cells become rounded, surrounding cells swell, and neighboring cells form a ‘physiological gall’ (Kloft 1957). The characteristics of xylem ray parenchyma cells make them an ideal feeding substrate for a sessile feeder like BWA. The function of parenchyma cells in producing heartwood may be altered by trophically related enzymes produced in BWA introduced during the feeding process and lead to the production of rotholz systemically throughout the tree, eventually leading to tree death.

Observations of Mirid and Coreid feeding revealed that parenchyma feeding Homoptera are able to drain plant cells of nutrients that are far beyond the maximum reach of their stylets (Miles 1987). BWA stylets can reach up to 1.5mm, or 5x the length of their body (Balch 1952, Srivastava 1962, Forbes and Mullick 1970a). Plant-sucking bugs may release enzymes, such as amylase, which break down starch creating an osmotic imbalance between
the cell they are feeding on and surrounding intact cells (Miles and Taylor 1994). Miles (Miles 1987, Miles and Taylor 1994) suggests different types of feeding dependent on enzymes, such as pectinase, cause a larger lesion which spreads beyond the reach of the insects stylets (Miles 1987). Whole body homogenates of BWA have been shown to exhibit pectinase activity (Adams and McAllan 1958). The draining of nutrients from neighboring cells and breakdown of the middle lamellae allows parenchyma feeding homopterans to feed from one location for a longer period of time without penetrating a new cell (Miles 1987, Miles and Taylor 1994). BWA would also need to defend themselves against the myriad of plant defense chemicals produced by Fraser fir. The breakdown of the middle lamellae may be the first step in BWA shutting down the phenylpropanoid pathway by inhibiting the synthesis of phenylalanine ammonia lyase which is produced in the middle lamellae of ray parenchyma (Franceschi et al. 1998). In other insect species polyphenol peroxidase (Peng and Miles 1988, Miles and Peng 1989), peroxidase (Ahmad et al. 1989, Miles and Peng 1989, Mathews et al. 1997), and some proteases (Jongsma and Bolter 1997) may be involved in combating plant defense systems.

*Amylase:*

Many insects produce α-amylase which breaks down native starch or glycogen in a random manner, as opposed to β-amylase which removes successive maltose units or glucoamylase which removes successive glucose units (Terra and Ferreira 1994). Amylase may occur in the salivary glands (M.R. and J.B. 1967), midgut (R. Socha 1997), or in both locations (Takanona and Hori 1974) depending on the type of insect, pH of each tissue type, and diet of the insect. Based on the size of starch grains (0.5-10μm in Norway spruce
(Cabalkova et al. 2008) in comparison to BWA stylet food canals (1µm in diameter (Forbes and Mullick 1970a)) it is likely that amylase is expressed extra-orally for the digestion of starch within the plant cell prior to ingestion. It should be noted that amylase activity may be attributed to gut microbes (Srivastava 1962, Toenshoff et al. 2012a, Toenshoff et al. 2012b).

Amylase is also present in insect and other arthropod eggs because carbohydrates are a part of the energy requirement for embryogenesis relying on glycogen (Cohen and Patana 1985). In camel tick’s eggs α-amylase activity increased from day three to a peak activity on day 18 with parallel rapid increase in glycogen levels between days 12 and 18 (Mohamed 2000). Lipid metabolism is considered the main energy source for insect eggs during development (Cohen and Patana 1985). Where changes in fat content are low with losses near 10%, only about half of the energy expended is accounted for (Ludwig and Ramazzotto 1965). Glycogen stores, on the other hand, have been shown to lose 50-90% of their initial supply indicating that glycogen is also a major source of energy during mealworm embryogenesis (Ludwig and Ramazzotto 1965).

Protease:

Digestive proteases are most commonly found in the midgut of insect digestive tracts and function to release amino acids from dietary protein (Jongsma and Bolter 1997). Insects possess many different types of proteases depending on species, host plant, and gut pH. Gut pH is one of the most important properties in determining digestive enzyme function. Midgut pH changes throughout different regions of the midgut to accommodate different types of enzyme activity. The midgut of some Hemiptera is acidic with decreasing pH values
descending toward the posterior region (Terra and Ferreira 1994) while others may be alkaline, such as trypsin-like proteases (Cohen 1993).

Trypsin is a serine proteinase which cleaves proteins at lysine and arginine connections (Cohen 1993, Zeng et al. 2002) while chymotrypsin cleaves peptides at phenolic amino acid sites (Agusti and Cohen 2000). Lepidoptera and many Diptera have been shown to use serine proteases and have alkaline midguts to provide optimum activity for this class of proteinase (Jongsma and Bolter 1997). Other classes of proteases, including cysteine protease, present in Coleoptera, have acidic midguts. Cysteine proteases may have evolved to counter plant tissue, such as legume seeds, high in protease inhibitors. Insects using serine proteases can also survive on these plant tissues indicating there are a variety of adaptations to avoid a plants’ protease inhibitors (Jongsma and Bolter 1997).

It has been shown that parenchyma cells increase protein concentration after BWA infestation (Kloft 1957, Forbes and Mullick 1970a). Protein concentrations are also known to fluctuate for Fraser fir, and peak protein levels coincide with peak BWA production levels (Kloft 1957). Similar methods of an osmotic imbalance have been described for amylase in plant-sucking bugs (Miles 1987). A lack of free amino acids in plant tissue and the secretion of proteases may partially explain the 2-8 week aestivation of crawlers after insertion for some, but not all individuals during the same time of year (Amman and Speers 1965), and may also explain why BWA clump around some feeding sites (Newton pers. comm., personal obs.). Probing behavior of nymphs looking for feeding sites and/or preparation of future feeding sites would require secretion of digestive enzymes. Proteases secreted during probing may work to test a feeding site for protein availability, or release free amino acids
from stored proteins. If the site is not ideal and the crawler has everted her stylets beyond a point of retraction, she may continue to release protease enzymes into the parenchyma cells causing an osmotic imbalance which the plant would correct by transporting more amino acids for conversion to protein in the area. BWA may prepare a site for feeding by secreting initially high levels of protease after insertion, depending on the level of amino acid build up and protein recruitment they may continue to develop, aestivate for 2 to 8 weeks, overwinter, or die. The flexibility of BWA to alter diapause activity allows her to manipulate fir hosts, while conserving energy, and improving the feeding site for itself and future generations.

Peroxidase:

Reactive oxygen species (ROS) are a byproduct of aerobic metabolism in living organisms. ROS cause damage to important macromolecules in living organisms especially lipids, and proteins, including DNA, and RNA. They are also released by plants as part of a defense response against insects and pathogens, acting directly against the organism, or as signaling molecules for other plant defense pathways, including HR (Fernandes 1990, EnglishLoeb et al. 1997, Maffei et al. 2007). Insects, like all other living organisms, must remove ROS to prevent tissue damage.

Catalase, a well-known enzyme present in insects, scavenges hydrogen peroxide at high concentrations, but has less effectiveness at lower H$_2$O$_2$ concentrations (Mathews et al. 1997). Insects are also known to contain a glutathione peroxidase that is active against lipid peroxides, but not H$_2$O$_2$ (Ahmad et al. 1989). Ascorbate peroxidase is active against ascorbic acid, but also towards H$_2$O$_2$, and two lipophilic peroxides. Ascorbate peroxidase may be an important reducer of H$_2$O$_2$ when concentrations are not high enough for catalase to be
effective, and may aid glutathione peroxidase activity, especially since it is not inhibited by plant produced peroxidase inhibitors (Mathews et al. 1997).

Ascorbate peroxidase activity in the salivary glands, midgut, and regurgitate of Helicoverpa zea indicated that it is most likely important in removing ROS ingested from plant material during feeding (Mathews et al. 1997). Mathews et al (1997) also point out the importance of available ascorbate as a possible cofactor in this system. Ascorbate is regenerated by two insect enzymes, dehydroascorbate reductase (DHAR) and ascorbate free radical reductase (AFRR), whose importance should not be overlooked when considering the ability of ascorbate peroxidases function in removing ROS (Mathews et al. 1997).

The ability for phytophagous insects to detoxify plant allelochemicals partially determines host specificity. Three aphids demonstrated the ability to perform peroxidative functions on diets through the production of both sheath material and watery saliva (Miles and Peng 1989). These studies indicate the importance of sheath material in countering plant defenses, but the stylet sheath material and the role of peroxidases may play other roles for sessile feeding insects.

In plants, the proper rapid response to an insect or pathogen can be the difference between successful resistance and susceptibility. Reactions like the hypersensitive response (HR) work well against plant pathogens and insects if initiated quickly, especially, for galling insects or BWA which are limited in their ability to move to a new feeding site. (Miles 1968, Sopow et al. 2003, Chen 2008). H$_2$O$_2$ is one of the main signaling molecules for HR (Fernandes 1990, Mayar 2006, Maffei et al. 2007) and also has antimicrobial properties allowing it to act directly against introduced pathogens after wounding or feeding by an
insect herbivore, further protecting the plant from infestation. Chewing insects and those with piercing and sucking mouthparts damage the plant in different ways and are perceived by the plant differently. Recent evidence suggests plants respond to aphid feeding with gene regulation more similar to that of fungal or bacterial pathogen defense than genes associated with chewing insects (Maffei et al. 2007).

BWA clearly delays or inhibits HR in Fraser fir as has been hypothesized in previous studies (Hain 1988, Fernandes 1990), especially with evidence that resistant fir species form HR much faster than susceptible species (Mullick and Jensen 1976b). Due to the various roles of H$_2$O$_2$, neutralization of this molecule may also affect downstream defense pathways such as the phenyl propanoid pathway (PAL1) which has been shown to be down regulated in Fraser fir infested with BWA (Emerson 2012).

Most ROS are involved in rapid response upon initial infestation (Maffei et al. 2007). After the initial defense systems of a plant have been neutralized, cell maintenance and suppression of other defense pathways may be more important. Torsten and Aart (2006) proposed that a possible function of the salivary sheath is to plug the insertion point of plant sucking insects to prevent the leakage of Ca$^{2+}$ and consequent plugging of sieve tube elements within the plant. One function of sieve tube plugging involves the cross-linking of phenol-proteins which, is also triggered by oxygen during plant cell invasion by a stylet. Peroxidases may similarly work to reduce ROS to keep the tubes from plugging, however, polyphenol oxidases may also play a role (Torsten and Aart 2006).

*Polyphenol oxidase:*
Polyphenol oxidases (PPOs) have been described as a sclerotizing agent in insect cuticle, were hypothesized to be involved in stylet sheath formation of homopterous insects (Miles 1965), and have since been shown to function mainly as a detoxifying agent for phenolic substances (Miles 1999) (but this does not preclude their actions in stylet sheath setting or gelling), and affect plant growth via gall formation. The flavonoid, catechin, is a common plant defense compound elicited to act as a feeding deterrent, however, when oxidized by rose aphid polyphenol oxidase becomes a phagostimulant or non-deterrent for the aphids (Peng and Miles 1988). Susceptible rose buds contain lower quantities of phenolic constituents, which increase significantly in resistant buds (Peng and Miles 1988).

Hattori et al (2005) describe two types of phenoloxidases in the green rice leafhopper: that are present in the salivary glands and secreted in the watery saliva during feeding. In showing that laccase-type phenoloxidase is secreted by leafhoppers in the watery saliva during probing it is suggested that its major function is detoxifying phenolic substances released from cell vacuoles whose acidic pH is more favorable to the function of the PPO activity, as opposed more basic phloem (Hattoria et al. 2005). Phenolic compounds perform many defense functions within the plant, including production of quinones which are highly toxic as protein alkylating agents (Mayar 2006). Laccase, therefore may oxidize these substances into non-toxic polymers. Another function of the PPO, laccase, may be to degrade lignin (Hattoria et al. 2005).

All of the enzymes mentioned above work in concert within and secreted by an insect. Pectinase would give the insect access to cell contents by breaking down the middle lamellae, while amylase and protease would liberate stored starch and protein into usable
glucose and amino acid constituents. Peroxidases and polyphenol oxidases act to regulate metabolic functions within the insect, but also neutralize defense compounds produced by the plant. We hypothesize that the pectinase released by BWA breaks down the middle lamellae disrupting the production of PAL and consequently the phenylpropanoid biosynthetic pathway responsible for the production of defense compounds. The other enzymes present are responsible for reactions observed in Fraser fir, including rotholz production, gouting, and swelling which are discussed in more detail in chapter two.

**CHEMICAL CONTROL OF BWA:**

Christmas tree growers spend ~$1.5 million annually in chemical control of the BWA in the southern Appalachians. Chemical control is currently the only form of management for this invasive insect pest. Unfortunately, the use of broad-spectrum insecticides to treat for BWA has several negative side effects. Broad-spectrum insecticides not only reduce natural predator populations, but also eliminate competition for other pests, which also require chemical control. The reduction of competition and natural predators causes an increase in secondary pest populations. A new control method is desirable for short term management of BWA while resistance breeding is conducted for Fraser fir insect pests.

An alternative to broad spectrum insecticides would be to implement a more specific chemical that can be used in conjunction with biological control methods. Growth regulators such as methoprene have a lower toxicity, and negligible impacts on predatory/beneficial insects, and the environment. Use of these two strategies would reduce chemical pesticide inputs on Fraser fir Christmas tree farms.
The $1.5 million spent annually to control for BWA does not include associated costs with damage loss and secondary pest control. Reactions to BWA infestation include loss of apical dominance, gouting, rotholz production, and tree death. Christmas tree growers often cull BWA infested trees because they are unsalable, especially once the tops have been compromised. BWA control requires 300-800 gallons of pesticide per acre depending on tree size and density. (Potter et al. 2005) Although, recent pesticide application rates have decreased from 4.15 pounds active ingredient (ai)/acre, to 2.09 pounds ai/acre, there has been an overall increase in the use of organophosphates as they have replaced recently banned pesticide such as Lindane.

In 2006 Dimethoate was the number one pesticide used by 34.8% of Christmas tree growers (Sidebottom 2007). Dimethoate is an organophosphate acting on the nervous system of insects. It is highly acutely toxic to birds, mammals, and beneficial insects like bees. (Edwards 2006) Treatments like these will knock down predator insects, which would normally control late season pests like cinara aphids. One advantage to using organophosphates is that they kill insects quickly, however, the problem is that not all pests are controlled with a single application, and it does not kill mite eggs or BWA and elongate hemlock scale (EHS) by itself so another product must be added, or another pesticide application will have to be made later to control other pests.

Balsam woolly adelgid management with broad spectrum insecticides has increased the number of other pest species, and eliminated the ability to utilize biological control. BWA are consistently listed as the main insect pest in Christmas tree farms (Sidebottom 2007). Since the introduction of BWA, several predator release programs have been proposed
and implemented in an attempt to control the invasive insect. None have been successful due to a variety of factors (for review see Potter 2006). Chemical control in natural stands was impractical because it involved either injection, or drenching of the entire bole of the tree.

A native generalist predatory lacewing (*Chrysopa oculata*) are considered a possible biological control agent for a variety of Fraser fir pests including balsam twig aphid (BTA) (Fondren et al. 2004), BWA, cinara aphids, and mites. Unlike the commercially available lacewing predator, *Chrysoperla rufilabris*, the golden-eyed lacewing, (*Chrysopa oculata*), is known to be predaceous in both larval and adult life-stages (McEwen et al. 2007). There is no known artificial diet for rearing *C. oculata*, but the development of one, along with a factitious diet is currently under way. Preliminary predation tests involving *C. oculata* on BWA in the lab have shown both adult and larval life stages feed on BWA. *Chrysopa. oculata* naturally occur in Christmas tree plantations in western North Carolina and are readily available from April through November of any given year. Other species of lacewings provide a working method for rearing of this species as an augmentative biological control agent in the control of BWA in conjunction with methoprene treatments. (Gardner, pers.comm.)

Methoprene has been shown to have negligible effects on *Chrysopa* spp. (Westigard 1974a). Methoprene also affects all stages of insect development acting as an ovicide, and lowering fecundity of adults, while preventing immature stages from developing (Staal 1975b). This type of control will have much better long-term effects, because reducing fecundity of a pest is predicted to cause the population to crash (McClure 1991b). Methoprene is an alternative pesticide, which can be used in conjunction with augmentative
biological control of several pest species. If Christmas tree farmers implement a successful biological control program they would require fewer pesticide applications (Toth et al. 2004). Growers have already made great strides in providing habitat for predatory insects by spraying fewer herbicides, and allowing growth of groundcover plants (Sidebottom 2007). These practices make the Fraser fir Christmas tree a greener, more sustainable industry with fewer environmental impacts.

Methoprene is registered as a biochemical pesticide by the EPA. It has low toxicity with little risk to non-target species. Methoprene is biodegradable, breaking down quickly in sunlight, and on vegetation. It has low water solubility, so contamination of aquatic habitats is not expected to have lasting effects, especially since the half-life is so short (PMEP 1995). Methoprene was classified as a biochemical pesticide because it acts as an insect growth regulator, or juvenoid compound which mimics juvenile hormone in insects, preventing development of adults and fecundity. It has been shown to control a number of pest species while having negligible impacts on predators, particularly Chrysopa sp. (Westigard 1974a). Previous integrated pest management (IPM) strategies suffered environmental and economic costs. For instance, the use of broad-spectrum insecticides results in population outbreaks of spruce spider mite (SSM) and hemlock rust mite (HRM). It has been postulated that if growers did not have to treat for BWA that biological control for other pests would work (Potter et al. 2005). Use of methoprene as a biochemical treatment in conjunction with augmentative biological control has the potential to control multiple pest species which would reduce cost, and overall chemical input in to Christmas tree plantations.
GOALS

The goals of this research were to provide a better understanding of the interactions between BWA and its host Fraser fir in order to guide a breeding program that incorporates resistance traits into Fraser fir. Breeding resistant Fraser fir is a long-term research goal which will allow growers to reduce chemical inputs in the control of BWA. Ultimately, understanding the mechanism by which BWA kill Fraser fir should elucidate areas where resistance factors may occur in other species. Specifically, genes responsible for resistance factors which can be identified, selected for, and introduced transgenically or through traditional cross breeding methods with European fir species would accomplish the goal of producing BWA resistant Fraser fir.

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CHAPTER II:  
BWA FEEDING ENZYMES  

ABSTRACT:  

Whole body homogenates of balsam woolly adelgid (*Adelges piceae*) adults, crawlers, and eggs were tested for various types of enzyme activity. All samples showed amylase, protease, peroxidase, and polyphenol oxidase activity. Possible roles of each enzyme are discussed with relation to plant defense and feeding biology of BWA. Occurrence of these enzymes in crawlers as well as adults indicate they are not a byproduct of feeding but are produced in the insect and most likely introduced into the tree during feeding. Whole body homogenates of BWA have also been shown to possess pectinase, an enzyme important in the digestion of the middle lamella of plant cell walls. If BWA introduce amylase, pectinase, protease, peroxidase, and polyphenol oxidase into xylem ray parenchyma when feeding, it would explain, in part, the local swelling of cells, disruption of phenylalanine ammonia lyase synthesis (PAL), and the abnormal formation of heartwood, termed "rotholz". Here we present the possible mechanism for rotholz production likely caused by the introduction of certain enzymes. This study provides evidence for potential impacts of the introduction of these enzymes on Fraser fir by the action of digestive enzymes present in BWA.

INTRODUCTION:  

Feeding by balsam woolly adelgid (BWA, *Adelges piceae*) on Fraser fir (*Abies fraseri*) causes hypertrophy in nearby plant cells, swelling, gouting, loss of apical dominance, and production of suberized impervious tissue near and far from the feeding site. It has been
suggested that the saliva introduced by BWA during feeding causes these reactions (Balch et al., 1964; Forbes and Mullick, 1970; Mullick, 1969a, b; Mullick and Jensen, 1976a). These reactions to BWA feeding in Fraser fir reduce the transduction of water and place the tree in a state of physiological drought (Mullick and Jensen, 1976b; Puritch, 1971). Prolonged infestations can cause tree death in as little as 2-5 years and has caused 100% mortality of some mature Fraser fir populations (Amman and Speers, 1965).

Adelgids (Hemiptera: Homoptera) are sessile feeders with styles up to 1.5x the length of their bodies (Balch, 1952). They insert their styles through bark tissue on the bole of the tree through inter- and (rarely) intra-cellular spaces in search of xylem ray parenchyma cells where they begin feeding (Balch, 1952). Two types of saliva are secreted by homopterans and some heteroptera, including a watery liquid, for digestion, and more viscous material, for stylet sheath formation (Miles, 1960, 1968). In BWA, stylet sheath material has been observed in intercellular spaces where branching is a presumed result of the insect searching for a suitable feeding site, or leakage into the apoplastic space (Balch, 1952). Leakage of sheath material into intercellular spaces may be a sign of resistance factors in the host plant (Balch, 1952). Reactions to feeding by BWA in balsam fir include, but are not limited to, gouting, swelling, and the production of abnormal wood, and occur a distance much greater than the length of the stylets from the feeding site (Balch, 1952; Balch et al., 1964).

Observations of mirid and coreid feeding revealed that these parenchyma feeding Hemiptera are able to drain plant cells of nutrients that are far beyond the maximum reach of their styles (Miles, 1987). Miridae do not use a stylet sheath, but produce a pectinase
enzyme which breaks down the pectin present in the middle lamella (Miles, 1987; Miles and Taylor, 1994). Coreidae produce a stylet sheath and a sucrase enzyme in a process termed ‘osmotic pump feeding’ where accumulation of intercellular glucose causes parenchyma cells to unload amino acids and other nutrients to adjust for the osmotic imbalance (Miles and Taylor, 1994). Phloem unloading is controlled by plant invertases, the coreid sucrase enzyme appears to function similarly, allowing the insects to feed on nutrients recruited to the feeding site without having to access another parenchyma cell directly (Miles and Taylor, 1994). The draining of nutrients from neighboring cells and breakdown of cell walls allows parenchyma-feeding Hemiptera to feed from one location for a longer period of time without penetrating a new cell (Miles, 1987; Miles and Taylor, 1994).

BWA have been shown to possess pectinase (Adams and McAllan, 1958) and recruit sugars and proteins (Kloft, 1957) to feeding sites. Balch reported that even after removal of settled BWA nymphs abnormally swollen parenchyma and cambial cells form (Balch, 1952) indicating that salivary enzymes introduced are responsible for these changes. Parenchyma cells where BWA feed are long-lived, metabolically active cells important for the storage of starch (Islam et al., 2012; Nakaba et al., 2006; Sauter and Cleve, 1994), transport of nutrients (Bel, 1990), synthesis of defense chemicals (Franceschi et al., 1998; Franceschi et al., 2000), and are responsible for lignification and heartwood formation (Nakaba et al., 2006; Nakaba et al., 2014).

Xylem ray parenchyma cells (XRP) are an optimal feeding site for BWA based on observations of their feeding habits, biology, and inferred physiology. As sessile feeders BWA must obtain all required nutrients from a single site to complete development from a...
crawler to an adult and produce eggs. Eggs of tobacco budworms are comprised of are metabolically expensive materials; from highest to lowest concentrations: protein, shell, lipid, and carbohydrates (Cohen and Patana, 1985). These macronutrient levels found in tobacco budworm eggs are similar to those of other insects (Cohen and Patana, 1985) and it is reasonable to expect BWA eggs to contain similarly expensive materials. When examining unhatched eggs of adelgids under a microscope Cohen observed oil droplets indicating a high level of lipids in the eggs (Cohen and Cheah, 2011) and BWA have been observed producing five eggs per day with as many as 248 eggs (average=96) per female, which is impressive considering eggs are about half the length of an adult female (Balch, 1952). The reproductive demands of BWA dictate that they locate an optimal feeding site.

Eggs are not the only metabolic expense for BWA. Many insects secrete “waxy” substances which function to regulate water loss, but larger “blooms” may also function as predator deterrents (Waku and Foldi, 1984). Although insect cuticle secretions are colloquially referred to as waxes, they may be comprised of a variety of lipids, proteins, fatty acids, alcohols, sterols, waxes, resins, free amino acids, and hydrocarbons (Waku and Foldi, 1984) which would be metabolically expensive for BWA to produce. It is possible that the woolly mass is a process for the excretion of excess nutrients. Stylet sheath material is also produced by BWA as they feed this material has been observed branching and appears to leak into intercellular spaces (Balch, 1952). Sheath material is composed of Lipoprotiens (Miles, 1960) and continuous production by BWA would be metabolically expensive, especially if branching or leaking into intercellular spaces were caused by resistance factors within the tree.
In order to overcome these metabolic deficits other homoptera have evolved feeding habits commensurate with the nutritional value of their diets. Aphids feed on phloem sap which is under positive pressure and requires little to no active sucking by the insect (Auclair, 1963; Douglas, 2003). However, phloem sap is unbalanced nutritionally consisting of mostly carbohydrates (sucrose 0.5-1.5M), and very low concentrations of free amino acids (50-800mM) with some lipids and sterols (Douglas, 2003) requiring huge amounts of material to be consumed. Aphids may consume as much as 10-133% of their body weight hr⁻¹ depending on species and instar (Auclair, 1963). Severed aphid stylets exude phloem sap at 1µl hr⁻¹, equal to emptying 235 sieve elements per minute (Douglas, 2003). Other homopterans such as leafhoppers, feed on xylem tissue under negative pressure requiring large clypeus muscles for suction (Leopold et al., 2003). These insects consume as much as 100-300 times their dry body weight per day and subsequently produce copious amounts of honeydew (Brodbeck et al., 1993). Leafhoppers also have a complex gut composed of several filter chambers which are highly corrugated, extending many times the length of the insect when straightened (Cohen et al 2003). The BWA on the other hand does not produce honeydew, but a single sap-like globule as an adult, and has a simple gut (Jarial, 1998).

Similar to other aphids, BWA do not possess malphigian tubules, and the anterior midgut performs the dual function of storage-excretion and production of digestive enzymes (Jarial, 1998). The cells of the anterior midgut of BWA contain several concretions rich in iron, silicon, chlorine, calcium, magnesium, manganese, and sulfur indicating that BWA feeding sites contain a surplus of these elements relative to the nutritional needs of BWA (Jarial, 1998). Jarial (1998) also found an abundance of rough endoplasmic reticulum in the
anterior midgut indicating these cells are responsible for the production of large amounts of digestive enzymes. In closely related species, hemlock woolly adelgid (Adelges tsugae, HWA), was found to possess several trophically related enzymes in whole body homogenates, including; amylase, protease, peroxidase, and polyphenoloxidase (Oten et al., 2014). Several important bacterial symbionts were also found in HWA which may be responsible for the production of digestive enzymes, the breakdown of plant defense compounds, or defense against predators (von Dohlen et al., 2013).

In this study we examine BWA for the presence of protease, amylase, peroxidase, and polyphenol oxidase in wholly ground egg, nympha first instars, and adults. We hypothesize BWA will contain these enzymes in all life stages, as they are important for feeding and combating plant defenses. Finding these enzymes in crawlers as well as the adults is critical in determining whether the adelgid is producing the enzymes or if their presence is a byproduct of feeding because as stated above these enzymes are also present in plants. These enzymes can act as elicitors of plant defense and may be ingested by the adelgid, or secreted by the adelgid to modify the feeding site. Adelgids may produce these enzymes in response to plant defense in order to neutralize harmful compounds that may negatively affect the adelgid. Since we are interested in the BWAs production of certain enzymes relative to one another, quantitation with spectrophotometric analysis is necessary. Based on these levels we may be able to predict changes in plant chemistry, or insect nutrition related to the physiology of the host-insect interaction.
METHODS:

We used colorimetric and fluorescence assays to determine the presence of various enzymes in wholly ground BWA. As explained by Oten (2014) dissection and separation of salivary glands in adelgids is difficult, and determination of presence or absence of tropically related enzymes can satisfactorily be achieved by using whole body homogenates (Adams and McAllan, 1958).

BWA typically produce two generations per year, with a peak in crawler activity in May-June and August-September depending on elevation and temperature (Arthur and Hain, 1984; Hain and Arthur, 1985). We collected Adults (n=300), crawlers (n=300), and eggs (n=300) from a mature abandoned Fraser fir plantation (Newland, NC, elevation 1200m) in August-September, 2013. Live BWA were collected and returned to the lab before processing. The woolly masses of BWA adults were removed prior to placing ten individuals in a 2mL microcentrifuge tube for storage at -80°C. Neonate Lygus lineolaris (Palisot de Beauvois) (Hemiptera: Miridae) were stored similarly for use as controls in the following assays. Methods for the amylase enzyme, peroxidase, polyphenol oxidase (PPO), and protease assay were adapted from Oten (2011). We compared levels of enzymes among adults, crawlers, and eggs to determine at what stage enzyme production occurred and whether some enzymes were present in the insect before feeding on Fraser fir.

Insects:
Balsam woolly adelgid (Adelges piceae):

BWA adults, crawlers, and eggs were collected at the height of their abundance during the year 2013. We collected adults (n=300), crawlers (n=300), and eggs (n=300) from
a bark of infested trees. The woolly masses of BWA adults were removed and eggs and crawlers were collected individually prior to placing ten individuals in a 2mL microcentrifuge tube for storage at -80°C.

*Lygus lineolaris:*

Neonate *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) were provided from a rearing facility and stored similarly to the procedure used for BWA for use as controls. In order to vary control concentrations either one or five *L. lineolaris* were placed in microcentrifuge tubes. These are similar to methods outlined by Cohen (2000) and Oten (2014; 2011).

**Green peach aphids (*Myzus persicae)*:

Neonate and adult green peach aphids (GPA) were obtained from a greenhouse reared colony at NCSU (Travis McClure, Steve Frank Lab). They were reared on black pearl pepper (*Capsicum annuum*) plants in an incubator set at 25 ºC (21-25 ºC) with RH 40-60 % and 13:11 L:D cycle. Assays were conducted on green peach aphids in order to assess their viability for future assays as a positive control.

**Total body weight:**

Groups of 10 BWA adults, crawlers and eggs were weighed 24 hours after freezing to determine total body weight. This was also done for *L. lineolaris* by weighing either one or five individuals, similar to how insects were processed for the enzyme assays. Weights were then averaged and normalized to a per individual weight amount (Table 1).

**Sample preparation:**
Insect protein was partially purified by microfiltration. Each frozen insect sample was ground in 200 µL of the corresponding assay buffer using a cordless motor pellet pestle. Samples were purified using a 3 kDa microcentrifuge filter (Millipore Corporation, Billerica, MA) spun for 10 minutes at 14,000 g which resulted in ca. 50 µL purified protein left in the filter. Filters were inverted and centrifuged for two minutes at 1000 g to recover the purified sample and 150 µL of the appropriate buffer were added. All samples were prepared in the same way for each assay except that different buffers were used according to each assay protocol.

Bradford assay:

A colorimetric assay using the Bio-Rad protein assay following the microtiter plate protocol (Bio-Rad Laboratories Inc., Hercules, CA) was used to determine total soluble protein content of BWA and L. lineolaris. This protocol can be used to detect protein concentrations from 1-20 µg, and the assay produces a linear curve for concentrations from 50 to 500 µg/mL (Bio-Rad Laboratories Inc., Hercules, CA). Two standard curves were created to account for samples where higher or lower concentrations were tested. Standard curves employed bovine serum albumin (BSA) at 50, 100, 500, and 1000 ng dissolved in diH₂O where either 10 µL (low) or 50 µL (high) of BSA were used. Standard curves were produced and used to calculate, by least squares analysis, the protein content of BWA and L. lineolaris samples with corresponding high or low volumes analyzed.

The standard curves were used to calculate the total soluble protein (ng) which was then used in the corresponding enzyme assays to calculate protein activity per µg protein. An
average of all samples was taken and divided by the insect body weight to determine the approximate percentage of soluble protein per insect (table 1).

Amylase assay:

An EnzChek® Ultra amylase kit (MP 33651, Invitrogen, Eugene, OR) was used to determine the presence of amylase in wholly ground samples of BWA adults, crawlers, eggs, *L. lineolaris*, and GPA. One or five GPA neonates and adults, and *L. lineolaris* neonates were used while 10 BWA adults, crawlers, and eggs were used. Samples were purified as described above. Negative controls included a buffer only solution of 50 mM sodium acetate buffer (pH 4.0). Each treatment was replicated three times.

Samples were analyzed using a 96-well microplate reader (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA) running Multi analysis software (v. 3.4.0.25). The microplate reader was set to record fluorescence intensity with an excitation wavelength of 485 nm and emission wavelength 535 nm while holding a temperature of 37°C. Recommended incubation time for this protocol is 30 min, or due to the continuous nature of the reaction readings can be made at multiple time points to determine rate of fluorescence increases. Relative fluorescent units (RFU) were continuously recorded for 90 readings over one hour (figure 1). Maximum RFU values were evaluated during this time period. BWA crawler and egg as well as *L. lineolaris* samples reached maximum RFU values near the end of the sampling period indicating that a longer incubation period may increase values; however, the rate of activity is unlikely to increase as the slope did not appear to be changing. BWA adults reached their maximum RFU value near 50 minutes.

Protease assay:
An EnzChek® protease assay kit using green fluorescence (MP E6638, Invitrogen, Eugene, OR) was used to detect protease activity in wholly ground samples of BWA adults, crawlers, eggs, *L. lineolaris*, and GPA. One or five GPA neonates and adults, and *L. lineolaris* neonates were used while 10 BWA adults, crawlers, and eggs were used. Samples were purified as described above. Positive control standards were included using trypsin protease (type II-S from porcine pancreas; Sigma-Aldrich, St. Louis, MO) diluted to 0.06, 0.125, 0.25 units activity/mL to create a standard curve. Negative controls included a buffer only solution of 1X phosphate buffer solution (PBS, pH 7.4). Each treatment was replicated three times.

Samples were analyzed using a 96-well microplate reader (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA) running Multi analysis software (v. 3.4.0.25). The microplate reader was set to record fluorescence intensity with an excitation wavelength of 485 nm and emission wavelength 535 nm while holding a temperature of 37 °C. Recommended incubation time for this protocol is one hour protected from light, or to increase sensitivity 24 hour incubation may be required. Readings were made at multiple time points to determine rate of fluorescence. Relative fluorescent units (RFU) were continuously recorded for 90 readings over a 4 hour period. Maximum RFU values were evaluated during this time period. The samples continuously increased at a constant rate until the end of the experiment indicating a longer incubation would have yielded higher values; however, without a change in reaction rate further incubation was considered unnecessary.
Perioxidase assay:

An Amplex® red hydrogen peroxide/peroxidase kit using protocol for the peroxidase assay (MP 22188, Invitrogen, Eugene, OR) was used to detect peroxidase activity in ground samples of BWA adults, crawlers, eggs, as well as positive controls using *L. lineolaris*, and GPA. One or five GPA nymphs and adults, and *L. lineolaris* nymphs were used while 10 BWA adults, crawlers, and eggs were used. Samples were purified as described above. Negative controls were comprised of a buffer only solution, e.g. 0.25 M sodium phosphate, pH 7.4. A standard curve treatment was created using horseradish peroxidase (HRP) by diluting the 10 mU/mL stock solution to 2.5, 1.25, and 0.6 mU/mL aliquots. Each treatment was replicated three times.

Samples were analyzed using a 96-well microplate reader (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA) running Multi analysis software (v. 3.4.0.25). An excitation wavelength of 535nm and emission wavelength 595 nm were recorded for 90 readings at 37 ºC. Recommended incubation time for this protocol is 30 min, or due to the continuous nature of the reaction reads can be made at multiple time points to determine rate of fluorescence. Relative fluorescent units (RFU) were recorded continuously for 90 readings over one hour (figure 2). RFU values were evaluated at 15 minutes. BWA crawler and egg as well as *L. lineolaris* samples reached maximum RFU values near the end of the sampling period indicating that a longer incubation period may increase values; however, the peak rate of activity, as indicated by the asymptote of the line, appeared to occur earlier, near 15 minutes. This was also the time point used by Oten (2014; 2011), which would allow for direct comparison.
Polyphenol Oxidase assay:

Wholly ground samples of BWA adults, crawlers, eggs, as well as positive controls using *L. lineolaris*, and GPA were prepared as with previously described assays. Polyphenol oxidase (PPO) activity was determined as described by Oten (2014; 2011). Briefly, each sample was divided in half so that half would be treated with a PPO inhibitor, phenylthiourea (PTU), and half without inhibitor. The use of a specific PPO inhibitor would help affirm the authenticity of polyphenol oxidase. Wells were first filled with 50 µl of each sample, replicated three times per treatment. Treatment groups included; one or five GPA neonates and adults, and one and five *L. lineolaris* neonates, and 10 each of BWA adults, crawlers, and eggs. Negative controls included a buffer only (PBS) and buffer with inhibitor (PBS+PTU). For the substrate 50 µL of 5.5 mM L-DOPA was used. After each sample was loaded 10 µL of PBS buffer was added to half the sample treatments, while the other half received 10 µL of PTU. Samples were then incubated, protected from light for 20 minutes at 37 ºC prior to adding 50 µL of substrate to each well.

Samples were analyzed using a 96-well microplate reader (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA) running Multi analysis software (v. 3.4.0.25). Absorbance was recorded at 450 nm continuously for 30 readings over the course of ten minutes while holding a constant temperature of 37 ºC. Absorbance values at the end of the assay were used to compare PTU to non-inhibitor samples, and between treatment comparisons.

Enzyme activity quantification and data analysis:
Amylase activities (RFU) at 50-60 minutes, protease activities (RFU) at 221 minutes, peroxidase activities (RFU) at 15 minutes, and PPO activities (abs) at 10 minutes were first corrected by subtracting buffer value readings at the same time point. Protease and peroxidase assays both included a standard curve to calculate units of enzyme activity relative to the standard. Amylase and PPO assays did not include standard curves and values are reported as RFU or absorbance rates. Using protein concentrations obtained from the Bradford assay for each sample the rate of enzyme activity (U/min, mU/min, RFU/min, abs/min) was divided by the total soluble protein concentration present in the sample to determine rate of enzyme activity per unit of protein. These values were then divided by the number of insects present in the sample to analyze protein activity per unit of protein per insect. The amylase RFU/µg protein/min/insect values were log transformed to normalize the data for comparison.

Enzyme activity per unit of protein per insect was analyzed using a one-way ANOVA where comparisons between samples were drawn using a Tukey-Kramer HSD, except for the PPO data which were analyzed using a repeated measures MANOVA to compare differences between samples and within samples with respect to the presence of the PPO inhibitor, PTU. All analysis was performed using JMP ® Pro v.10.0.0 (SAS, 2012).

RESULTS:

Bradford assay:

Protein content differed between *L. lineolaris* samples as well as *L. lineolaris* and BWA samples, but were not different between BWA samples (F=22.14, df=44, p<0.0001) (table 1).
Amylase:

There were not significant differences in log transformed amylase activity (RFU/µg protein/min/insect) between all samples or time (seconds) to reach maximum RFU. Adult BWA samples demonstrated similar levels of amylase activity compared to *L. lineolaris*, however neither exhibited RFU values as high as those of BWA crawler and egg samples (table 2). Green peach aphids also showed amylase activity.

Protease assay:

There were significant differences between samples for protease activity (U/µg protein/min/insect) with BWA eggs exhibiting the highest activity levels, more than three times those of *L. lineolaris*. BWA crawler and adult samples were approximately two times higher than *L. lineolaris*, but not significantly different (table 3).

Peroxidase assay:

There were significant differences between samples for peroxidase activity (mU/µg protein/min/insect). Adult BWA showed the lowest levels of activity but were not different from the crawler samples. *Lygus lineolaris* and BWA crawlers were not different from one another, but both were significantly less than BWA egg samples. BWA eggs exhibited more than 4.5 times peroxidase activity than BWA adults, two times more than crawlers, and 1.25 times more peroxidase activity than *L. lineolaris* samples (table 4). It is possible that higher levels of peroxidase activity could result in lower RFU because excess peroxidase breaks down the byproduct rosuferin reducing fluorescence activity. However, this is unlikely due to
the fact that BWA adult and crawler samples fell within the range of the standard curve (0.6-2.5 mU/mL).

Polyphenol Oxidase assay:

There were no significant differences between samples for PPO activity (abs/ugprotein/min) (F=1.65, Ndf/Ddf=3/8, p=0.25), despite BWA adult activity being more than 13 times greater than L. lineolaris protein samples. BWA crawler and egg samples were also higher than L. lineolaris samples both with and without PTU present (table 5). The PTU inhibitor caused a significant difference in PPO activity for samples with PTU compared to those without (F=12.9, Ndf/Ddf=1/8, p=0.007), and there was a slight difference (at α=0.10) for the magnitude of the PTU effect by sample (F=3.9, Ndf/Ddf=3/8, p=0.0549). The PTU inhibitor had the greatest effect on the BWA crawler sample, with the least effect on L. lineolaris PPO activity. (This section is confusing to me. It is not clear whether or not your comparisons are accounting for the protein content of each sample. In other words, is it reported as units of activity per mg of protein? This is the only way that comparisons can be made meaningfully.)

**DISCUSSION:**

Balsam woolly adelgid adults, crawlers, and eggs exhibited varying levels of protease, amylase, peroxidase, and PPO activity, verifying the presence of these enzymes during all life stages. This indicates that they produce these enzymes themselves rather than acquisition through feeding. Insects were not dissected so this activity may not be limited to feeding or salivary enzyme production; however, the presence of these enzymes in BWA
adults and crawlers suggests they are important for feeding activity or combating plant defense systems.

Adams and McAllan (1958) showed that whole body homogenates of BWA show pectinase activity. We propose that this enzyme is responsible for several symptoms exhibited by Fraser fir infested with BWA, including disruption of PAL1. The middle lamellae of xylem ray parenchyma (XRP) where BWA feed is comprised mainly of pectin (Romberger et al., 1993). This is also the site of phenylalanine ammonia lyase (PAL) production in Norway spruce (Franceschi et al., 1998), and important elicitor of the phenylpropanoid biosynthetic pathway (Ferrer et al., 2008). It has already been shown that BWA-infested Fraser fir down regulate PAL1 compared to noble fir (*Abies procera*) which upregulate this gene (Emerson, 2012). It is likely that the introduction of pectinase into the middle lamellae causes the downregulation of PAL1. Furthermore, the ability for noble fir to upregulate this gene, despite BWA infestation, indicates a possible mechanism for resistance. One possibility for noble fir resistance is if the production of PAL occurs at higher levels in the cytosol rather than the middle lamellae, but evidence in norway spruce indicate the primary site of production as the middle lamellae (Franceschi et al., 1998). The pectin of the middle lamellae is most likely differentially methylated to prevent BWA pectinase from disrupting its production of PAL, conferring resistance through anti-manipulation and allowing noble fir to produce defensive chemicals using the phenylpropanoid pathway.

The activities of BWA amylase and protease may be responsible for swelling of XRP as observed by Balch (1952) as well as the recruitment of proteins and carbohydrates to the feeding site, as shown by Kloft (1957). The production of these two enzymes would allow
BWA to feed from the same cell similar to ‘osmotic pump’ feeding described by Miles and Taylor (1994). Xylem ray parenchyma are important sites for storage of starch grains, production of defense compounds, and formation of heartwood as well as several other functions (Bel, 1990; Franceschi et al., 1998; Islam et al., 2012; Nakaba et al., 2006; Nakaba et al., 2014; Sauter and Cleve, 1994). Based on the size of starch grains (0.5-10µm in Norway spruce (Cabalkova et al., 2008) in comparison to BWA stylet food canals (1µm in diameter (Forbes and Mullick, 1970) it is likely that amylase is expressed extra-orally for the digestion of starch within the plant cell prior to ingestion. It should be noted that amylase activity may be attributed to gut microbes (Srivastava, 1962; Toenshoff et al., 2012a; Toenshoff et al., 2012b).

As amylase digests starch grains an osmotic imbalance is created causing an immediate influx of water to the cell from the apoplastic space, in conjunction with pectinase activity this would cause the cell to swell. As BWA continue to release amylase to break down starch, while removing freed saccharides, they may also secrete protease to break down large storage proteins to free amino acids for consumption. The removal of saccharides and amino acids by BWA would cause Fraser fir to reallocate those resources to the site where BWA are feeding as evidenced by Kloft (1957). These actions may present as gouting or swelling at the site of infestation producing a ‘gall’ effect where the area contains more nutrients.

BWA crawler samples showed high protease activity compared to all other samples. This may be explained by probing behavior of nymphs foraging for feeding sites and/or preparation of future feeding sites. Proteases secreted during probing may work to test a
feeding site for protein availability. If the site is not ideal and the crawler has everted her styles beyond a point of retraction, she may continue to release protease enzymes into the parenchyma cells causing an osmotic imbalance which the plant would correct by transporting more protein to the area. Protein concentration has been shown to increase near BWA feeding sites (Kloft, 1957) and similar methods of an osmotic imbalance have been described for amylase in plant-sucking bugs (Miles, 1987). A lack of initial protein in plant tissue and the secretion of proteases may partially explain the 2-8 week aestivation of crawlers after insertion for some, but not all individuals during the same time of year (Amman and Speers, 1965), and may also explain why BWA clump around some feeding sites (Newton pers. comm., personal obs.). BWA may prepare a site for feeding by secreting initially high levels of protease after insertion, depending on the level of amino acid build up and protein recruitment they may continue to develop, aestivate for 2 to 8 weeks, overwinter, or die. The flexibility in BWAs level of diapause activity allows it to manipulate its host while conserving energy, improving the feeding site for itself and future generations.

The loss of starch grains in XRP may also act as a trigger inducing premature heartwood formation which would present as rotholz. The exact mechanism for induction of heartwood is not completely understood, but recent evidence shows that is starts with the depletion of starch granules, followed by the accumulation of phenolics (Islam et al., 2012). This effect may be delayed with the disruption of the phenylpropanoid pathway, and as BWA produce polyphenol oxidase (PPO) and peroxidase enzymes to combat Fraser fir defensive chemicals.
Whole body homogenates of BWA adults, crawlers, and eggs all showed varied levels of PPO activity with crawlers having the highest activity, and the highest level of suppression by PTU. Crawler BWA most likely encounter the highest levels of phenolic defense compounds when penetrating the bark of Fraser fir, and subsequent down regulation of those chemicals, compounds [4], [6], and [8] in particular, may be a direct result of PPO activity (see chapter III). Compound [8] may be related to regulation of the PAL1 defense pathway. PPO activity in BWA may play a crucial role in detoxifying plant defense compounds, reducing clogging of sieve tubes facilitating the transport of nutrients to the feeding site, and repressing downstream defense pathways.

Polyphenol oxidases (PPOs) have been described as a sclerotizing agent in insect cuticle, were hypothesized to be involved in stylet sheath formation of homopterous insects, but have since been shown to function mainly as a detoxifying agent for phenolic substances, and affect plant growth via gall formation. Catechin is a common plant defense compound elicited to act as a feeding deterrent, however, when oxidized by rose aphid polyphenol oxidase becomes a phagostimulant or non-deterrent for the aphids. Susceptible rose buds contain lower quantities of phenolic constituents, which rise significantly in resistant buds (Peng and Miles, 1988).

Hattori et al (2005) describe two types of phenoloxidases in the green rice leafhopper present in the salivary glands and secreted in the watery saliva during feeding. In showing that laccase is secreted in the watery saliva, which is ejected during probing, of the green rice leafhopper, it is suggested that its major function is detoxifying phenolic substances released from cell vacuoles whose acidic pH is more favorable to the function of the PPO activity as
opposed phloem which is much more basic (Hattoria et al., 2005). Phenolic compounds perform many defense functions within the plant, including production of quinones which are highly toxic as protein alkylating agents (Mayar, 2006). Laccase, therefore may oxidize these substances into non-toxic polymers. Another function of the PPO, laccase, may be to degrade lignin (Hattoria et al., 2005).

The role of peroxidase in BWA is, in all likelihood, also associated with combating plant defensive responses. BWA clearly must delay or inhibit HR in Fraser fir as has been hypothesized in previous studies (Fernandes, 1990; Hain, 1988) especially with evidence that resistant fir species form a HR much faster than susceptible species (Mullick and Jensen, 1976a). Due to the various roles of H$_2$O$_2$, neutralization of this molecule may also affect downstream defense pathways such as the mevalonate pathway responsible for sesquiterpene synthesis.

Rotholz production appears to be systemic and appears far from BWA feeding sites, other symptoms such as the loss of apical dominance also appear to occur as a result of a systemic affect from BWA feeding. Proteins present in aphid saliva range in size from 30-240kDa and can easily be transported through the phloem system. Radio-labeled saliva has been shown to be transported through apple trees and ingested by non-labeled aphids. The transport of these proteins may act to enhance feeding sites for future generations of insects, and may act as a form of communication between feeding insects (Torsten and Aart, 2006). In BWA the transport of digestive enzymes to different parts of the tree may aid in accumulation of nutrient resources at the feeding site as described earlier.
CONCLUSIONS:

The composition and activity of BWA saliva with regard to feeding, detoxification of plant toxins, and altering of host physiology are important in understanding the interactions between BWA and Fraser fir. Amylase activity in BWA may function to break down starch grains stored in xylem ray parenchyma cells and may work in conjunction with pectinase (Adams and McAllan, 1958) and proteases to accumulate nutrients forming a ‘physiological gall’ at the feeding site aiding in ‘osmotic pump feeding’ or macerate and flush feeding as described by Miles (1994). Furthermore, pectinase, amylase, and protease may all play a crucial role in triggering rotholz production. Presence of protease in BWA may play a role in not only digesting dietary proteins, but also creating a more hospitable feeding site for other BWA by recruitment of protein through osmotic imbalances. Peroxidases and PPOs likely perform many functions within BWA in combating Fraser fir defense systems, especially with relation to down regulation of defensive compounds.

These enzymes were also found in HWA using similar methods, and it should be noted that this study, as well as the HWA study only represent a snapshot of enzyme activity in these two adelgid species because insects were only collected at one time point. It is probable that these enzyme levels vary throughout the year depending on nutrient availability within the plant, exogenous factors such as temperature and precipitation, and phenology of the insect itself. Comparisons of HWA and BWA enzymes are probably not meaningful at this time, but the presence of these enzymes in two different adelgid species is notable (Table 6). Both BWA and HWA tested were collected during peak reproductive periods June/September and January respectively.
Future work for regarding Fraser fir resistance to BWA should involve the characterization and study of enzyme inhibitors (EI) present in Fraser fir, Turkish fir, and Veitch fir, three species representing the range of BWA susceptibility from highly susceptible to highly resistant, respectively. In order to determine if different fir species possess BWA specific enzyme inhibitors, isolation of all EIs from Fraser, Turkish, and Veitch fir and subsequent characterization and testing against all of the above enzymes in BWA would determine if resistance to BWA were dependent on enzyme inhibition. If BWA enzyme inhibition did occur in these resistant and tolerant fir species, and they were characterized, transgenic introduction of multiple enzyme inhibitors would be the most efficient form of providing BWA resistant Fraser fir to the Christmas tree industry and regeneration of natural stands without long term traditional breeding. As mentioned previously, it is also possible resistance factors may rely on differential methylation of the middle lamellae, i.e. noble fir, allowing for normal defense response through the phenylpropanoid pathway.

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Oten, K. L. F., 2011b, Host-Plant Selection by the Hemlock Woolly Adelgid, Adelges tsugae Annand: Sensory Systems and Feeding Behavior in Relation to Physical and
Chemical Host-Plant Characteristics, North Carolina State University, Raleigh, North Carolina, 265 p.


SAS, 2012, JMP (R) Pro.


**TABLES AND FIGURES:**

Table 1. Total protein content and weight of BWA adults, crawlers, and eggs as determined by the Bradford protein assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight/n^a (µg)</th>
<th>Std Dev</th>
<th>n</th>
<th>Protein content (ng)</th>
<th>Std Dev</th>
<th>T-K HSD*</th>
<th>n</th>
<th>%protein</th>
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*Samples connected by the same letter are not significantly different at α=0.05 level for protein content by sample using the Tukey-Kramer HSD.
^aug/n is the calculated weight of an individual insect based on the weight of 10 individuals.
Figure 1. Enzyme kinetics for BWA amylase activity.
Table 2. Amylase activity of BWA adults, crawlers, and eggs as determined by the EnzChek ultra amylase assay kit (E-33651). Relative fluorescent units µg protein⁻¹ min⁻¹ insect⁻¹ (RFU/µg protein/min/insect) and Time to reach max RFU.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RFU/µg protein/min/insect</th>
<th>*RFU/ug protein/min/insect</th>
<th>*Std Dev</th>
<th>F Ratio df</th>
<th>p-value</th>
<th>Time (seconds) to max RFU</th>
<th>Std Dev</th>
<th>F Ratio df</th>
<th>p-value</th>
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<tbody>
<tr>
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<td>2960</td>
<td>1039.23</td>
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</table>

*values log transformed for comparison
Table 3. Total protease activity of BWA adults, crawlers, and eggs as determined by the EnzChek protease assay kit (E-6638).

<table>
<thead>
<tr>
<th>Sample</th>
<th>U/µg protein/min/insect</th>
<th>Std Dev</th>
<th>T-K HSD*</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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</tbody>
</table>

*Samples connected by the same letter are not significantly different at $\alpha$=0.05 level for protein content by sample using the Tukey-Kramer HSD.
Figure 2. Enzyme kinetics for peroxidase activity. Adult, Lygus 5, and Lygus 1 follow the primary axis on the left, while the crawler and egg samples follow the secondary axis on the right.
Table 4. Peroxidase activity of BWA adults, crawlers, and eggs as determined by the Amplex red hydrogen peroxide/peroxidase assay kit (A-22188).

<table>
<thead>
<tr>
<th>Sample</th>
<th>mU/µg protein/min/insect</th>
<th>Std Dev</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>T-K HSD</th>
<th>F Ratio</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BWA Egg</td>
<td>4.25</td>
<td>0.24</td>
<td>3.66</td>
<td>4.84</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lygus</td>
<td>3.03</td>
<td>0.998</td>
<td>0.55</td>
<td>5.11</td>
<td>AB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BWA Crawler</td>
<td>2.26</td>
<td>0.02</td>
<td>2.04</td>
<td>2.48</td>
<td>BC</td>
<td>14.77</td>
<td>11</td>
<td>0.0021</td>
</tr>
<tr>
<td>BWA Adult</td>
<td>0.92</td>
<td>0.57</td>
<td>-0.49</td>
<td>2.33</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Samples connected by the same letter are not significantly different at α=0.05 level for protein content by sample using the Tukey-Kramer HSD.
Figure 3. Polyphenol oxidase (PPO) activity with and without phenylthiourea (PTU) a PPO inhibitor.
Table 5. Polyphenol oxidase (PPO) activity with and without phenylthiourea (PTU) a PPO inhibitor.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PPO w/oPTU (abs/ugprotein/min)</th>
<th>PPO w/PTU (abs/ugprotein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>0.233652</td>
<td>0.133563</td>
</tr>
<tr>
<td>Crawler</td>
<td>0.721366</td>
<td>0.018656</td>
</tr>
<tr>
<td>Egg</td>
<td>0.19288</td>
<td>-0.08711</td>
</tr>
<tr>
<td>Lygus</td>
<td>0.017958</td>
<td>-0.00513</td>
</tr>
</tbody>
</table>
Table 6. HWA and BWA enzyme activity comparison.

<table>
<thead>
<tr>
<th>Source</th>
<th>Protease (U/µg protein/min)</th>
<th>Amylase (mU/µg protein/min)</th>
<th>Peroxidase (mU/µg protein/min)</th>
<th>PPO w/PTU (abs/µg protein/min)</th>
<th>PPO w/o PTU (abs/µg protein/min)</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>HWA (Adults)</td>
<td>0.259</td>
<td>0.1088</td>
<td>0.2323</td>
<td>0.00323</td>
<td>0.0569</td>
<td>(Oten 2011b, Oten et al. 2014)</td>
</tr>
<tr>
<td>Lygus (neonate)</td>
<td>0.8405</td>
<td>0.7748</td>
<td>0.1283</td>
<td>nr</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>BWA (Adult)</td>
<td>1.27534</td>
<td>6.73123</td>
<td>0.92</td>
<td>0.13356</td>
<td>0.23365</td>
<td></td>
</tr>
<tr>
<td>BWA (Crawler)</td>
<td>1.82654</td>
<td>8.08495</td>
<td>2.26</td>
<td>0.01865</td>
<td>0.72136</td>
<td>This study</td>
</tr>
<tr>
<td>BWA (Egg)</td>
<td>2.70033</td>
<td>8.07085</td>
<td>4.25</td>
<td>-0.08710</td>
<td>0.19288</td>
<td></td>
</tr>
<tr>
<td>Lygus (neonate)</td>
<td>0.87585</td>
<td>7.64304</td>
<td>3.03</td>
<td>-0.00513</td>
<td>0.01795</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER III:

FRASER FIR JUVABIONE AND BARK CHEMICAL COMPOSITION WITH RELATION TO BALSAM WOOLLY ADELGID INFESTATION

ABSTRACT:

Trees in the genus *Abies* contain a variety of chemicals synthesized in defense against insects and pathogens. In particular, juvabione, has been studied as a potential factor in *Abies* resistance to balsam woolly adelgid infestations. Juvabione is an insect juvenile hormone mimic which can be extracted from *Abies* spp. with petroleum ether. In order to examine differences between infested and healthy Fraser fir (*Abies fraseri*), petroleum ether extracts were made from different portions of wood, and bark of infested and healthy trees as part of an analysis of defensive compounds. Juvabione was present in juvenile wood and mature wood in both infested and healthy samples, but no juvabione was identified in the extracts from bark. In agreement with previous studies, juvabione and related compounds were more concentrated in infested Fraser fir, than healthy samples. Juvabione and related compounds occurred at much higher concentrations in juvenile wood than mature wood. This is significant because BWA does not come into contact with mature wood. In examining the role of juvabione and other defensive compounds found in the bark of Fraser fir, the following hypotheses regarding defense of *Abies* against BWA were proposed: 1) Juvabione is a defense evolved to combat fungi and associated bark beetles so production is limited to inner wood tissues where it would be most effective against larval insects and fungi, 2) juvabione synthesis occurs in the juvenile wood and transport to the bark and mature wood sections is hindered by rotholz caused by the adelgid infestation, 3) BWA produce enzymes
that combat the production of juvabione and other defense compounds at the feeding site. Petroleum ether extracts were tested in a bioassay using live insects and an inhibitory concentration (IC50) was found to occur within the range of natural levels found in healthy and infested Fraser fir. Several other defensive compounds were identified in the bark tissue, including four antifungal compounds, which provides some support for the first hypothesis.

**INTRODUCTION:**

Fraser fir (*Abies fraseri*) is highly susceptible to BWA and succumbs to infestation due to a hypersensitive response and formation of non-conductive tissue which restricts water and nutrient movement throughout the tree (Mitchell 1967b, Hollingsworth and Hain 1994). Fraser fir also produces defense compounds, such as juvabione, in response to BWA infestation (Fowler et al. 2001a). Conifers are well known for producing other defense chemicals in response to insect attack; those most studied are terpenoids, flavenoids, lignans, phenols, and steroids (Yang et al. 2008).

Conifers in general have evolved defenses against a variety of pests. Most literature concerning conifer defensive reactions focuses on bark beetles and fungi, and the defensive systems of conifers against bark beetles and related pathogens is well documented. Production of secondary defensive compounds is a keystone to this “arms race” (Franceschi et al. 2005).

Bark is the first line of defense against insect and pathogen attack. Bark periderm acts as a mechanical barrier, but also contains constitutive defensive compounds as part of chemical protection. In Fraser fir, resin blisters contain many defensive compounds including
alpha-pinene, 3-carene, beta-phenylalline, and montoterpenes which are hypothesized to prevent insect attack, especially from bark beetles (Sutton et al. 1997).

All conifers studied contain polyphenolic parenchyma-containing (PP) cells. Polyphenolic parenchyma cells are considered part of the constitutive defensive system for conifers that can be induced to produce more resin. Traumatic resin ducts (TRD) are part of an induced defensive response. Constitutive resin ducts exist to store and produce resin, while TRDs form after an attack and may contain different types of resin than pre-existing ducts. (Franceschi et al. 2005)

Both systems employ resin, which contains terpenes, phenolics, and resin acids. Phenolics are important in conifer defense against pests and pathogens, so it is logical that phenolics are also abundant. Phenols bind proteins, inactivating them and hindering the ability of pests/fungi to use digestive/feeding enzymes. Phenolics and tannins are also toxic to insects and pathogens, reducing feeding ability and nutritive value of the plant. Presence of phenols also aids in lignification of cell walls to help prevent the spread of pathogens, or close off the feeding area for some insects. (Franceschi et al. 2005)

Juvabione was first discovered in the wood of Abies, in the 1960’s and showed great potential as an insect growth regulator (Sláma and Williams 1965, Bowers 1966, 1968, 1971). Induced defensive compounds, including juvabione, have been observed in relation to BWA attack, but to date no correlations to resistance have been derived (Arthur and Hain 1987, Sutton et al. 1997, Fowler et al. 2001a).

Juvenile hormone mimics, such as juvabione, prevent metamorphosis when applied to the pre-metamorphic instar of insects such as mealworms and bugs. These compounds,
referred to as insect growth regulators, disrupt the balance of two naturally occurring insect hormones, juvenile hormone and ecdysone. Juvabione mimics juvenile hormone, which is normally down-regulated in the pre-metamorphic instar to allow development into an adult. (Bowers 1981, IRAC 2013). The presence of juvabione prevents complete metamorphosis, and insects will experience supernumerary molts, or incomplete metamorphosis which can lead to death, as observed in Tenebrio molitor (Bowers 1966).

One hypothesis was that juvabione is an important defensive compound in relation to BWA resistance (Arthur and Hain 1987, Fowler et al. 2001a). It has been shown to vary between Fraser fir provenances (Arthur and Hain 1987), and to vary between trees at different infestation levels (Fowler et al. 2001a). Juvabione has also been shown to decrease fecundity of BWA (Fowler et al. 2001b), possibly by decreasing the size of the ovarioles as it does in other adult insects (Fockler and Borden 1973, Prabhu and John 1975). The quantity of juvabione varies between Fraser fir trees, and was also shown to vary within a single tree vertically, with higher concentrations near the tops of trees compared to the middle and bottom (Fowler et al. 2001a). However horizontal distribution of juvabione has not been assessed in Fraser fir. Horizontal distribution of juvabione, especially in juvenile wood, mature wood, and bark may elucidate where it is biosynthesized within the tree and how it is transported in response to BWA infestation. Puritch and Nijholt (1973) showed that juvabione seemed to be localized to wood adjacent to BWA infestation, and may only be synthesized in response to BWA attack. However, other studies regard juvabione as innocuous with respect to insect defense due to its limited activity and specificity (Staal 1975). Juvabione may be more effective, as it has also been shown to act, as an antifungal
agent (Willför et al. 2007, Yoneyama et al. 2013). Nonetheless, its function within the tree, with regard to tree physiology, is unknown.

Bark defense compounds found in oleoresin have been studied with relation to BWA, but no correlations to resistance have been made (Amman 1969, Arthur and Hain 1985, 1987, Christiansen et al. 1987, Hollingsworth and Hain 1992, Franceschi et al. 2005). Bark is the interface between BWA and their feeding site. Defensive compounds present in the bark are important in the initial defense against BWA as well as induced changes which may help tolerate BWA infestations. For instance, Turkish fir (Abies bornmuelleriana) and silver fir (Abies alba), are considered tolerant/resistant to BWA, and these species of fir have been observed to produce thicker bark in response to BWA infestations (Mitchell 1966, Hollingsworth and Hain 1992). Fraser fir shows little increase in bark thickness when infested by BWA.

The current research tested several hypotheses regarding juvabione concentration, its efficacy, and other defensive compounds present in Fraser fir. It was predicted that Fraser fir juvabione concentrations would vary between juvenile wood, mature wood, and bark tissue in healthy and BWA infested trees. Compounds found within the bark were also analyzed and compared between healthy and infested trees. It was hypothesized that Fraser fir would show an increase in defensive compounds in the bark in response to BWA. In order to test the biological relevance of compounds extracted from the trees, a bioassay using live insects was used to test the hypothesis that there is a dose dependent response of these defensive chemicals.
METHODS:

Plant materials:

We collected six heavily infested and six healthy Fraser fir bolts (1.8m) in Ashe County, NC. Wood disks (7.6cm) were cut from each bolt at a height of 91.4cm and placed in a drying oven (at 105°C) for 48 hours before weighing, measuring, and separating each into juvenile wood, mature wood, and bark sections (Table 1). Heavily infested trees had more than 10 BWA/2.2cm² and exhibited signs of infestation such as loss of apical dominance and a thinning crown. Upon inspection of the wood disks, rotholz were observed on 2-3 consecutive growth rings. Healthy trees did not exhibit any signs of infestation and no BWA were observed on the bolts.

The pith and first three/four rings were considered juvenile wood, identified by poor differentiation between early and latewood. Rings four/five were discarded. Rings six and above were identified as mature wood, as there were clear distinctions between early and latewood growth. Bark was separated from the outside ring and care was taken not to include any cambium in the bark sample (and vice versa). A chisel was employed to chop each wood section into 3mm³ chunks. Trees 4 (infested) and 10 (healthy) were used in a separate study with green peach aphids to observe the effects of extracts on feeding behavior and fecundity. Mature wood and juvenile wood, in trees 4 and 10, were combined for extraction because differences in juvabione, not wood section, were being analyzed.

Extraction:

Methods from Puritch and Nijholt (1974) were adapted for petroleum ether extraction of the dried wood samples. Samples were processed as previously described; however, the
extract was not methylated or washed using NaOH in an effort to expedite the analysis of samples for possible development of resistance screening methods. Methylation and washing with NaOH also alters the structures of the resin and fatty acids (Puritch and Nijholt 1974), and might have compromised the goals of the bioassay. Briefly, after drying, separating, and chopping the wood into 3mm³ chunk 3-8 g of each sample was placed in 25x80 mm single thickness cellulose extraction thimbles for use in a Soxhlet apparatus. Petroleum ether (150 mL) was boiled in a 250 mL round bottom flask with glass boiling stones. All glassware was cleaned with warm soapy water and rinsed three times in acetone between uses. Soxhlet cycles occurred every 15 minutes and were run continuously for a 12 hour period. Petroleum ether and extract were transferred to beakers through glass filter paper, where petroleum ether was allowed to evaporate for 48 hours under a fume hood leaving a clear or yellow oil resin. Extracts were dissolved in 20mL acetone for transfer to preweighed 40mL vials with Teflon screw caps. Acetone was evaporated for 48 hours under the fume hood and dried in a gravity convection incubator (45 ºC) with anhydrous calcium sulfate (Drierite) for 48 hours before weighing.

GC-MS analysis:

We used a gas chromatograph (HP 6899) with mass spectrometer (HP 5973) using Chemstation software. We injected 1 μL using an autosampler with helium carrier gas in a 30 m column (HP-5MS, I.D. 25 mm). The GC oven was programmed to run from 80-200 ºC at 5 ºC·min⁻¹, 200-230 ºC at 1ºC·min⁻¹, and 230-300 ºC at 30ºC·min⁻¹ between samples temperature was ramped down from 300 ºC to 80 ºC at 30 ºC·min⁻¹ and held at 80 ºC for 4 minutes before the next sample was injected. The flame ionization detector was on between
4 and 30 minutes, and turned off during the rest of the program to avoid accumulation of extraneous data.

A calibration curve for juvabione was created using five concentrations of an external juvabione standard. Concentrations of juvabione standard included 15, 30, 60, 90, and 120 ng·μL⁻¹. The external juvabione standard curves showed strong linear correlations between peak area and concentration (r²=1.0, Figure 1). Concentrations of injected samples varied to keep peak area/height within the range of our standard curve. All calibrations of standards and samples were performed in triplicate with an acetone blank between each set of sample injections.

Identification of compounds within samples was performed after integrating the curves and using the National Institute of Standards and Technology (NIST) mass spectra database (Wiley v.7) to compare our samples to those of the library. Juvabione peaks had retention times around 25 minutes for all samples where it was present, and contributed to most of the peak area on each chromatogram between 24 and 30 minutes for juvenile and mature wood samples. Peaks identified as juvabione or dehydrojuvabione were combined for further analysis and are herein referred to as juvabione related compounds (JRCs) as identified by Fowler (2001a). Bark samples contained many other compounds, not identified as juvabione or dehydrojuvabione, discussed herein.

The percentages as well as the absolute amounts of JRCs in the mature and juvenile wood of infested and healthy wood samples were compared using a Proc Glimmix in SAS Enterprise Guide (EG-v5.1 2012). Bark samples did not contain juvabione and were not included in these analysis. Percentage for juvabione was calculated using the standard curve
for each sample based on the known amount of extract injected into the GC-MS. Percentages for juvabione and dehyrdrojuvabione were combined for total JRC. The µg amount of JRCs present per gram wood sample was calculated by first, dividing the amount of extract obtained by the amount of wood sampled (extract yield) and conversion to µg resulting in extract yield. Then, extract yield was multiplied by the percent JRC to calculate the µg JRC present per gram of wood sampled.

The solvent front in bark sample chromatograms eluted between 0-6 minutes. Between 41 and 55 peaks were identified for each bark sample chromatogram after autointegration using the Chemstation software. Peaks that eluted during the first 6-20 minutes included 5-12 peaks and accounted for 6-8% of the total peak area for peaks eluting between 6 and 30 minutes. Bark sample peaks that eluted between 20 and 27 minutes accounted for 7-22% of the total peak area for peaks eluting between 6 and 30 minutes and included 20-32 peaks. From 27-30 minutes 5-12 peaks occurred which accounted for 34-88% of the total peak area for peaks eluting between 6 and 30 minutes, however many of these peaks occurred outside the measureable range of the detector. Peaks that eluted between 20 and 27 minutes were selected for further analysis because their masses would be most similar to juvabione and were expected to include defense compounds similar in size to juvabione.

In order to further narrow down the 20-32 compounds identified in the bark sample, peaks that eluted between 20 and 27 minutes were compared by subtracting chromatogram peak heights of infested bark samples from those of healthy trees. Differences between peak heights were ranked from highest to lowest and those in the top one percent and bottom ten percent were selected for further analysis (Figure 2). The top one percent are those
compounds constitutively higher in healthy trees which may provide initial defense against BWA attack or are down-regulated in infested samples, although the latter is harder to prove. Negative values indicate those samples which were higher in infested samples than healthy samples and may be up-regulated in response to BWA infestation. In order to examine these changes caused by BWA, a larger proportion of negative peak height differences were examined by analyzing the bottom ten percent. Differences between healthy and infested peak heights were calculated as well as the standard deviation for peak retention time, peak area, and percent area. A literature search was conducted for each compound to determine possible functions.

Insects for Bioassays:

Mealworms (*Tenebrio molitor*) were obtained from Timberline Mealworm Company, Marion IL 62959 to start a colony. They were reared on a diet of homogenized whole wheat flour (125 g), wheat germ (70 g), and brewer’s yeast (5 g) (Cohen 2011). Mealworms were housed in plastic containers in an incubator set at 27 °C (20-26 °C) with RH 20-60% and 24 hour dark cycle.

Milkweed bugs (*Oncopeltus fasciatus*) were obtained from Carolina Biological Supply, Burlington, NC 27215 to start a colony. They were reared on organic sunflower seeds and water *ad libitum* in an incubator set at 25 °C (21-25 °C) with RH 40-60% and 13:11 L:D cycle.

Green peach aphids (*Myzus persicae*) were obtained from a lab colony at NCSU (Travis McClure, Steve Frank Lab). They were reared on black pearl pepper (*Capsicum*...
*annuum*) plants in an incubator set at 25 °C (21-25 °C) with RH 40-60% and 13:11 L:D cycle.

Bioassays:

Procedures outlined in Bowers (1966, 1968) were used to provide a framework for the bioassay. Topical applications of extract (10 μL) dissolved in acetone were applied to mealworms, milkweed bugs, and green peach aphids within 24-48 hours of molting to their final nymphal instar. Samples were dissolved in 10 mL, 5 mL, 3 mL, or 2 mL aliquots of acetone to vary the amount of extract applied to each insect. As a positive control we applied 10 μL of 1.2% methoprene, a commercially available insect growth regulator, to groups of insects, as well as juvabione. Negative controls included an acetone only treatment and another control group where nothing was applied. We then recorded the number of insects that molted normally for each treatment as well as those that died, underwent abnormal metamorphosis, or supernumerary molts. Insects that failed to molt, underwent abnormal metamorphosis, or took longer than the average time to molt were considered to suffer from treatment effects. More than 80% of controls pupated within 11 days +/- 2.67 (13 days) so those whose pupation times were greater than 13 days were considered affected by treatment. Using these parameters we calculated half maximal inhibitory concentration (IC₅₀) as oppose to the typical lethal dose (LD₅₀), as we were interested in the effectiveness of the substance in preventing or delaying normal growth and development of the test insects. Insects from each treatment that did molt into normal looking adults were combined per treatment and allowed to lay eggs for 10-20 days before we counted the number of total offspring produced.
We evaluated the concentration of extract applied to each insect as well as the juvabione using the standard curves from the GC-MS; log transformed the concentrations, and calculated the IC$_{50}$ by plotting the log transformed concentrations by the percent mortality, transformed by the normalized expected distribution. The same functions were performed for the bark samples using total extract concentrations and juvabione standards. An F-test was performed to examine the fit of the IC$_{50}$ model based on five concentrations tested for each treatment (Table 2). We compared the effects of petroleum ether extracts applied to mealworms to negative and positive controls containing nothing (i.e., no application of solvent), acetone only, or methoprene (1.2 %) using an ANOVA with a Tukey-Kramer HSD accounting for both wood type and infestation level.

RESULTS:

Petroleum ether extracts of juvenile wood, and mature wood contained both juvabione and dehydrojuvabione, but no JRCs were identified in the petroleum ether extracts of the bark (Figure 3). There were significant differences in percent JRCs between wood sections, with juvenile wood having a much higher percent JRC (F=6.46, df=1,16, p=0.0218). There were not significant differences between infestation levels (F=2.66, df=1,16, p=0.1223), although infested samples consistently contained higher percentages of JRCs than healthy samples. The infested juvenile wood extract had the highest percent of JRCs compared to mature wood (t=4.53, df=15, p=0.0004). Healthy juvenile wood extracts contained the second highest percent JRC, which were different from healthy mature wood, but not infested juvenile or mature wood samples (t=2.14, df=15, p=0.0492). Infested mature wood
(t=1.01, df=15, p=0.3304) and healthy mature wood (t=0.29, df=15, p=0.7721) extracts had the lowest percent JRC, respectively, and were not different by infestation level (Table 3).

Bark extracts contained a variety of compounds. Eight peaks were examined using the methods described. Chemicals which were more abundant in healthy bark samples, the top one percent of those differentially abundant between healthy and infested trees, i.e. defensive chemicals at higher concentration in healthy trees, included three peaks which accounted for a small portion (ca. 10 %) of the total peak area for peaks eluting between 6 and 30 minutes. Extracts that were more abundant in infested bark samples, the bottom ten percent, included five peaks, and comprised one-third (ca. 30%) of the total peak area for peaks eluting between 6 and 30 minutes. The largest differences occurred between healthy and infested sample peaks four and seven (Table 3).

Manoyl oxide [4], forskolin [6], and salviaalane/oxazolidinedione [8] were found at higher concentrations in healthy trees than infested trees. 2,5-cyclohexadien-1-one, 4-methyl-4-propyl- [1], scleral [2], abietane [3], 18-norabeta-8,11,13-trien-4-ol [5], and sclareolide [6] were found at higher concentrations in infested Fraser fir bark compared to healthy bark. (Table 3): Compounds are referred to by their peak number [in brackets] throughout the rest of the text.

The petroleum ether extracts were tested in a bioassay using newly molted pupae of mealworms (Coleoptera: *Tenebrio molitor*), pre-metamorphic aphids (Hemiptera: Homoptera: *Myzus persicae*), and milkweed bugs (Hemiptera: *Oncopeltus fasciatus*) in order to assess the biological activity of these extracts and efficacy as a defense mechanism.
In experiments performed by Mester (2012), aphids in all treatments, except controls, fell off leaves and died within seven days of being treated. Aphid feeding was also examined using extracts from healthy and infested trees (Erin Mester, personal communication). Aphid feeding was apparent by the production of honeydew. When added to aphid diets, infested wood and healthy bark extracts increased aphid feeding, however, reproduction appeared to be delayed in these treatments. These results imply that healthy wood and infested bark extracts may contain compounds that affect feeding in aphids (Mester 2013).

Milkweed bugs treated with methoprene (n=20) died after 7 days without molting into adults. Milkweed bugs treated with extracts of juvenile wood (n=20), mature wood (n=20), and bark (n=20) did not molt into adults after 7 days, but containers became infected with mold around day 9, after several had molted into adults. There is no way to determine if the insects died from the mold exposure, or treatments. Since mold appeared near the same time that the milkweed bugs were molting into their final instar the experiment was corrupted so these data were not analyzed any further. Transferring the milkweed bugs to smaller containers for the experiment resulted in mold growth. Control insects in the colony showed no signs of mold growth.

Under the generalized linear model testing for treatment on mealworms (methoprene, bark, juvenile wood, etc.) and infestation level effects, there was no statistical support for infestation level effects ($X^2=0.6$, df=1, p=0.4382), but there was a significant treatment effect ($X^2=63.53$, df=6, p<0.0001). Methoprene and juvabione standard control treatments had the highest mortality followed by juvenile wood, bark, mature wood, respectively, with acetone, and control treatments having the lowest mortality (Table 5.)
The IC₅₀ for inhibition of molting in mealworms for total extract applied was 72 µg for samples containing JRCs, and 1 µg for bark samples. When percent juvabione was calculated for total extract, the IC₅₀ for inhibition of molting in mealworms was 23 µg for those samples containing JRCs. The standard juvabione used produced an IC₅₀ of 5 µg for inhibiting complete metamorphosis when applied to pupal mealworms. Dosages were not varied for methoprene, so an IC₅₀ was not calculated. In general, inhibition of molting from pupae to adult in mealworms treated with methoprene, juvabione, juvenile wood, mature wood, and bark did occur. Supernumerary molts into adults with undeveloped abdomens were also observed (Figure 6).

**DISCUSSION:**

These studies show the concentration of JRC is higher in BWA infested juvenile wood than any other type of wood or bark, and differences between trees infested with BWA and healthy trees varied in magnitude by wood type (e.g. concentration juvabione in infested juvenile wood is two times that of healthy juvenile wood, but juvabione in mature wood is nearly four times higher in infested trees). When calculating juvabione concentration (µg g⁻¹), my results were similar to those observed by Fowler (1999), in that juvabione concentrations were higher in infested wood than healthy wood for both juvenile and mature wood sections.

In order to assess potential for resistance between *Abies* species based on JRCs, controlled experiments involving potentially resistant species need to be performed. However, Fowler (1999) and others studying juvabione in *Abies* (Bowers 1966, Manville 1974, Puritch and Nijholt 1974, Manville 1975) were able to extract JRCs from either whole
wood (containing both juvenile and mature wood), or mature wood only with varied results within trees and between species. It was shown that *A. fraseri* may contain the most JRC, and that younger trees may contain more JRC than older trees. In review of the literature, there is wide variation in JRC concentration for Fraser fir, as with other species (Table 6.)

In examining the role of JRCs and other defense compounds found in the bark of Fraser fir, the following hypotheses, which are not mutually exclusive, regarding defense of *Abies* against BWA are proposed: 1) juvabione is a defense evolved to combat fungi and associated bark beetles so production is limited to inner wood tissues where it would be most effective against larval insects and fungi, 2) juvabione synthesis occurs in the juvenile wood and transport to the bark and mature wood sections is hindered by rotholz caused by the adelgid infestation, 3) BWA produce enzymes that combat the production of juvabione and other defense compounds at the feeding site.

Juvabione has been studied extensively for its activity (Bowers 1966, Manville 1974, Staal 1975, Yoneyama et al. 2013), presence in *Abies* (Manville 1974, Puritch and Nijholt 1974, Manville 1975, 1976, Manville et al. 1977, Manville and Kriz 1977), its synthesis (Ficini et al. 1974, Negishi and Sabanski 1976, Miles and Brinkman 1992), and somewhat to its relation to BWA (Puritch and Nijholt 1974, Fowler 1999, Fowler et al. 2001a); however, its function within the tree, and physiology are poorly understood. Our work indicates the main site for juvabione production is limited to the juvenile wood. We hypothesize this is due to its specific role against pathogenic fungi (Yang et al. 2008), and furthermore its transport to mature wood may be hindered by rotholz induced by BWA feeding.
Juvabione biosynthesis begins with the mevalonate pathway, in the cytosol, where sesquiterpenes are formed (Trapp and Croteau 2001). From this pathway farnesyl diphosphate (FPP) is formed which can be turned into a variety of sesquiterpene compounds. Conversion of FPP to (E)-α-bisabolene two reactions achieved by generation of nerolyldyl diphosphate (NPP) after the terminal diphosphate is moved, followed by a bisabolene synthase directed reaction resulting in the formation of the six-member ring (Bohlmann et al. 1998b). One possibility for lower juvabione concentrations in mature wood compared to juvenile wood is that BWA introduce digestive enzymes into the cytosol, such as peroxidase and polyphenol oxidase which reduce and oxidize thiols and phenols present in the mevalonate pathway.

It is not known how (E)-α-bisabolene is converted to juvabione, but similarity in structure and inducible production of juvabione via this pathway indicate it is the only precursor (Figure 7). Bisabolene synthase is unique in Abies in that its only product is (E)-α-bisabolene and its production is inducible by stem wounding. Other sesquiterpenoid synthases are constitutive and produce a variety of different sesquiterpenes (Bohlmann et al. 1998a, Steele et al. 1998). Another unique feature, is that juvabione/todomatuic acid appear to be the only products of (E)-α-bisabolene and the increased production of juvabione/todomatuic acid have been shown to be induced responses to BWA (Puritch and Nijholt 1974) and fungal attack (Yoneyama et al. 2013) in Abies. The mevalonate pathway and subsequent production of juvabione is highly specific compared to other defense compounds. The metabolic cost to produce secondary metabolites, such as juvabione, is relatively high (Gershenzon 1994), supporting the second hypothesis, that infested trees
invest in juvabione defense by synthesizing large amounts of JRC, but it is not transported to
the site of attack.

Here we show that extracts from bark and juvenile wood inhibit metamorphosis of
mealworms from the final instar at similar levels, despite juvenile wood containing high
levels of JRCs. Juvabione has been shown to exhibit high levels of anti-fungal activity
(inhibits fungal growth) as well as fungicidal (kills fungi) activity (Yang et al. 2008,
Yoneyama et al. 2013). It has also been shown to increase in response to fungal attack (Raffa
and Smalley 1995). Interactions with BWA have shown trends toward increased juvabione in
infested fir trees (Puritch and Nijholt 1974, Fowler 1999), and juvabione has been shown to
work as an effective insect growth regulator (IGR) on mealworms (Tenebrio molitor) and
Pyrrhocoris apterus (Bowers 1966, 1968) while also affecting the fecundity of BWA
(Fowler 1999) and other insects (increase: (Garcia and Furtado 1980) decrease/increase:
(Rogers et al. 1974). Our results indicate JRC found in Fraser fir do inhibit metamorphosis,
but not at any higher levels than compounds found in bark extract, and not as well as other
IGRs such as methoprene, or pure juvabione.

An interesting finding is the difference between the IC$_{50}$ obtained from pure juvabione
compared to the petroleum ether extracts, and the calculated JRC for those extracts. The IC$_{50}$
for the petroleum ether extracts was more than 14 times that of the juvabione standard, and
when calculating JRC for those extracts the juvabione standard still had an IC$_{50}$ more than
four times greater than Fraser fir wood extracts. These results indicate there may be other
compounds present working as antagonists to the juvenile hormone mimicking activity. The
bark IC$_{50}$ was very low, indicating high insecticidal activity.
Juvabione concentration was higher in the juvenile wood than in the mature wood regardless of infestation level. No juvabione was found in the bark nor were any precursors involved in its synthesis, indicating that it is synthesized in the juvenile wood. The amount of juvabione (µg) in the infested mature wood was not different from that of healthy mature wood, indicating that even though concentrations increased in the juvenile wood in response to infestation, they were not transported to the mature wood, possibly due to the production of rotholz. Previous studies have shown rotholz to affect translocation of materials throughout the wood of Fraser fir in response to BWA infestation (Mitchell 1967b, Mitchell 1967a, Puritch 1971, Puritch and Mullick 1975). Rotholz production is also correlated to BWA density in A. fraseri, but not in A. alba (Hollingsworth and Hain 1992), and differences in water potential occur between infested and healthy trees (Arthur and Hain 1986). Mitchell (1966) studied several species of Abies infested with BWA and found variation in severity of response to BWA attack. This suggests that there may be genetic potential for breeding resistance to BWA with species that do not produce rotholz.

Juvenile wood has also been shown to have lower relative water conductivity (Phillips et al 1996). Juvabione is fairly soluble in water with an estimated Log Kow of 4.22 (values less than 10 tend to be hydrophyllic) (Leo et al. 1971). Low sapflow coupled with the production of rotholz may have inhibited transport of juvabione from juvenile wood to mature wood in BWA infested trees. Unfortunately, little is known about the transport of sesquiterpenes defense compounds in conifers, even though advances have been made in this area of research (Keeling and Smith 2006).
Bark extracts did inhibit pupation of mealworms, and the IC$_{50}$ was very low (1.2 µg), indicating a high specificity for insect defense. When examining the possible functions of the compounds present in the bark, only compounds [1] and [4] were related to those with insecticidal activity (Lajide et al. 1996, Petrakis et al. 2005, Ybarra et al. 2005). Compounds [2], [4], [5], [7], and [8] have been reported as having antimicrobial or antifungal activity (Cutler et al. 1977, Ankli et al. 2000, Jasiński et al. 2001, da Silva et al. 2008, Ugur et al. 2010, Seo et al. 2012, Maskovic et al. 2013). Compounds [3] and [6] may be responsible for regulation of plant growth and defense signal transduction (Alkio et al. 2005, Jiang et al. 2005, Hamberger et al. 2011). Compounds [2] and [4] may also have antioxidant activities (Maskovic et al. 2013) associated with them. The antimicrobial activity of compounds present in the bark may account for its high insecticidal activity if it removes beneficial bacteria or symbiotic fungi from the feeding substrate and/or gut of the insect.

Compound [1]: 2,5-Cyclohexadien-1-one, 4-methyl-4-propyl-

Infested Fraser fir contained relatively higher amounts of [1] than healthy trees indicating a positive insecticidal response to BWA infestation. Compound [1] is similar in structure to a known insect insecticide, jacaranone (figure 8), which has been extracted from several plant species, and is shown to inhibit larval growth (Lajide et al 1996), aid in thrips resistance (Leiss et al 2009), act as an insect anti-feedant, and insecticide (Xu et al 2003). Jacaranone is also active against several lines of human cancer cells including melanoma (Wang et al 2010, massaoka et al 2012) as well as promastigotes responsible for Leishmaniasis, Chagas, and malarial diseases (Morais et al, 2012). (for review see: Gachet and Schuhly 2009). Typical hypersensitive response in plants involves programmed cell
death (apoptosis) around the feeding site to restrict movement of nutrients to the invader and begin the process of forming a barrier between the insect/pathogen and healthy plant tissue. Jacaranone activity against cancer cells may be due to involvement in the process of programmed cell death. These findings indicate that Fraser fir is responding to BWA attack with some success and efficacy of this chemical on BWA mortality should be examined.

Compounds [2],[7]: Labdanes

Both [2] and [7] are relatively more abundant in BWA infested Fraser fir than healthy trees indicating that the trees might be perceiving BWA infestation similar to that of a fungal or bacterial infection, or combating symbiotic bacteria and/or fungi associated with BWA. Many diterpenoids possess the labdane skeleton and have been identified in several plant families, including Pinaceae. Sclareol is an antifungal, antibacterial, plant growth inhibitor, and plant diterpene with a labdane skeleton (Costas and Dimas 2001, Figure 9). Sclareolid lactol has been identified as a genetic signaling defense related molecule, and is used to induce plant defense regulating the ATP-binding cassette (ABC) transporters and activating defense related compounds through mitogen-activated protein kinase (MAPK) systems (Seo et al 2012). Presence of this compound in Fraser fir infested with BWA demonstrate its ability to detect the insect and initiate defense responses. However, survival of the insect, and subsequent tree death indicate the response is not fast enough, or downstream defensive chemicals that would be effective against BWA are disrupted.

Compounds [3], [4], [5], [6]: Abietanes

Abietanes are a diterpene resin acid with a phananthrene skeleton comprised of three benzene rings with a dimethyl group in the first position, a methyl group on the 5th carbon,
and a trimethyl group on the 7th position. Abietanes are part of the inducible defense system in plants activating systemic acquired resistance (SAR) in plants (Chaturvedi et al. 2012). They are responsible for inducing hypersensitive response as well as possessing antimicrobial activity and insecticidal activity (Bonito et al 2011, Dempsey et al 2012, Chaturvedi et al 2012, Kusmoto et al 2010, Radulovic et al 2010). Abietane is responsible for plant hypersensitive response which is a defense mechanism used by plants to disrupt nutrient flow to infected areas through the production of necrotic tissue and imbuing defensive chemicals to the area of infection (Franceschi et al. 2002, Hudgins et al. 2004, Alkio et al. 2005). By producing necrotic periderm preceded by impervious tissue, the plant is able to wall off an infection to prevent its spread (Fernandez 1990).

Interestingly both [4] and [6], compounds responsible for insect resistance were relatively higher in healthy trees than infested trees indicating that BWA may be affecting the production of these chemicals in support of the 3rd hypothesis. Compounds [4] and [6] are similar to other norditerpenoids, which have been shown to have direct insecticidal activity in Spodoptera frugiperda when extracted from Grindelia scorzonerifolia (Ybarra et al 2005). Compound [6] is also very similar to forskolin which may be responsible for activating the MAPK cascades transcriptionally regulating the phenylalanine ammonia-lyase (PAL). PAL is the initial step in instigating production of defense compounds via the phenylpropanoid pathway and is regulated by MAPK and H2O2 (Allwood et al 2002). PAL1 has been shown to be down regulated in Fraser fir infested with BWA (Emerson, 2010), and may be inhibited by BWA feeding activity (see enzyme chapter). Compound [5] on the other hand is relatively higher in infested Fraser fir compared to healthy trees. This compound shares a similar
structure to those responsible for antimicrobial activity (Ankli et al 2000, Dakir et al 2005). In further analysis of BWA feeding enzymes we show that pectinase disrupts the production of phenylalanine ammonia lyase (PAL) an important enzyme responsible for initiating the phenylpropanoid pathway. The down regulation of these compounds support the 3rd hypothesis and other findings regarding the role of BWA trophically related enzymes discussed in the previous chapter.

Compound [8]: Salvialane/Oxazolidinedione

Compound [8] was relatively more abundant in healthy Fraser fir bark extract than in extract from BWA infested trees indicating this compound is negatively affected by BWA infestation. Oxazolidinedione is similar to the bark extract compound [8] and may act similarly as a fungicide (Hoveland et al 1996). Oxazolidinedione is also well known as an anticonvulsant and GABA agonist. GABA antagonists are part of the group 2 class of insecticides which act on GABA-gated ion channels (IRAC 2013) and compound [8] may act similarly by disrupting GABA-gated ion channels in Fraser fir. Salvialane is also similar to [8] and has been shown as a possible antimicrobial agent active against bacteria, yeast, and other fungi (Kuzma et al 2009, Bonito et al 2011). (Figure 11) Depending on the route of biosynthesis within Fraser fir, the down regulation of compound [8] by BWA may be related to enzymes introduced by the insect during feeding as discussed previously.

**CONCLUSIONS:**

BWA affect Fraser fir chemistry in interesting ways. These results support three hypotheses regarding Fraser fir reaction to BWA infestation. Combined, these hypotheses form what we call the ‘confused defense hypothesis’ wherein Fraser fir perceive BWA attack
the same as a fungal elicitor. In doing so, Fraser fir increases defense compounds responsible for antimicrobial defense, while down-regulating (either self-induced or BWA-influenced) insect defensive compounds. The increase in antimicrobial compounds may also affect BWA symbionts.

These results show that the concentration of juvabione increases from 17 % to 36 % in juvenile wood extract where it is highest, and from 2-8 % in mature wood extracts, for a two and four fold increase in the concentration of juvabione present in petroleum ether extracts of healthy Fraser fir compared to those infested with BWA. The transport of juvabione may be hindered by differences in conductivity between juvenile and mature wood as well as rotholz production. Juvabione is most likely an antifungal compound based on its activity (Yoneyama et al 2013) and increase in response to fungal attack has been shown in other studies (Raffa and Smalley 1995).

In BWA-infested Fraser fir, more defensive chemicals were observed in this study that relate to fungal defense than those known (or shown) to be responsible for insect defense. As part of our proposed ‘confused defense hypothesis’ the ability of Fraser fir to produce insecticidal compounds may be hindered by BWA feeding enzymes via down-regulation the mevalonate pathway, PAL1 and the phenylpropanoid pathway. Other defense pathways related to fungal defense should be studied in Fraser fir infested with BWA.

Future investigations should focus on other fir species with known resistance to BWA to compare chemical defensive compounds produced in response to BWA attack. Tests involving insect and fungi specific elicitors should be tested on Fraser fir to determine how it perceives BWA infestations. More assays should be conducted to test the specificity of the
compounds found in Fraser fir for relevant biological activity. Fraser fir resistance screening should involve fungal elicitors in comparison to BWA to see if the responses are similar.

LITERATURE CITED:


Fowler, G. 1999. The potential of juvabione as a host resistance mechanism against the balsam woolly adelgid (Adelges piceae [Ratzeburg]). North Carolina State University.


Mitchell, R. G. 1967b. Abnormal ray tissue in 3 true firs infested by the balsam woolly aphid. Forest Science 13: 327-&.


REFERENCES FOR TABLE 4:


(2) Maskovic, P.; Radojkovic, M.; Ristic, M.; Solujic, S. Natural Products Communications 2013, 8. 667-670.


Table 1. Wood disk sample summary from healthy and infested Fraser fir.

<table>
<thead>
<tr>
<th>Infestation level</th>
<th>Tree ID</th>
<th># of Rings with Rhotholz</th>
<th>Rings (tree age)</th>
<th>Diameter (cm)</th>
<th>Height (cm)</th>
<th>Dry Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infested</td>
<td>1</td>
<td>3</td>
<td>10</td>
<td>9.5</td>
<td>2.8</td>
<td>77.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>10</td>
<td>9.5</td>
<td>2.6</td>
<td>77.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>9.7</td>
<td>3</td>
<td>113.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>9</td>
<td>6.9</td>
<td>3.1</td>
<td>66.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>9</td>
<td>6.6</td>
<td>3</td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
<td>9</td>
<td>6.4</td>
<td>3</td>
<td>52.3</td>
</tr>
<tr>
<td>Healthy</td>
<td>7</td>
<td>0</td>
<td>9</td>
<td>8.1</td>
<td>3</td>
<td>61.2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>9</td>
<td>8.5</td>
<td>3</td>
<td>70.3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0</td>
<td>11</td>
<td>8</td>
<td>2.2</td>
<td>43.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>8</td>
<td>6.4</td>
<td>2.5</td>
<td>29.106</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0</td>
<td>8</td>
<td>6.4</td>
<td>2.5</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0</td>
<td>9</td>
<td>6.4</td>
<td>3.5</td>
<td>41.648</td>
</tr>
<tr>
<td>Average</td>
<td>n/a</td>
<td>2.67*</td>
<td>9.16667</td>
<td>7.68333</td>
<td>2.85</td>
<td>59.7962</td>
</tr>
<tr>
<td>±stdev</td>
<td>n/a</td>
<td>0.51</td>
<td>0.83485</td>
<td>1.36637</td>
<td>0.34772</td>
<td>23.6148</td>
</tr>
</tbody>
</table>

*Infested bolts only
Figure 1. Juvabione standard curve for samples 3, 5, 6, 11, and 12 with standard deviation indicated by vertical bars.
Figure 2. Chromatogram for healthy minus the infested bark sample between 20 and 27 minutes. Values displayed correspond to the top one (black line) and bottom ten percent (red line) of peaks.
Table 2. Petroleum ether extracts of Fraser fir EC$_{50}$ for mealworm pupae.

<table>
<thead>
<tr>
<th>Sample</th>
<th>µg applied</th>
<th>µg EC$_{50}$ dose</th>
<th>$r^2$</th>
<th>F-ratio</th>
<th>n</th>
<th>df</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile and Mature wood</td>
<td>2-329</td>
<td>72.416</td>
<td>0.769</td>
<td>9.966</td>
<td>113</td>
<td>3,4</td>
<td>0.051</td>
</tr>
<tr>
<td>Bark</td>
<td>0.13-2.4</td>
<td>1.243</td>
<td>0.700</td>
<td>7.012</td>
<td>63</td>
<td>3,4</td>
<td>0.077</td>
</tr>
<tr>
<td>Calculated juvabione</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile and Mature wood</td>
<td>0.28-77.3</td>
<td>22.868</td>
<td>0.635</td>
<td>5.225</td>
<td>113</td>
<td>3,4</td>
<td>0.106</td>
</tr>
<tr>
<td>Juvabione standard</td>
<td>0-120</td>
<td>5.106</td>
<td>0.665</td>
<td>5.956</td>
<td>141</td>
<td>3,4</td>
<td>0.093</td>
</tr>
</tbody>
</table>

* Probability that the linear model is a good fit based on the F-test, significance based on $\alpha=0.10$. 
Figure 3. Chromatograms for infested juvenile wood, mature wood, and bark from one of the trees sampled, the gray line indicates the external juvabione standard at 60.04ng/ul.
Table 3. Mean percent and µg/g JRCs from petroleum ether extracts calculated per juvenile and mature wood samples compared between infested and healthy trees.

| Wood Section | Infested | Mean % JRC\(^a\) ±Standard error | LS-means* | Pr > |t|  | Juv/wood\(^b\) (µg/g) ±Standard error | LS-means* |
|--------------|----------|----------------------------------|-----------|------|----|-----------------------------|-----------|
| Juvenile wood | Yes      | 36.1363 ± 7.974                   | A         | 0.0004 | 2161.02 ± 206.04 | A         |
|               | No       | 17.067 ± 7.974                    | AB        | 0.0492 | 361.79 ± 206.04  | B         |
| Mature wood   | Yes      | 8.9678 ± 8.9152                   | B         | 0.3304 | 146.8 ± 206.04    | B         |
|               | No       | 2.3514 ± 7.974                    | B         | 0.7721 | 148.39 ± 206.04   | B         |

\*LS-means with the same letter are not significantly different at α=0.05.

\(^a\) %Juvabione includes peaks identified as juvabione and dehydrojuvabione.

\(^b\) Calculated weight of juvabione extracted/dry weight of wood.
Table 4. Defensive compounds differentially abundant in the bark of infested and healthy Fraser fir.

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Peak</th>
<th>RT (average stdev)</th>
<th>PH Healthy</th>
<th>PH Infested</th>
<th>PH difference</th>
<th>Area % (average stdev)</th>
<th>Possible Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5-Cyclohexadien-1-one, 4-methyl-4-propyl-</td>
<td>1</td>
<td>21.86</td>
<td>2.76x10^5</td>
<td>2.25x10^5</td>
<td>-5.11x10^4</td>
<td>0.32</td>
<td>Insect Growth Regulator(1)</td>
</tr>
<tr>
<td>Sclaral (Sclareolide lactol)</td>
<td>2</td>
<td>23.98</td>
<td>1.26x10^6</td>
<td>1.52x10^6</td>
<td>-2.6x10^5</td>
<td>5.37</td>
<td>Antimicrobial, Antifungal, Antioxidant(2)</td>
</tr>
<tr>
<td>Diterpene resin acid (abietane)</td>
<td>3</td>
<td>24.66</td>
<td>5.73x10^5</td>
<td>7.44x10^5</td>
<td>-1.71x10^5</td>
<td>1.21</td>
<td>Plant defense diterpenoid(4, 3), Plant growth inhibitor, Hypersensitive response(5)</td>
</tr>
<tr>
<td>(+)- Manoyl oxide</td>
<td>4</td>
<td>24.80</td>
<td>3.46x10^6</td>
<td>1.85x10^6</td>
<td>1.61x10^6</td>
<td>6.39</td>
<td>Antioxidant(6), Antifungal(8, 7), Antimicrobial(9), Insecticidal(10), Anti-feedant(11)</td>
</tr>
<tr>
<td>18-Norabieta-8,11,13-trien-4-ol</td>
<td>5</td>
<td>25.07</td>
<td>8.67x10^5</td>
<td>9.11x10^5</td>
<td>-4.42x10^4</td>
<td>2.17</td>
<td>Diterpenoid, Antibacterial(12, 13)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>6</td>
<td>25.29</td>
<td>1.11x10^6</td>
<td>6.96x10^5</td>
<td>4.1x10^5</td>
<td>3.12</td>
<td>Regulates SA/AC/PAL inducible defense against fungi(16, 15, 14)</td>
</tr>
<tr>
<td>Sclareolide</td>
<td>7</td>
<td>26.59</td>
<td>4.26x10^6</td>
<td>5.04x10^6</td>
<td>-7.76x10^5</td>
<td>19.66</td>
<td>Antifungal defense(17, 18), Antibacterial defense(19)</td>
</tr>
<tr>
<td>Salvialane / Oxazolidinedione</td>
<td>8</td>
<td>26.65</td>
<td>1.16x10^6</td>
<td>7.67x10^5</td>
<td>3.96x10^5</td>
<td>0.74</td>
<td>Antibacterial, Antifungal(23, 20, 21, 25, 24, 22)</td>
</tr>
</tbody>
</table>

* = Peak numbers are associated with chromatogram figure 5, RT = Retention time, PH = Peak height, a,b = Largest differences between healthy and infested bark samples. Stdev between Healthy-infested PH were <0.0001.
Figure 4. Chromatogram of healthy and infested bark between 20-27min where 8 peaks were identified as being different between the two samples.
Table 5. Mean percent mortality of Mealworm pupae in juvabione bioassay, compared using a Tukey-Kramer HSD.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean % Mortality</th>
<th>Std Dev</th>
<th>mealworms treated (n)</th>
<th>Means comparisons*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoprene</td>
<td>96.2%</td>
<td>19.6%</td>
<td>75</td>
<td>A</td>
</tr>
<tr>
<td>Juvabione</td>
<td>49.4%</td>
<td>33.0%</td>
<td>86</td>
<td>B</td>
</tr>
<tr>
<td>Juvenile wood</td>
<td>35.8%</td>
<td>42.7%</td>
<td>61</td>
<td>BC</td>
</tr>
<tr>
<td>Bark</td>
<td>28.2%</td>
<td>36.9%</td>
<td>63</td>
<td>BC</td>
</tr>
<tr>
<td>Mature wood</td>
<td>25.0%</td>
<td>38.4%</td>
<td>59</td>
<td>BC</td>
</tr>
<tr>
<td>Acetone</td>
<td>9.6%</td>
<td>26.4%</td>
<td>55</td>
<td>C</td>
</tr>
<tr>
<td>Control</td>
<td>2.0%</td>
<td>4.7%</td>
<td>65</td>
<td>D</td>
</tr>
</tbody>
</table>

*Means with the same letter are not significantly different at $\alpha=0.05$
Table 6. Summary of findings from studies examining JRCs in Abies.

<table>
<thead>
<tr>
<th>Tree species</th>
<th>Tree age (Mean, stdev)</th>
<th>n</th>
<th>Infestation level</th>
<th>% JRC from petroleum ether solubles</th>
<th>ug/g juvabione</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. balsamea</td>
<td>66 17</td>
<td>6</td>
<td>Unknown</td>
<td>61-88</td>
<td>Not reported</td>
<td>Manville, 1975</td>
</tr>
<tr>
<td>A. grandis</td>
<td>66 64</td>
<td>45</td>
<td>Nil-Heavy</td>
<td>not reported</td>
<td>0-80</td>
<td>Puritch and Nijholt, 1974</td>
</tr>
<tr>
<td>A. amablis</td>
<td>53 55</td>
<td>19</td>
<td>Nil-Heavy</td>
<td>not reported</td>
<td>0-10</td>
<td>Manville and Kriz, 1977</td>
</tr>
<tr>
<td>A. lasiocarpa</td>
<td>111 21</td>
<td>9</td>
<td>Unknown</td>
<td>54-80</td>
<td>Not reported</td>
<td>Manville and Kriz, 1977</td>
</tr>
<tr>
<td>A. fraseri</td>
<td>8</td>
<td>10</td>
<td>Healthy</td>
<td>not reported</td>
<td>30-138</td>
<td>Fowler, 2001</td>
</tr>
<tr>
<td>A. bornmulleriana</td>
<td>not reported</td>
<td>1</td>
<td>Healthy</td>
<td>2.6-3.9</td>
<td>76-570</td>
<td>Willfor et al, 2007</td>
</tr>
<tr>
<td>A. cilicia</td>
<td>1</td>
<td>1</td>
<td>Healthy</td>
<td>2.6-3.9</td>
<td>28-4200</td>
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<tr>
<td>A. fraseri</td>
<td>9</td>
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<td></td>
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<td>Infested</td>
<td>6-28</td>
<td>0-3402</td>
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</table>
Figure 5. Showing *Tenebrio molitor* that did not complete the final molt (left), underwent an incomplete molt (right), and successfully molted (bottom right).
Figure 6. Mevalonate pathway and conversion of FPP to (E)-α-bisabolene in the production of juvabione (reproduced with permission (Bohlmann et al. 1998b)).
Figure 7. Comparison in structure of compound [1] extracted from the bark of Fraser fir to jacaranone.

Figure 8. Comparison in structure of labdanes extracted from Fraser fir ([2],[7]) and Sclareol.
Figure 9. Comparison in structure of abietane and those extracted from Fraser fir ([3], [4], [5], [6]).
Figure 10. Structures of [8] salvialane skeleton and oxazolidinedione
CHAPTER IV:

DIFFERENCES IN FRASER AND TURKISH FIR TOTAL SOLUBLE CARBOHYDRATES, LIPIDS, PHENOLICS, AND XYLEM RAY PARENCHYMA ANATOMY AFTER BWA INFESTATION

ABSTRACT:

Fraser (Abies fraseri) and Turkish (A. bornmuelleriana) fir are both susceptible to infestation by the balsam woolly adelgid (Adelges piceae, BWA). However, Turkish fir does not exhibit the same high levels of mortality observed in BWA infested Fraser fir indicating that Turkish fir is tolerant to this insect. Total soluble carbohydrates (TSC), lipids, and soluble phenolics (TSP) were compared between these two species at two different times during a controlled infestation with BWA. Samples were also taken from trees that were prepared for histological analysis and examined microscopically to compare xylem ray parenchyma (XRP) cell characteristics between species and over time. Lipids were significantly different between species and infestation level with higher lipid concentrations in Fraser fir and infested samples. There were positive correlations between TSC and TSP for both species not infested, but only infested Turkish fir maintained this relationship, while infested Fraser fir did not. More XRP cells were present in Turkish fir and positive correlations between XRP cells and lateral pits between cells existed in Fraser fir. Other correlations among anatomical characteristics for each species are discussed.

INTRODUCTION:

Complex combinations of tactile and chemical inputs determine selection of a host by an insect. For a sessile feeding insect like BWA to choose a host, the combination of nutrients must be ideal in order to ensure future generations. Phenol, carbohydrate, lipid, and

Xylem ray parenchyma (XRP) cells are an important site for storage of starch (Sauter and Cleve 1994), but are also unique due to their longevity (Nakaba et al. 2006) and important role in radial transport of nutrients (Bel 1990), biosynthesis of materials (Franceschi et al. 1998), and heartwood formation (Nakaba et al. 2006, Islam et al. 2012). In black locust starch is stored in parenchyma with peak accumulation occurring prior to the dormant season; this starch is metabolized during the winter (Yamada et al. 2011). From December to June starch decreased in storage cells and living wood fibers died after starch depletion, while parenchyma cells, which are long lived, did not (Yamada et al. 2011). Although the mechanism for cell death in parenchyma cells may be different than tracheids and other short-lived cells (Nakaba et al. 2006), starch depletion is observed prior to cell death and formation of heartwood (Islam et al. 2012). When BWA feed on Fraser fir tissue changes occur where formation of non-conductive tissue like compression wood forms called rotholz (Balch et al. 1964, Mitchell 1967, Hollingsworth and Hain 1994b) which may be
similar to heartwood formation controlled by XRP cells and influenced by starch levels as discussed in previous chapters.

Another key element to XRP and conifer defense are lipids. XRP are the primary storage site for starch granules, lipids, and glycolipids (Saranpää 1988). Glycolipids are an essential constituent of outer sapwood responsible for preventing cell lyses during freezing and thawing during the winter (Saranpää 1988, Sauter and Cleve 1994). The storage of lipids and starches in XRP cells provide insects with an ideal feeding site, but these constituents may also play a role in tree metabolism and activation of insect defense pathways (Franceschi et al. 2000, Franceschi et al. 2005, Nagy et al. 2006, Lahr and Krokene 2013).

Conifers are well known for the ability to produce phenolic compounds used in defense against insects and pathogens (Puritch 1977, Franceschi et al. 1998). Parenchyma cells play an important role in phenolic production and accumulation of phenolic compounds in response to insect and pathogen attack (Franceschi et al. 1998, Franceschi et al. 2000, Martin et al. 2002, Hudgins and Franceschi 2004, Franceschi et al. 2005). Polyphenolic parenchyma (PP) and XRP work in concert to produce and store phenolic compounds (Franceschi et al. 2000, Franceschi et al. 2002, Islam et al. 2012). XRP are integral in communication and nutrient transport between PP and phloem and cambium/xylem (Bel 1990, Sauter and Cleve 1994). Upon wounding by an insect or pathogen jasmonates and other elicitors activate the phenyl propanoid pathway (PAL) important in lignification defense, as well as phenolic response in PP cells (Franceschi et al. 1998). XRP produce signaling molecules for downstream regulation of phenolic production in resin ducts and PP cells while communicating with the xylem to produce traumatic resin ducts (TD) (Hudgins
and Franceschi 2004). Phenolics act directly on the insect either as a toxic substance, antifeedant, or hormone mimic, and some polyphenols and terpenoids are also used in the formation of defensive structures and lignification of cells walls (Franceschi et al. 2005, Cao et al. 2007).

Studies involving BWA have shown anatomical and chemical changes in TD, PP, and XRP cells. These studies have all shown changes in size and shape of XRP and PP with accumulation of TD. Differences between total monoterpenes in oleoresin have been observed between Fraser fir provenances, but no differences were observed between infested and uninfested trees. Most of these studies involved established Fraser fir populations which had been infested for some time. (Arthur and Hain 1985, 1986, 1987, Hollingsworth and Hain 1992, 1994a, b, Shepard et al. 1995, Sutton et al. 1997) One study involved artificial wounding and application of defense elicitors and found similar results with no differences in monoterpenes concentration between controls and treatments. Differences in specific monoterpenes existed between infested and uninfested trees existed at Mt. Rogers, but not the other two study sites (Arthur and Hain 1987), indicating a need to further study these phenomenon controlling for environmental and possible genetic effects.

We hypothesize that BWA infested Fraser and Turkish fir will differ in response with regard to TSC, lipid, and phenolics. Xylem ray parenchyma cell morphology is also an important indicator for Abies response, as swelling of these cells has been observed in response to BWA infestation (Balch et al. 1964). Comparing changes in these cell structures between a susceptible species like Fraser fir and a tolerant species like Turkish fir (Mitchell 1966, Hain 1988, Newton et al. 2009, 2010, Newton et al. 2011) should elucidate clues in
BWA resistance traits. We expect to see differences in the number of xylem ray parenchyma cells between species.

**METHODS:**

Plant material:

Fraser and Turkish fir seedlings were moved from transplant beds near Boone, NC to pots in a greenhouse where they were maintained for two years. Prior to the experiment trees were acclimated to incubators for 30 days. Incubators were set at 17ºC and 75% humidity with a 12:12 light/dark cycle. Temperature and humidity highs and lows were recorded daily for the duration of the experiment. Two incubators were used in the experiment with five trees per species in each incubator. Treatment trees were infested as described below, while control trees were not, and all other procedures were performed on both control and treatment trees. We infested trees in one incubator using the rain down technique (Newton et al. 2011). Control trees were protected in a separate incubator set at the same temperature and light settings. Briefly, a 60 cm section of BWA infested Fraser fir was suspended 60cm above the tops of the trees. Adelgid crawlers rained down onto the trees and the number of BWA falling were estimated by placing a sticky trap (73cmx5cm) along the tops of the trees. Adelgid counts were made after two weeks and at the end of the experiment.

Sample collection:

Prior to infestation two samples (10cm terminal branches) were taken from each tree to determine total soluble carbohydrates (TSC), lipids, phenolics (TSP), and microtomy for xylem ray parenchyma (XRP) cell analysis. After 21 days of the rain down technique and infestation with BWA a similar branch sample was taken for TSC, lipid, and TSP analysis,
while another branch sample and stem samples were taken to observe XRP characteristics of each tree.

Total Soluble Carbohydrates and lipids:

For the extraction of TSC and lipids we used a modified colorimetric phenol-sulfuric acid method (Dubois et al. 1956, Tissue and Wright 1995). Briefly, we dried plant material before grinding to a fine powder. We extracted the soluble sugars from the ground sample by washing it three times with 2mL methanol:chloroform:water (12:5:3 v/v) solution and centrifugation. The 6mL of supernatant was allowed to separate overnight incubated at 4°C in the dark. The top methanol:water fraction contained sugars and the chloroform fraction contained the lipids and pigments (Dickson 1979). 200µL was taken from the top fraction and reacted for 30 minutes with 800 µL H₂O, 1 mL phenol (5%), and 5mL H₂SO₄. A standard curve using a sucrose solution ranging from 20 to 200 µg was created in triplicate to compare absorbance spectra at 450nm to determine TSC quantities. Three absorbance readings were taken for each sample and standard curve sample and averaged. Buffer only absorbance readings were averaged and subtracted from tree and standard curve samples before calculating absolute sucrose quantities. %TSC was calculated by correcting sucrose values by the dilution factor and dividing by the original sample weight. Chloroform fractions were evaporated and dried lipids and pigments were weighed to determine quantities which were divided by sample weight for %lipid values.

Total Soluble Phenolics:

We used the Folin-Ciocalteu method as a colorometric analysis of total phenols. Variations in time, wavelength, and standard may change specificity of the test, however, it is
fairly robust, simple, and accurate in determining total phenols (Bueno et al. 2012). Briefly, we dried plant material before grinding to a fine powder. We extracted the soluble phenolics from the ground sample with 2mL methanol (95%) agitated for 48hr in the dark at 25ºC. Samples were centrifuged at 13,000 rpm for five minutes and the supernatant was extracted for analysis. 100 µL of the sample was diluted with 100 µL methanol (95%). A standard ranging from 0 to 50 µg protocatechuic acid was created in triplicate to quantify phenolic activity. Folin-Ciocalteu reagent (10%, F9252 Sigma-Aldrich) was added followed by 800 µL of Na$_2$CO$_3$ (700mM) to 20µL and incubated at 25ºC for one hour in complete darkness. After the incubation period absorbance at 760nm was read three times for each sample and standard. Buffer values were subtracted from sample and standard curve absorbance readings and the standard curve was used to calculate µg total soluble phenolics (TSP, protocatechuic acid) for each sample. Calculated sample TSP values were divided by the original sample weights to determine %TSP.

Xylem ray parenchyma characteristics:

Branch and stem samples collected prior to and 21 days after infestation with BWA by the rain down technique were cut into 1 cm sections and soaked in H$_2$O$_2$ (10%) for 48 hours at room temperature. Samples were then washed in water three times before fixing in ethyl alcohol (95%). A few drops of stain (0.2% Acid fuchsin, 1% aniline blue, and 0.1% malachite green) was added to the samples and continued to soak in ethyl-alcohol-(95%)-stain-solution for 48 hours. Samples were removed from staining solution and washed with ethyl alcohol (95%) three times and allowed to air dry before embedding them in wax. Wax embedded samples were sectioned to produce radial, longitudinal, and cross-sectional slices.
(10-25 µm) for each sample. Slices were placed in glycerol and observed at 40 and 100x magnification using a light microscope.

Anatomical characteristics recorded for plant samples included the apparent number of rings in branch and stem samples as well as the number of radial rays in cross sections, the number of xylem ray parenchyma (XRP) cells in 5 clusters of each radial section, as well as the number of lateral and longitudinal pits between 5 different XRP cells. Image J was used to measure the area of 5 XRP cells per sample, as well as the bark thickness in 5 places for each sample. XRP cells, pits, area, and bark were averaged across the 5 counts/measurements taken. Final values assessed for each anatomical characteristic included rings, rays, XRP cells, XRP area, pits, and bark thickness for each species at each infestation level and sampling period (figure 1).

Balsam woolly adelgid development:

Samples collected for TSC, lipids, and TSP were examined for BWA abundance and development. BWA counts included the development stages present on each 10cm section. We calculated the number of adults/settled 1st instars/cm to assess the adelgids ability to develop on each species.

Statistical considerations:

Two data points were not considered for all analyses due to mold growth on the branch samples for one of the control Turkish fir trees. %TSC, %Lipids and %TSP data were first analyzed using a distribution analysis to determine normality. The normal quantile plot for %TSC indicated these data were skewed (see appendix). %TSC data were normalized by taking the differences between day 21 and 0 to calculate the Δ%TSC. Negative values were
made positive by adding 20, and data were transformed using the Box Cox method with a lambda (λ) of 0.7 (see appendix). Transformed Δ%TSC data were analyzed using an ANOVA by species, infestation level, and species crossed with infestation level to determine any significant differences, raw %TSC data and Δ%TSC data were reported. The distribution analysis of %Lipids and %TSP showed the data were normally distributed without transformation (see appendix). %Lipids and %TSP were analyzed by species, infestation level, sample day, and species crossed with infestation level, day, infestation level and day, and infestation level crossed with day using an ANOVA. Comparisons using ANOVA with two or more factors crossed with significant differences were further analyzed using a Tukey–Kramer HSD. %TSC, %Lipids, and %TSP were also analyzed using multivariate methods. Pairwise comparisons were performed to determine any significant correlations between variables. Correlations were run for all variables, variables by species, and variables by species and infestation level. Only pairwise comparisons with significant values were reported (see appendix). Similar to the above analysis ANOVAs were performed for rings, rays, XRP cells, pits, XRP area, and bark thickness between species, infestation level, and species by infestation level, however, because stem samples were included in the 21 day sampling period and not the initial sampling, species by infestation level by day were not considered. Multivariate analyses were also run on all anatomical characteristics by species and infestation level. All analyses were performed in JMP 11 Pro (SAS 2012).

RESULTS:

Total Soluble carbohydrates:
There were no significant differences in %TSC between Fraser and Turkish fir species infested with BWA compared to healthy trees or between sample periods (F=1.06, df=37, p=0.41). Transformed Δ%TSC data were still not significant among species (F=2.72, df=18, p=0.1178) at α=0.1 level, and when infestation levels were factored in, the level of significance declined (F=2.17, df=18, p=0.1332). The trends in the data show that %TSC declined between sample periods in control trees for both tree species and in BWA infested Fraser fir. However, %TSC increased between sample periods in Turkish fir trees infested with BWA (figure 2a).

Lipids:

There were significant differences in %Lipids (F=3.21, df=37, p=0.0117) in comparisons between species, infestation level, and sample period. The largest differences in %Lipids occurred between species (F=18.42, df=37, p=0.0001). Trends in the data show that %Lipids increased over the 21 day period in all but control Fraser fir (table 1). These differences are especially profound between species by day (F=6.53, df=37, p=0.0013) as well as species by infestation level (F=6.32, df=37, p=0.0016) (table 1, figure 2b).

Total soluble phenolics:

Although no significant differences in %TSP were observed between species across infestation level and sampling period (F=0.61, df=37, p=0.7465), there were interesting trends as well as correlations present. A positive correlation was present between %TSC and %TSP for both infested and control Turkish fir trees, as well as control Fraser fir trees, but not infested Fraser fir (table 1). Although not significant, Turkish fir contained higher %TSP when compared by species (F=0.48, df=37, p=0.49), and across both species infested trees

125
had higher percent phenolics than non-infested trees (F=0.32, df=37, p=0.58) (Tables 3 & 4). Most interesting were the trends seen for each species in the non-infested compared to infested trees over time. Both species show a decrease in %TSP in non-infested controls, but an increase in %phenolics in BWA infested samples (figure 1).

Fraser and Turkish fir xylem ray parenchyma characteristics:

There were no differences in the number of apparent growth rings between species at the beginning (F=3.4272, df=15, p=0.0853) indicating the trees were of approximately the same age (table 2). Neither species displayed a significant increase in the number of rings at the end of the experiment either. Ray number was not different between species (F=0.5507, df=35, p=0.4631). There is also no significant difference in ray number between species and infestation level (F=0.6098, df=35, p=0.6136), however trends in the data for both species show that infested trees had lower ray numbers compared to control trees (table 2). There were significant differences in the number of xylem ray parenchyma cells (XRP) per cluster between species (f=25.1871, df=35, p<0.0001). Samples taken from trees of each species at the beginning of the experiment prior to infestation did not show significant differences in the number of XRP cells/cluster (F=3.3761, df=16, p=0.0514), but did show differences at the end of the experiment after being infested for 21 days (F=4.9182, df=18, p=0.0142). Infested trees of both species showed an increase in the number of XRP cells/cluster, but the significant differences were only between species (F=13.9196, df=18, p=0.0017), not infestation level (F=0.7365, df=18, p=0.4027) (table 2). There were no significant differences in the number of latitudinal or longitudinal pits between species for control or infested trees regardless of sampling day, however, correlations existed between the number of pits and
XRP cells, and ray number for Fraser fir. This correlation was especially strong in Fraser fir infested with BWA. In Turkish fir similar correlations existed especially pits with XRP area in BWA infested Turkish fir (table 5). Correlations between anatomical characteristics and %TSC, lipids, and %TSP were also analyzed, but not taken into consideration because anatomical characteristics of the 21 day samples were observed in branches and stems of trees, while %TSC, %Lipids, and %TSP were only analyzed in branches only. For these reasons bark thickness was also left out of the analysis as stem samples were not available at the initial sampling period. One notable correlation between anatomical characteristics and plant metabolites were %TSC and XRP cells in BWA infested Turkish fir (see appendix).

BWA settling:

The rain down method was successful in infesting the Fraser and Turkish fir with ca. 1 crawler per cm$^2$ every two weeks, an estimated 2600 BWA crawlers came into contact with the treatment trees during the 21 day period. On the branches sampled more BWA settled on Fraser (n=52, 0.96 BWA/cm) compared to Turkish (n=23, 0.4 BWA/cm) fir (p=0.029) consistent with previous studies (Newton et al. 2009, 2010, Newton et al. 2011). The number of BWA/cm were correlated with anatomical and physiological characteristics, but only pits were significant for Turkish and Fraser fir at $\alpha=0.1$. Although not significant, correlations between certain traits and BWA/cm did exist (Table 6). In a study performed with Erin Mester (2013) location of settled BWA and subsequent development into 2$^{nd}$, 3$^{rd}$, and final instars and egg production were assessed on trees from the same cohort in a similar study. On whole Fraser fir 2$^{nd}$ instars were most common on undeveloped buds and needle cushions and total adelgid counts were highest on undeveloped buds (appendix).
DISCUSSION:

In Fraser fir %Lipids were more than 1.5 times higher than Turkish fir. In both species %Lipids increased between sampling periods except control Fraser fir trees. In Turkish fir TSC and TSP showed strong positive correlations, but only control Fraser fir trees showed the same correlation, while BWA infested Fraser fir exhibited a slight negative correlation between TSC and TSP. Although not significant, trends in TSP data indicate that both species conserve defensive resources when not under attack, but increase phenolic production when infested with BWA. Plants use lipids (Maskovic et al. 2013) and phenols (Miles and Oertli 1993) in insect defense and carbohydrate metabolism is essential to synthesis of defense compounds (Keeling and Smith 2006). We expected to see an increase in phenolic production in BWA infested trees not only as they produced defensive compounds, but also in the production of rotholz, as discussed in previous chapters.

The number of XRP cells differed between tree species, with Turkish fir having more XRP cells than Fraser fir. This may be a way for Turkish fir to compartmentalize nutrients into more fragmented packets, limiting access to potential pests/pathogens. As the primary site for nutrient storage it is reasonable that a correlation between XRP and TSC exists. However, by dividing those nutrients among more compartments Turkish fir may gain an advantage by dividing access to nutrient pools, especially against a sessile feeder like BWA. Xylem ray parenchyma are long lived storage cells important for radial transport (Bel 1990, Sauter and Cleve 1994) and storage of starch and other materials as well as the formation of heartwood in Abies (Nakaba et al. 2006, Islam et al. 2012). Balsam woolly adelgid have been described as feeding on these XRP cells and cause changes in protein, starch, and
morphology of these cells (Kloft 1957, Miles 1959, Balch et al. 1964, Mullick 1969b, a, Forbes and Mullick 1970, Mullick and Jensen 1976). The size and number of XRP cells in Fraser and Turkish fir provide evidence for morphological differences between species that may contribute to tolerance of BWA infestation by Turkish fir.

Lipids and starch granules are stored in XRP and may play an essential role in heartwood formation. Time of starch granule disappearance and formation of heartwood coincide indicating the breakdown of starch granules is either a signal or source of energy in the formation of heartwood. Lipids in the XRP may also play a role in phenolic production aiding in cell wall lignification and heartwood formation (Saranpää 1988, Islam et al. 2012). The role of XRP cells, starch content, lipid, and phenolic production align with BWA infestation symptoms and the formation of ‘rotholz’. Adult BWA produce amylase which breaks down starch in XRP where they feed. The breakdown of starch may act as a signal in early heartwood formation leading to the production of rotholz. Adult BWA also produce other digestive enzymes, such as, PPO and protease, which may disrupt this process or cause a systemic reaction reducing conductivity of water and impeding nutrient flow.

When BWA feed on Fraser fir they insert their stylet bundle up to 1.5mm below the surface of the bark and feed on XRP cells (Forbes and Mullick 1970). During feeding, BWA may release a variety of enzymes for the external breakdown of starches, lipids, and proteins (see chapter II). As starch granules, and lipids are broken down in a XRP cell a reaction may be triggered which leads to the development of heartwood formation (Islam et al. 2012) at the same time defensive phenolic compounds as well as those involved in lignification of cells walls is induced (Franceschi et al. 1998, Franceschi et al. 2000). In Fraser fir this can result
in the formation of rootholz, and a state of physiological drought, eventually killing the tree (Saigo 1976, Hain et al. 1983, Hain 1988, Hollingsworth and Hain 1994b). The process of heartwood formation may be temporarily impeded by BWA feeding if feeding enzymes such as PPO are released to degrade certain phenolic compounds. Preventing death of XRP cells would allow BWA to feed longer on a particular XRP cell recruiting nutrients to that site passively (see chapter II). In Turkish fir these reactions have not been observed, making this species tolerant to BWA infestation. Differences between Fraser and Turkish fir lipid, carbohydrate, and phenolic production in response to BWA infestation indicate that Turkish fir may more efficiently convert starches to defensive phenolic compounds compared to Fraser fir (Table 3).

Anatomical characteristics may also give Turkish fir an advantage over Fraser fir in defending against BWA attack. Turkish fir have more XRP cells per cluster than Fraser fir. If XRP cells are plugged when fed upon by BWA more cells would benefit Turkish fir in allowing nutrient flow to continue while in Fraser fir it may be completely hindered or diminished below a healthy level (Bel 1990, Torsten and Aart 2006). One analogy might by the Titanic compartment concept, where compartments on the ship were created so that if one filled with water it could be closed off from the others to prevent the ship from sinking. The same compartment concept may apply more successfully here, where having more XRP compartments benefits Turkish fir by allowing it to shut off those infested by BWA, while still performing normal functions with the remaining cells.

It is also possible that Turkish fir is tolerant to BWA infestation because the salivary enzymes (PPO, peroxidases) produced by BWA do not interfere with phenolic polymer
cross-linking responsible for the plugging of XRP tubes (Torsten and Aart 2006). It is likely that Turkish fir having evolved with adelgids has developed differential chemistry in response to adelgid feeding enzymes and may be more efficient at plugging tubes than a naive host such as Fraser fir. This would allow Turkish fir to successfully starve BWA, by compartmentalizing nutrients as is evidenced in decrease in BWA fecundity when feeding on Turkish compared to Fraser fir (Newton et al. 2009, 2010). Fraser fir on the other hand, not having evolved with adelgids would not be able to successfully plug XRP tubes allowing passive diffusion of nutrients to BWA feeding sites, and when XRP cells are finally killed or converted to heartwood (rotholz), Fraser fir would not have enough open XRP cells to maintain healthy nutrient flow.

CONCLUSIONS:

Turkish and Fraser fir differ in their ability to withstand BWA infestation. Turkish fir may use lipid and carbohydrate resources more efficiently to produce defensive phenolic compounds; however, our results show that both species increased TSP in response to BWA infestation. Enzymes produced by BWA may react differently with phenols produced by Turkish fir compared to Fraser fir resulting in hindered defensive response in the latter. Fraser fir infested with BWA are known to increase nutrients (starches, lipids, proteins) locally at BWA feeding sites which may be a result of an inability to successfully plug XRP tubes. Turkish fir, having more XRP cells, can either withstand the infestation because tubes connected to BWA feeding sites are successfully plugged, and/or nutrient flow within the tree is not hindered by BWA feeding due to compartmentalization.
Future work should include studies of changes in %TSC, %lipids, and %TSP throughout the season in order to determine whether nutrient availability coincides with BWA phenology. Specific phenol and BWA enzyme interactions should be studied to see if differences between Turkish and Fraser fir defense pathways exist and are being exploited by the insect. Different ages of trees should be observed to determine if other trade-offs between defense and growth occur between species when infested with BWA.

Anatomical studies should also be studied in the future. The plugging of sieve tube elements may be visualized by staining calcium. Recruitment of calcium to plug these elements may exist in Turkish, but not Fraser, fir and act as a mechanism of host plant tolerance to BWA. Other anatomical characters and various staining methods should be pursued to better understand the feeding biology of BWA and nutrient recruitment to feeding sites between different fir species.

LITERATURE CITED:


SAS 2012. JMP (R) Pro computer program, version 10.0.0.


Figure 1. Diagram of how anatomical characteristics were measured in cross (top) and radial (bottom) sections of fir tree branches and stems.
Table 1. Total soluble carbohydrates (TSC), lipids, and Total soluble phenolics (TSP) in Fraser and Turkish fir prior to and after 21 days infestation with BWA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Infested</th>
<th>Day</th>
<th>%TSC</th>
<th>Δ%TSC</th>
<th>%Lipids</th>
<th>Δ%Lipids</th>
<th>%TSP</th>
<th>Δ%TSP</th>
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<td>Fraser</td>
<td>No</td>
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<td>Δ-3.13</td>
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<tr>
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<td></td>
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<td>0</td>
<td>8.68</td>
<td>-4.14</td>
<td>9.95^AB</td>
<td>1.35</td>
<td>0.097</td>
<td>0.0014</td>
</tr>
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<td>21</td>
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<td></td>
<td>11.30^AB</td>
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<tr>
<td>Turkish</td>
<td>No</td>
<td>0</td>
<td>7.48</td>
<td>-1.30</td>
<td>6.24^AB</td>
<td>1.87</td>
<td>0.11</td>
<td>-0.014</td>
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<td></td>
<td>21</td>
<td>6.18</td>
<td></td>
<td>8.11^AB</td>
<td></td>
<td>0.097</td>
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<tr>
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<td>Yes</td>
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<td>3.95</td>
<td>1.59</td>
<td>5.48^B</td>
<td>1.45</td>
<td>0.084</td>
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<td>21</td>
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<td>6.94^AB</td>
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<td>0.087</td>
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</tbody>
</table>

^A^B = Connecting letters report from Tukey-Kramer HSD, values with the same letter are not significantly different at α=0.05.
Figure 2. %TSC (a), %Lipids (b), and %TSP (c) for Fraser (green) and Turkish (purple) fir samples at two time points for BWA infested (solid) and control (dotted) trees.
Table 2. Xylem Ray parenchyma (XRP) anatomical characteristics in stems and branches of Fraser and Turkish fir prior to and after 21 days infestation with BWA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Infested</th>
<th>Day</th>
<th>Apparent rings</th>
<th>Ray number</th>
<th>XRP cells/cluster</th>
<th>number of pits (lateral)</th>
<th>Number of pits (longitudinal)</th>
<th>XRP area (mm²)</th>
<th>Bark thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraser</td>
<td>No</td>
<td>0</td>
<td>4</td>
<td>87.4</td>
<td>3.85&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.4</td>
<td>2.6</td>
<td>133456.5</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>5.5</td>
<td>146.6</td>
<td>3.92&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>233838.8</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0</td>
<td>5.4</td>
<td>96.92</td>
<td>3.58&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.15</td>
<td>3.1</td>
<td>487939.8</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>5.75</td>
<td>136.02</td>
<td>4.04&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.75</td>
<td>3.13</td>
<td>180586.0</td>
<td>2.55</td>
</tr>
<tr>
<td>Turkish</td>
<td>No</td>
<td>0</td>
<td>5.56</td>
<td>110.76</td>
<td>5.72&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4</td>
<td>3.15</td>
<td>259766.1</td>
<td>1.13</td>
</tr>
<tr>
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<td></td>
<td>21</td>
<td>5.75</td>
<td>175.76</td>
<td>6.04&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.93</td>
<td>3.13</td>
<td>175784.2</td>
<td>4.15</td>
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<tr>
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<td>Yes</td>
<td>0</td>
<td>5.4</td>
<td>110.2</td>
<td>5.32&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.73</td>
<td>3.07</td>
<td>115707.5</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>5.96</td>
<td>124.02</td>
<td>6.36&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.25</td>
<td>2.55</td>
<td>215957.7</td>
<td>2.38</td>
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<sup>AB</sup> = values connected by the same letter are not significantly different at α=0.05.
Table 3. Correlations between %TSC, %lipids, and %TSP.

<table>
<thead>
<tr>
<th>Species</th>
<th>Infested</th>
<th>Variable by Variable</th>
<th>Correlation</th>
<th>Count</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Signif Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkish</td>
<td>No</td>
<td>%TSP %TSC</td>
<td>0.7046</td>
<td>8</td>
<td>-0.0001</td>
<td>0.9417</td>
<td>0.0510*</td>
</tr>
<tr>
<td>Turkish</td>
<td>Yes</td>
<td>%TSP %TSC</td>
<td>0.6207</td>
<td>10</td>
<td>-0.0147</td>
<td>0.8990</td>
<td>0.0555*</td>
</tr>
<tr>
<td>Fraser</td>
<td>No</td>
<td>%TSP %TSC</td>
<td>0.5870</td>
<td>10</td>
<td>-0.0676</td>
<td>0.8883</td>
<td>0.0744*</td>
</tr>
<tr>
<td>Fraser</td>
<td>No</td>
<td>%TSP %lipids</td>
<td>0.4724</td>
<td>10</td>
<td>-0.2237</td>
<td>0.8494</td>
<td>0.1679</td>
</tr>
<tr>
<td>Turkish</td>
<td>Yes</td>
<td>%TSC %lipids</td>
<td>-0.1926</td>
<td>10</td>
<td>-0.7333</td>
<td>0.4973</td>
<td>0.5940</td>
</tr>
<tr>
<td>Fraser</td>
<td>No</td>
<td>%TSC %lipids</td>
<td>0.1917</td>
<td>10</td>
<td>-0.4981</td>
<td>0.7329</td>
<td>0.5958</td>
</tr>
<tr>
<td>Fraser</td>
<td>Yes</td>
<td>%TSC %lipids</td>
<td>0.1389</td>
<td>10</td>
<td>-0.5378</td>
<td>0.7067</td>
<td>0.7019</td>
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<tr>
<td>Turkish</td>
<td>Yes</td>
<td>%TSC %lipids</td>
<td>0.1237</td>
<td>10</td>
<td>-0.5487</td>
<td>0.6989</td>
<td>0.7336</td>
</tr>
<tr>
<td>Turkish</td>
<td>No</td>
<td>%TSP %lipids</td>
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<td>-0.7502</td>
<td>0.6524</td>
<td>0.8198</td>
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<tr>
<td>Fraser</td>
<td>Yes</td>
<td>%TSP %lipids</td>
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<td>-0.6706</td>
<td>0.5849</td>
<td>0.8458</td>
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<tr>
<td>Turkish</td>
<td>No</td>
<td>%TSC %lipids</td>
<td>-0.0824</td>
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<td>-0.7439</td>
<td>0.6606</td>
<td>0.8462</td>
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<tr>
<td>Fraser</td>
<td>Yes</td>
<td>%TSP %TSC</td>
<td>-0.0307</td>
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<td>-0.6478</td>
<td>0.6107</td>
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*significant at α=0.1
Table 4. Correlations between anatomical characteristics by species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Variable</th>
<th>by Variable</th>
<th>Correlation</th>
<th>Count</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Signif Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraser</td>
<td>bark</td>
<td>rays</td>
<td>0.5794</td>
<td>19</td>
<td>0.1699</td>
<td>0.8183</td>
<td>0.0093**</td>
</tr>
<tr>
<td>Turkish</td>
<td>bark</td>
<td>rays</td>
<td>0.5678</td>
<td>17</td>
<td>0.1198</td>
<td>0.8236</td>
<td>0.0174**</td>
</tr>
<tr>
<td>Fraser</td>
<td>pitslat</td>
<td>rays</td>
<td>0.7187</td>
<td>9</td>
<td>0.1044</td>
<td>0.9360</td>
<td>0.0291**</td>
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<tr>
<td>Fraser</td>
<td>pitslat</td>
<td>xrpcells</td>
<td>0.6804</td>
<td>10</td>
<td>0.0889</td>
<td>0.9171</td>
<td>0.0303**</td>
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<tr>
<td>Fraser</td>
<td>bark</td>
<td>rings</td>
<td>0.5951</td>
<td>13</td>
<td>0.0656</td>
<td>0.8631</td>
<td>0.0319**</td>
</tr>
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<td>rays</td>
<td>rings</td>
<td>0.5427</td>
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<td>-0.0118</td>
<td>0.8419</td>
<td>0.0553*</td>
</tr>
<tr>
<td>Turkish</td>
<td>pitslong</td>
<td>rings</td>
<td>0.5646</td>
<td>12</td>
<td>-0.0138</td>
<td>0.8599</td>
<td>0.0558*</td>
</tr>
<tr>
<td>Fraser</td>
<td>pitslong</td>
<td>xrpcells</td>
<td>-0.7515</td>
<td>6</td>
<td>-0.9709</td>
<td>0.1541</td>
<td>0.0850*</td>
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</table>

*significant at α=0.10 level.
**significant at α=0.05 level.
Table 5. Correlations between anatomical characteristics by species and infestation level.

<table>
<thead>
<tr>
<th>Species</th>
<th>Infested</th>
<th>Variable by Variable</th>
<th>Correlation</th>
<th>Count</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Signif Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraser</td>
<td>No</td>
<td>bark rings</td>
<td>0.9470</td>
<td>6</td>
<td>0.5850</td>
<td>0.9943</td>
<td>0.0041**</td>
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<tr>
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<td>No</td>
<td>bark rays</td>
<td>0.8156</td>
<td>9</td>
<td>0.3305</td>
<td>0.9598</td>
<td>0.0074**</td>
</tr>
<tr>
<td>Fraser</td>
<td>Yes</td>
<td>pitslat xrpcells</td>
<td>0.8294</td>
<td>8</td>
<td>0.3002</td>
<td>0.9682</td>
<td>0.0109**</td>
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<tr>
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<td>No</td>
<td>rays rings</td>
<td>0.8968</td>
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<td>0.3133</td>
<td>0.9887</td>
<td>0.0154**</td>
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<tr>
<td>Turkish</td>
<td>Yes</td>
<td>xrparea pitslat</td>
<td>0.8392</td>
<td>7</td>
<td>0.2341</td>
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<td>0.0182**</td>
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<tr>
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<td>Yes</td>
<td>pitslat rays</td>
<td>0.7062</td>
<td>8</td>
<td>0.0030</td>
<td>0.9421</td>
<td>0.0503*</td>
</tr>
<tr>
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<td>No</td>
<td>rays rings</td>
<td>0.7377</td>
<td>7</td>
<td>-0.0346</td>
<td>0.9584</td>
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<td>Yes</td>
<td>pitslong rings</td>
<td>0.6904</td>
<td>7</td>
<td>-0.1304</td>
<td>0.9497</td>
<td>0.0859*</td>
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<tr>
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<td>No</td>
<td>bark rays</td>
<td>0.6377</td>
<td>8</td>
<td>-0.1216</td>
<td>0.9262</td>
<td>0.0889*</td>
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<tr>
<td>Turkish</td>
<td>Yes</td>
<td>pitslong xrpcells</td>
<td>-0.6717</td>
<td>7</td>
<td>-0.9462</td>
<td>0.1647</td>
<td>0.0985*</td>
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</table>

*significant at $\alpha=0.10$ level.
**significant at $\alpha=0.05$ level.
Table 6. Correlations between BWA/cm anatomical characteristics, %TSC, %lipids, and %TSP by species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Variable</th>
<th>by Variable</th>
<th>Correlation</th>
<th>n</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Signif Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkish</td>
<td>Bark</td>
<td>BWA/cm</td>
<td>0.2528</td>
<td>5</td>
<td>-0.8102</td>
<td>0.9281</td>
<td>0.6816</td>
</tr>
<tr>
<td>Fraser</td>
<td>Bark</td>
<td>BWA/cm</td>
<td>0.1479</td>
<td>5</td>
<td>-0.8446</td>
<td>0.9113</td>
<td>0.8124</td>
</tr>
<tr>
<td>Turkish</td>
<td>Δ%TSC</td>
<td>BWA/cm</td>
<td>-0.1253</td>
<td>5</td>
<td>-0.9073</td>
<td>0.851</td>
<td>0.8409</td>
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<tr>
<td>Fraser</td>
<td>Δ%TSC</td>
<td>BWA/cm</td>
<td>0.0157</td>
<td>5</td>
<td>-0.8787</td>
<td>0.8857</td>
<td>0.98</td>
</tr>
<tr>
<td>Turkish</td>
<td>%Lipids</td>
<td>BWA/cm</td>
<td>-0.7159</td>
<td>5</td>
<td>-0.9795</td>
<td>0.4516</td>
<td>0.1738</td>
</tr>
<tr>
<td>Fraser</td>
<td>%Lipids</td>
<td>BWA/cm</td>
<td>0.5955</td>
<td>5</td>
<td>-0.6042</td>
<td>0.9688</td>
<td>0.2893</td>
</tr>
<tr>
<td>Turkish</td>
<td>%TSP</td>
<td>BWA/cm</td>
<td>-0.5946</td>
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<td>-0.9687</td>
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<td>0.2903</td>
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<tr>
<td>Fraser</td>
<td>%TSP</td>
<td>BWA/cm</td>
<td>-0.3025</td>
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<td>-0.9352</td>
<td>0.7908</td>
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<td>Turkish</td>
<td>Pits (lat.)</td>
<td>BWA/cm</td>
<td>0.854</td>
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<td>-0.5975</td>
<td>0.9969</td>
<td>0.146</td>
</tr>
<tr>
<td>Fraser</td>
<td>Pits (lat.)</td>
<td>BWA/cm</td>
<td>0.0213</td>
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<td>-0.9594</td>
<td>0.9627</td>
<td>0.9787</td>
</tr>
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<td>Turkish</td>
<td>Pits (long.)</td>
<td>BWA/cm</td>
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<td>4</td>
<td>-0.3745</td>
<td>0.9983</td>
<td>0.0836*</td>
</tr>
<tr>
<td>Fraser</td>
<td>Pits (long.)</td>
<td>BWA/cm</td>
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<td>-1</td>
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<td>0.7862</td>
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<td>Rays</td>
<td>BWA/cm</td>
<td>0.5808</td>
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<td>-0.6183</td>
<td>0.9674</td>
<td>0.3045</td>
</tr>
<tr>
<td>Fraser</td>
<td>Rings</td>
<td>BWA/cm</td>
<td>-0.6485</td>
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<td>-0.9916</td>
<td>0.8297</td>
<td>0.3515</td>
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<tr>
<td>Turkish</td>
<td>XRP area</td>
<td>BWA/cm</td>
<td>0.1631</td>
<td>5</td>
<td>-0.84</td>
<td>0.9139</td>
<td>0.7932</td>
</tr>
<tr>
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<td>XRP area</td>
<td>BWA/cm</td>
<td>0.3692</td>
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<td>-0.7609</td>
<td>0.944</td>
<td>0.5408</td>
</tr>
<tr>
<td>Turkish</td>
<td>XRP cells</td>
<td>BWA/cm</td>
<td>0.1822</td>
<td>5</td>
<td>-0.8342</td>
<td>0.917</td>
<td>0.7693</td>
</tr>
<tr>
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<td>XRP cells</td>
<td>BWA/cm</td>
<td>0.6723</td>
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<td>0.9758</td>
<td>0.2137</td>
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<tr>
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<td>XRP cells</td>
<td>BWA/cm</td>
<td>-0.3943</td>
<td>5</td>
<td>-0.9471</td>
<td>0.7483</td>
<td>0.5113</td>
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</table>

*significant at α=0.10 level.
CHAPTER V:

CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS:

BWA affect Fraser fir chemistry, physiology, and anatomy through the introduction of feeding enzymes and other feeding activities. As a sessile feeder it is likely BWA stylets are perceived by Fraser fir as both insect and pathogenic fungi. Defense compounds that are found in higher concentrations in Fraser fir bark infested with BWA are related to those responsible for antifungal colonization, while some insect defense compounds are at lower concentrations. Juvabione and related compounds seem to have little effect on BWA despite higher concentrations in infested juvenile and mature wood indicating JRCs do not come into contact or are not sufficiently ingested to work against BWA infestation. It is possible JRCs are a more apt defense against wood boring beetles and pathogenic fungi than BWA.

There is sufficient evidence that enzymes present in BWA are likely the cause of the down regulation of some defense pathways, including the PAL1 pathway responsible for producing insect defensive chemicals. Peroxidase and polyphenol oxidase (PPO) are important enzymes for insect neutralization of plant defense chemicals and defense pathways reliant on reactive oxygen species (ROS). BWA also possess feeding enzymes such as amylase and protease which may allow them to recruit nutrients to the feeding site much like a galling insect through the depletion of starch and protein in local XRP cells. Disruption of the osmotic balance in XRP cells may explain some of the anatomical changes observed in these cells after BWA infestation.
Turkish and Fraser fir differ in their ability to withstand BWA infestation. Turkish fir, having more XRP cells, can either withstand the infestation because tubes connected to BWA feeding sites are successfully plugged, and/or nutrient flow within the tree is not hindered by BWA feeding. Turkish fir may use lipid and carbohydrate resources more efficiently to produce defensive phenolic compounds; however, our results show that both species increased TSP in response to BWA infestation. Enzymes produced by BWA may react differently with phenols produced by Turkish fir compared to Fraser fir resulting in hindered defensive response in the latter. Fraser fir infested with BWA are known to increase nutrients (starches, lipids, proteins) locally at BWA feeding sites which may be a result of an inability to successfully plug XRP tubes.

Methoprene is a viable alternative biopesticide to broad spectrum organophosphates currently used to control BWA and other Fraser fir pests in Christmas tree plantations. Methoprene would work well with augmentative biological control due to its low impact on predatory insects, especially *Chrysopa spp.* The use of methoprene in Christmas tree plantations would reduce overall chemical inputs by controlling multiple pest species and increasing benefits of biological control saving the industry money, and reducing environmental impact.

**FUTURE WORK:**

Future investigations should focus on other fir species with known resistance to BWA to compare chemical defense compounds produced in response to BWA attack. Tests involving insect and fungi specific elicitors should be tested on Fraser fir to determine how it perceives BWA infestations. More assays should be conducted to test the specificity of the
compounds found in Fraser fir for relevant biological activity. Fraser fir resistance screening should involve fungal elicitors in comparison to BWA to see if the responses are similar.

Studies of changes in %TSC, %lipids, and %TSP throughout the season should also be conducted, in order to determine whether nutrient availability coincides with BWA phenology. Specific phenol and BWA enzyme interactions should be studied to see if differences between Turkish and Fraser fir defense pathways exist and are being exploited by the insect. Different ages of trees should be observed to determine if other trade-offs between defense and growth occur between species when infested with BWA.

The characterization and study of BWA enzymes, and corresponding inhibitors (EI) in Fraser fir, Turkish fir, and Veitch fir, would present future studies with possible mechanisms for host plant resistance to BWA. The three species represent the range of BWA susceptibility from highly susceptible to highly resistant, respectively. In order to determine if different fir species possess BWA specific enzyme inhibitors, isolation of all EIs from Fraser, Turkish, and Veitch fir and subsequent characterization and testing against all of the above enzymes in BWA would determine if resistance to BWA were dependent on enzyme inhibition. If BWA enzyme inhibition did occur in these resistant and tolerant fir species, and they were characterized, transgenic introduction of multiple enzyme inhibitors would be the most efficient form of providing BWA resistant Fraser fir to the Christmas tree industry and regeneration of natural stands without long term traditional breeding.

Future work should also involve looking at the above mentioned three species and anatomical changes that occur within each species. Sieve tube plugging may be an important factor regulating nutrient loss due to BWA feeding and resistant/tolerant species may be
better able to perform this defense mechanism. Studies should involve staining macronutrients such as calcium. Amino acid profiles of infested and uninfested trees of these species should also be examined.

Methoprene would reduce chemical input, control multiple pests, and allow the use of lacewing augmentative biological control within the Fraser fir system. Future studies regarding its use should be focused on impacts to other Fraser fir pest species and beneficial insects. Variations in formulations and applications should be tested for efficacy in reducing BWA populations on a large scale in the field with cost analysis and environmental impact studies. Biological control is a viable tool for Christmas tree IPM that has yet to be implemented because of the side effects associated with broad spectrum insecticides. Future work should focus on biopesticides like Methoprene as a viable alternative to broad spectrum organophosphates and pyrethroids that will work well with biological control of several pest species in Fraser fir plantations.

One common thread throughout this work is the relation of defensive mechanisms involved in the PAL pathway and BWA feeding. Enzymes produced by BWA appear to affect this pathway having major implications into its importance in defense against this particular insect pest. The presence of pectinase in BWA and the role of the middle lamellae in conifer production of PAL indicate this enzyme is primarily responsible for disruption of the phenylpropanoid pathway. Chemicals observed in the bark which appeared to be down regulated in BWA infested trees are potentially related to the induction of the PAL pathway. Polyphenol oxidase (PPO) is an enzyme present in BWA and may be responsible for down regulation of compounds [4], [6], and [8], all of which are involved in regulation of the PAL
pathway. It is no coincidence that this pathway is only down-regulated in Fraser fir, and not in other resistant firs, especially with respect to a plant's ability to differentially methylate the middle lamellae. Future work should include the detailed characterization of this pathway in Fraser as well as resistant fir species with respect to specific BWA feeding enzymes to elucidate mechanisms of down-regulation and possible means for resistance.

**LITERATURE CITED:**


Fowler, G. 1999. The potential of juvabione as a host resistance mechanism against the balsam woolly adelgid (Adelges piceae [Ratzeburg]). North Carolina State University.


SAS 2012. JMP (R) Pro computer program, version 10.0.0.


APPENDICES
APPENDIX A

Testing methoprene as a potential insect growth regulator for BWA

Abstract:

Christmas tree growers spend an annual $1.5 million in chemical control of the balsam woolly adelgid (*Adelges piceae*, BWA) in the southern Appalachians. Chemical control is currently the only form of management for this invasive insect pest. Unfortunately, the use of broad-spectrum insecticides to treat for BWA has several negative side effects. Broad-spectrum insecticides not only reduce natural predator populations, but eliminate competition for other pests which subsequently also require chemical control. We propose implementing new control methods for Fraser fir insect pests. The first method is testing an alternative pesticide in the control of BWA. Methoprene has a lower toxicity, and negligible impacts on predatory/beneficial insects, and the environment. In conjunction with the first method, the second strategy involves developing implementation of a biological control method. This study evaluates methoprene as a control treatment for BWA. Our overall goal is to reduce or eliminate chemical pesticide inputs on Fraser fir Christmas tree farms.

Introduction:

Fraser fir (*Abies fraseri*) is a keystone species occurring naturally in the high elevations of the Southern Appalachians. In 1951 Fraser fir comprised 34% of the total basal area in the Great Smokey Mountains, and outnumbered spruce 3-5 times in these stands (sources). Trees ranged in age from saplings to 125 years old, with the oldest recorded fir standing at 168 years old (Oosting and Billings 1951). Fraser fir, along with red spruce, was
the climax species for elevations above 1500m, providing habitat for several endemic biota, including bird, mammal, invertebrate, and plant species (Potter 2012).

In 1908, the balsam woolly adelgid (*Adelges piceae*) was introduced, probably on ornamental planting stock from Europe, to the northern spruce-fir stands of Maine. In 1958 the Southeastern Forest Service research station surveyed the Mt. Mitchell area in North Carolina and estimated that 11,000 trees had been killed by BWA. Aerial surveys in 1960 estimated more than 200,000 fir trees were killed. Throughout the 1960’s thousands of additional trees were killed and in some stands mortality of Fraser fir reached 75 to 100%. By 1965 over 1.5 million trees were killed by BWA. During this time, Fraser fir was considered ecologically important in protecting watersheds, but had low economic value. (Amman and Speers 1965) The loss of Fraser fir in these stands saw a dramatic decline in overall basal area and growth, with drastic changes in sunlight, temperature, moisture, water run-off, and nutrient regimes. These habitat changes were directly linked to several species becoming threatened or endangered. (Wagner and Van Driesche 2010, McManamay et al. 2011) In combination with climate change and other anthropogenic effects, animals and plants in the spruce/fir zone have continued to decline, with 307 currently listed as threatened, endangered, or of concern (Annkatrin 2012).

Production of Fraser fir Christmas trees began in the mountains of North Carolina in the 1950’s, and grew from 430,500 being planted between 1953-1964 to 42 million trees in recent years (Sidebottom 2009). This hundred-fold increase in Christmas tree production brought North Carolina to the forefront of the Christmas tree industry, ranking 2nd in the nation. Fraser fir are grown in nurseries and exported all over the world. In the 1950’s a tree
would sell for $7, today total Christmas tree sales average $100 million in North Carolina (NCDA 2012). North Carolina produced 18% of the nation’s Christmas trees, ranks first in dollars made per tree, and 96% of the trees grown and sold are Fraser fir (Potter et al. 2005).

The Christmas tree industry spends $1.5 million annually to control for BWA, not including associated costs with damage loss and secondary pest control. Reactions to BWA infestation include loss of apical dominance, gouting, rotholz production, and tree death (sources). Christmas tree growers often cull BWA infested trees because they are unsalable, especially once the tops have been compromised. BWA control required 300-800 gallons of pesticide per acre depending on tree size and density. (Potter et al. 2005) Although, recent pesticide application rates have decreased, from 4.15 pounds ai/acre, to 2.09 pounds ai/acre, there has been an overall increase in the use of organophosphates as they have replaced recently banned pesticides such as Lindane. In 2006 Dimethoate was the number one pesticide used by 34.8% of Christmas tree growers (Sidebottom 2007).

Dimethoate is an organophosphate acting on the nervous system of insects. It is highly acutely toxic to birds, mammals, and beneficial insects like bees. (Edwards 2006) Treatments like these will knock down predator insects, which would normally control late season pests like Cinara aphids. One advantage to using organophosphates is that they kill insects quickly, however, the problem is that not all pests are controlled with a single application, and it does not kill mite eggs, or BWA and elongate hemlock scale (EHS) by itself so another product must be added, or another pesticide application will have to be made later to control other pests.
Balsam woolly adelgid management with broad spectrum insecticides has increased the number of other pest species, and eliminated the ability to utilize biological control (sources). BWA are consistently listed as the main pest in Christmas tree farms (Sidebottom 2007). Since the introduction of BWA, several predator release programs have been proposed and implemented in an attempt to control the invasive insect. None have been successful due to a variety of factors (for review see (Potter 2006)). Chemical control in natural stands is impractical because it involves either injection, or drenching of the entire bole of the tree.

One proposed method for controlling BWA in Fraser fir Christmas tree plantation is to utilize a native generalist predatory lacewing, \textit{(Chrysopa oculata)}, in a management strategy for BWA. \textit{Chrysopa oculata} predators are considered a possible biological control agent for a variety of Fraser fir pests, including balsam twig aphid (BTA) (Fondren et al. 2004), BWA, Cinara aphids, and mites. Unlike the commercially available lacewing predator \textit{Chrysoperla rufilabris}, which also feeds on BWA, the golden-eyed lacewing (\textit{Chrysopa oculata}) is known to be predaceous in both larval and adult life-stages (McEwen et al. 2007). Preliminary predation tests of \textit{C. oculata} on BWA in the lab and have shown both adult and larval life stages feed on all BWA life stages, including adult, settled first instar, and eggs. In 2012 a reliable method for sourcing \textit{C. oculata} from the field in western North Carolina was developed. The procurement of lacewing adults and eggs from April through November would allow growers a long treatment and rearing season for biological control of BWA using \textit{C. oculata}. There is already progress in the development of a factitious diet for rearing \textit{C. oculata} (Micah Gardner, pers. comm.).
Methoprene has been shown to have negligible effects on *Chrysopa* spp. (Westigard 1974b). Methoprene also acts on all stages of insect development acting as an ovicide and lowering fecundity of adults, while preventing immature stages from developing (Staal 1975a). This type of control will have much better long-term effects, because reducing fecundity of a pest is predicted to cause the population to crash (McClure 1991a). We propose a plan to provide methoprene as an alternative pesticide to be used in conjunction with augmentative biological control of several pest species. If Christmas tree farmers could implement a successful biological control program they would require fewer pesticide applications (Toth et al. 2004). Growers have already made great strides in providing habitat for predatory insects by spraying fewer herbicides, and allowing growth of groundcover plants (Sidebottom 2007). These practices make the Fraser fir Christmas tree a greener, more sustainable industry with fewer negative environmental impacts.

I hypothesize that I would see significant differences between BWA treated with methoprene compared to control groups.

**Methods:**

*Sample collection:*

Methoprene effects on BWA development, fecundity, and mortality, excluding genetic and environmental effects, were assessed in the lab on BWA infested Fraser fir bark samples from a single tree collected from western North Carolina. The tree was a healthy 2.2m Fraser fir which had been infested for at least one year and, upon observation, hosted ca. 10 BWA ovisacs per 2.2cm² of bark. A core borer (1cm dia.) was used to cut pieces of infested bark, including the cambium and outer xylem, from the bole of the tree. Ovisacs on
each piece were evaluated under a microscope (100x) to ensure at least one live egg laying adult BWA was present. Bark samples were kept on a tray filled with moist sand covered with wet paper towels. BWA have been known to live on bark samples using this method for ca. 2 months, allowing adults to lay eggs, and crawlers to hatch, settle, and develop (John Strider, pers. comm.).

Counts were made on each sample of adult, egg, crawler, settled 1st instar, 2nd instar, and 3rd instar BWA. Third instar BWA were distinguishable from adults because they did not have a woolly mass or eggs present, and look much different than 2nd instars which are much smaller and have distinct wool patterns on the dorsal and ventral surfaces. Crawlers are the only motile stage of BWA and are easily distinguished from settled first instars which have inserted their mouthparts but have not molted or produced the characteristic wool of a 2nd instar. Adult BWA present were marked by placing an insect pin near the ovisac. BWA counts were made for 50 bark pieces prior to treatment with Methoprene.

**Methoprene treatment:**

1.2% methoprene (Meteor™ IGR concentrate, (S)-methoprene: CAS #65733-16-6) was mixed according to the label in a water solution. A 0.5µl glass repeat-pipette was used to apply 0.5µl of the methoprene solution to each ovisac and other BWA individuals at the various life stages on 25 pieces of bark. For control treatments 0.5 µl of water was applied to BWA ovisacs and individuals on 25 pieces of bark.

Bark pieces were stored in an incubator held at 20°C, 70% relative humidity, and a 14:10 hour light:dark cycle. Water was added to the sand in the tray as needed to keep the bark from drying out. After 8 days BWA counts were performed as before for each bark
sample and settled BWA were removed to determine if they were still alive. Live BWA would move their stylets or legs after removal, while dead BWA did not.

**Statistical methods:**

The proportion of total live BWA at the end of the experiment were compared between control and treatment groups using an ANOVA in JMP Pro 11 (SAS 2012). The Henderson-Tilton formula was also used to calculate the percent mortality caused by the methoprene treatment compared to the control (Henderson and Tilton 1955). Changes from one life stage to the next were calculated for 2nd instars developing from crawlers and eggs as a proportion of 2nd instars at the end of the experiment minus those at the beginning divided by the number of crawlers and eggs at the beginning. Fecundity (eggs/adult) was calculated for control and treatment groups before and after treatment and compared.

**Results:**

Methoprene treated BWA experienced 77% mortality which was significantly different than control BWA (F=31.53, df=49, p<0.0001). Control BWA showed an increase in total numbers over the course of the experiment with an overall increase of 1.5%. Methoprene treated 2nd instars failed to develop (0%) compared to controls (33%), which was statistically significantly (F=14.92, df=44, p=0.0004). Fecundity of BWA adults was affected by time, but not treatment (F=12.22, df=79, p<0.0001); both treated and control BWA showed a decrease in fecundity from 1.3 eggs/adult to 0.4 eggs/adult, the difference being in the number of crawlers that survived and molted to 2nd instars (F=39.5, df=20, p<0.0001) (Table 1).
**Discussion:**

Methoprene acts as an insect growth regulator, and our results show that developing BWA are the most susceptible life stages to this chemical. Methoprene completely prevented 2\textsuperscript{nd}, and 3\textsuperscript{rd} instars from continuing to develop. The H-T correction showed that mortality observed in 2\textsuperscript{nd} and 3\textsuperscript{rd} instar life stages was a result of the methoprene treatment and not just death from natural causes, especially since most of the controls exhibited an increase in individuals. These results support our hypothesis that methoprene is a suitable candidate for implementation in the control of BWA, and could possibly yield benefits in combination with biological control.

Previous IPM strategies suffered environmental and economic costs. For instance, the use of broad-spectrum insecticides results in population outbreaks of spruce spider mite (SSM) and hemlock rust mite (HRM. It has been postulated that if growers did not have to treat for BWA because biological control for other pests would take care of BWA as well (Potter et al. 2005). The Christmas tree industry in North Carolina was haunted by unsafe practices during the early years of pesticide use. Several growers were fined for using banned sprays, and cancer rates were higher throughout region compared to other areas in North Carolina. Now, cancer rates are lower, and pesticide quantities are decreasing, but there is still a room for improvement in environmental quality and human health (Sidebottom 2009).

Methoprene is registered as a biochemical pesticide by the EPA. It has low toxicity with little risk to non-target species. Methoprene is biodegradable, breaking down quickly in sunlight and on vegetation. It has low water solubility, so contamination of aquatic habitats is not expected to have lasting effects, especially since the half-life is so short (PMEP 1995).
Methoprene was classified as a biochemical pesticide because it acts as an insect growth regulator, or juvenoid compound, which mimics juvenile hormone in insects, preventing development of adults and fecundity. It has been shown to control a number of pest species while having negligible impacts on predators, particularly *Chrysopa sp.* (Westigard 1974b). For these reasons, methoprene could be an ideal candidate for a synergist working in concert with novel biological augmentation techniques as a management strategy for BWA on Fraser fir.

The cost of methoprene and biological augmentation will save growers money in treating for pests if it controls multiple pests. Methoprene has already been shown to control a number of Homoptera and other pestiferous insect species with negligible effects on beneficial insects (Staal 1975a). Most growers using a successful IPM strategy spray once every 2-3 years for BWA, however, many growers prophylactically apply pesticides on a yearly basis whether a pest is present or not, and spray for other pest species when BWA is not the primary target (Sidebottom 2007). Methoprene could potentially control all homopterous pest species present on Fraser fir. Sprayed at a rate consistent with the current label would cost a grower $200-300 per acre (1gal/1500sqft=29gal/acre*$8/gal=$232/acre), making methoprene as expensive as hiring a specialist at a cost of $300-500 an acre in 2005 (Potter et al. 2005). Dimethoate is the most commonly used organophosphate currently utilized by Christmas tree growers (Sidebottom 2007) and costs far less per acre ($38.25/gal*300-800gal/acre/446.85 treatments /gal=$25.68-$68.48/acre) but requires multiple treatments, and does not control multiple pests, and has negative impacts on beneficial insects. Methoprene would have to be manufactured for use in Christmas tree
plantations to be competitively priced with current treatments before it is implemented by growers.

There are no short-term costs associated with implementing the proposed IPM strategy. 97% of growers use backpack sprayers to apply pesticides at least once a year, while there has been an increase in tractors with enclosed cabs and air blast mist blowers to improve efficiency (Sidebottom 2007). The formulations proposed for the application of methoprene is compatible with methods already employed by growers. The novel method for augmentative release of lacewing eggs is also durable, and will work with current methods (Micah Gardner, pers. Comm.).

Methoprene would reduce chemical input, control multiple pests, and allow the use of lacewing augmentative biological control within the Fraser fir system. Biological control is a viable tool for Christmas tree IPM that has yet to be implemented because of the side effects associated with broad spectrum insecticides. This study shows methoprene is a viable alternative to broad spectrum organophosphates and pyrethroids that will work well with biological control of several pest species in Fraser fir plantations.
### TABLES AND FIGURES:

**are significantly different between treatments at $\alpha<0.0001$.

**are significantly different between treatments at $\alpha=0.004$.

† 2nd instars that developed into 3rd instars could not be accurately calculated without distinguishing between new 2nd instars and those previously counted.

Table 1. Mortality of BWA treated with Methoprene at all life stages.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Methoprene</th>
<th>Control</th>
<th>H-T correction for %mortality</th>
<th>Fecundity (eggs/adult)</th>
</tr>
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<tr>
<td><strong>BWA life stage</strong></td>
<td><strong>Date</strong></td>
<td><strong>n</strong></td>
<td><strong>%mortality</strong></td>
<td><strong>n</strong></td>
</tr>
<tr>
<td>Adult</td>
<td>9/23/2013 10/1/2013</td>
<td>24</td>
<td>29%</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Egg</td>
<td>9/23/2013 10/1/2013</td>
<td>44</td>
<td>85%</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Crawler</td>
<td>9/23/2013 10/1/2013</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2nd instar</td>
<td>9/23/2013 10/1/2013</td>
<td>27</td>
<td>100%***</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>3rd instar</td>
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<td>6</td>
<td>100%</td>
<td>0</td>
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<td></td>
<td></td>
<td>0</td>
<td></td>
<td>2</td>
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<tr>
<td>All stages</td>
<td>9/23/2013 10/1/2013</td>
<td>105</td>
<td>77%***</td>
<td>86</td>
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<td></td>
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<td></td>
<td>80</td>
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## Appendix B

Table describing %juvabione and dehydrojuvabione per tree sample and wood section.

<table>
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<th>Infested</th>
<th>Section</th>
<th>Tree ID</th>
<th>LibraryID</th>
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<th>Mean</th>
<th>Std Dev</th>
<th>Min.</th>
<th>Max.</th>
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<td>5.1</td>
<td>0.0</td>
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Full GC Chromatograms of Healthy and Infested Fraser fir Bark samples

Infested PH Average
Healthy PH Average
Table showing NIST library IDs for 41 peaks for Tree number 3 infested bark sample

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APPENDIX C

%TSC distribution:
Box Cox Transformed Δ%TSC, with Box Cox plot:
% lipid distribution:
Correlations between anatomical characteristics and %TSC, %lipids, and %TSP by species and infestation level.

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*significant at $\alpha=0.10$ level.

**significant at $\alpha=0.05$ level.
Correlations between anatomical characteristics, %TSC, %lipids, and %TSP by species.

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*significant at α=0.10 level.
**significant at α=0.05 level.
Correlations between anatomical characteristics and %TSC, %lipids, and %TSP.

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*significant at α=0.10 level.
**significant at α=0.05 level.