ABSTRACT

MESTER, ERIN CATHLEEN. Establishing the Requirements for Developing Artificial Rearing Techniques for the Balsam Woolly Adelgid, *Adelges piceae*. (Under the direction of Fred P. Hain).

The balsam woolly adelgid, *Adelges piceae* (BWA), is a small, exotic, piercing-sucking insect that threatens Fraser fir (*Abies fraseri*), an important resource and prominent Christmas tree species. Control of this pest is difficult because of complexities of its biology and feeding behavior. The objective of this study was to understand the necessary environmental conditions and nutritional requirements to develop artificial rearing techniques of BWA.

BWA were reared in the laboratory to determine the best substrate and optimum environmental conditions for development and reproduction. The optimal temperature was 20°C for BWA development and 25°C was the least favorable. Logs were the only substrate that reached full development in all five environments. Buds were the best position for BWA development in cuttings and seedlings, possibly because they contain more nutrients. Un-infested and infested Fraser fir bark was analyzed for texture and hardness to serve as a model for membrane development. Infested bark was found to be twice as hard as un-infested bark, suggesting an insect-induced response which is possibly part of the tree's defense mechanism against adelgid attack. The hardness of films made from unblended hardwood-beeswax (0.4 mJ) and blended hardwood-beeswax (0.35 mJ) had hardness levels between the un-infested and infested bark and are suggested as optimal for further testing of BWA preferences because initial feeding by BWA causes an induced response that increases protein content of the bark as well as increased fertility and survival of adelgids. Chemical properties of Fraser fir bark and wood were analyzed and based on ion chromatography (IC)

results, demonstrated glucose levels of infested bark were four times higher than un-infested bark, suggesting BWA modify the bark causing an increase in nutrient availability for the adelgids.

Artificial diets for BWA were evaluated and solid diets made from torula yeast and soy appeared to be the most promising for further development. The highest number of crawlers inserted into unblended Fraser fir and none inserted into blended hardwood or Parafilm. Many of the diets with film substrates never got mold growth on them, suggesting the films had antimicrobial properties. The soy liquid diet with hardwood unblended film appeared to be most susceptible to mold growth, but it took an average of 13.6 days until mold was visible. Overall, crawlers inserted into rubber (0.10) more than films (0.08). Furthermore, across all the diets, the soy diet had the highest insertion rate in rubber (0.24), which was also twice as high as the insertion rate across all films (0.12). Rubber may also provide a more favorable substrate than the films and should be used as a cover for the diets in future trials. Further research is needed to better understand the feeding mechanisms of BWA.

The efficacy of the green peach aphid, *Myzus persicae*, ingesting a host defense chemical (juvabione in Fraser fir) was tested as a model for BWA. The aphids fed readily on the infested wood diet, suggesting that infested wood induces a chemical response that encourages feeding. Results indicate that juvabione or other compounds present in Fraser fir petroleum ether extract delayed reproduction, which may be a form of resistance and a possible population control method. Juvabione levels in Fraser fir trees should be the focus of further research, including their contribution to host resistance to BWA, and its use as a convenient and environmentally safe control method for BWA.

Establishing the Requirements for Developing Artificial Rearing Techniques for the Balsam Woolly Adelgid, *Adelges piceae*

by
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DEDICATION

I would like to dedicate this thesis to my Pop, Warren Joseph Ambrose. Thank you for being such a loving and generous grandfather. You will be deeply missed.

BIOGRAPHY

Erin Cathleen Mester was born and raised in Maryland and is the youngest of three children. Erin's parents ingrained in her from an early age the importance of giving back to the community in which she lived and the value of a good education. Before she attended college, Erin decided to serve her country by serving two years in AmeriCorps, a domestic type "Peace Corps," where she helped impoverished communities throughout the U.S. and earned two education awards in return for her service.

After AmeriCorps, Erin started her college career at Northern Arizona University. During this time Erin gained research experience by working in the freshwater ecology and herpetology labs at school. Erin wrote a grant and received funding from the Hooper Grant Scholarship for an undergraduate research project to estimate the age of *Crotaphytus collaris* by skeletochronology. In December 2002, Erin graduated with honors and received her B.S. in zoology with a minor in chemistry.

After college, Erin spent several years as a seasonal employee with The Nature Conservancy and the U.S. Forest service as a wildland firefighter. In 2006, Erin was ready for a permanent job and began her teaching career as a 6th grade science and social studies teacher at Cottonwood Middle School in Cottonwood, Arizona. During this time, Erin also attended school fulltime in order to become a certified teacher. In March 2008, Erin graduated with honors from Rio Salado College with her post-bachelor teaching certificate.

In the summer of 2008, Erin decided to move back to the east coast to be closer to her family and ended up in Raleigh, North Carolina with a position as an 8th grade science teacher. After four years as a teacher, Erin decided to return to school and earn her Master's

degree at North Carolina State University. In January of 2011, Erin began pursuing her M.S. degree in entomology fulltime under the direction of Dr. Fred Hain. While a student, she also worked as a TA for entomology and biology undergraduate courses as well as a fundraiser for an environmental nonprofit.

Throughout her academic and professional career, Erin has continued to give back to her community by volunteering with different volunteer groups and local nonprofits. She developed an interest in fundraising through her volunteer work and while going to graduate school fulltime, she worked as the Development Officer for the Alliance for Saving Threatened Forests, a regional nonprofit her advisor founded that supports research on developing pest resistant trees and other means of controlling the adelgid, an invasive forest insect. After graduation Erin plans to pursue her passion with a career in fundraising.

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CHAPTER I

Introduction and Literature Review Relating to the Balsam Woolly Adelgid

LIFE HISTORY, IMPACT, AND ECOLOGICAL IMPORTANCE OF THE BALSAM WOOLLY ADELGID

The balsam woolly adelgid (BWA), *Adelges piceae* (Ratzeburg), is an invasive forest pest widely distributed throughout Europe and North America, and infests only true firs, *Abies* species (Mitchell, 1966). North American fir species are more sensitive to BWA attack, for example, balsam fir, *Abies balsamea* (L.) Miller, which is sensitive and has been greatly damaged by the BWA (Balch and Carroll, 1956). In its native range, western Europe, the balsam woolly adelgid's primary host is European silver fir (*Abies alba* Miller), a tree that can support a large population of BWA yet still remain relatively unharmed (Mitchell et al. 1970). BWA was introduced to North America in the early 1900s, presumably on infested nursery stock (Balch and Carroll, 1956) and is believed to have entered North America through eastern Canada near the southern end of Nova Scotia (Balch, 1952). From there, the BWA spread extensively over the Maritime Provinces and New England states, devastating thousands of acres of balsam fir (Mitchell, 1966). BWA appeared on the west coast in 1928 (Mitchell, 1966) and later in the southern Appalachians in 1955 on Mount Mitchell, North Carolina (Amman, 1962), although it was probably present earlier. In the southeastern United States mountainous regions of northern Virginia, western North Carolina, and eastern Tennessee, the BWA specifically infest Fraser fir, *Abies fraseri* (Pursh) Poir, trees (Mitchell et al. 1970).

Fraser fir is an important natural resource and a prominent Christmas tree species. Fraser fir occurs naturally only in the southern Appalachians where it obtains dominance above 1800-1900 m (Busing et al. 1993). Seventy-five percent of all natural stands are in the Great Smoky Mountains National Park (Dull et al. 1987) and before the devastation by BWA, was the dominant tree at the highest elevations (Dull et al. 1987). Shortly after the introduction of BWA, over 90 percent of old-growth or mature Fraser fir was killed (Hay and Johnson, 1980). In natural conditions, mature Fraser fir is very susceptible to BWA attacks, while younger trees do not appear to be as affected, although they can be heavily infested in plantations (Ragenovich and Mitchell, 2006), in part due to the effect of fertilizer treatments (Arthur and Hain, 1984). In plantations, all ages of fir are susceptible to BWA, especially Fraser fir. Trees that are pole-sized or larger seem most susceptible to mortality caused by BWA, although all sizes of trees are attacked (Ragenovich and Mitchell, 2006).

Extensive logging in the early 1900s (Pyle and Schafale, 1988), a majority of which occurred between 1912 and 1922 (Hollingsworth and Hain, 1991), as well as air pollution has contributed to the decline of Fraser fir forests (Hain, 1986). At the highest elevations where fir is the dominant tree, it is possible that greater inputs of atmospheric pollutants are present, due to higher precipitation and frequent cloud immersion and contribute to fir mortality (Dull et al. 1987, Hollingsworth and Hain 1991). However, BWA has demonstrated the most significant ability to annihilate and damage Fraser fir forests (Mitchell, 1966). Weather is an important factor affecting the spread of BWA as well its survival, particularly in the northern latitudes and higher elevations (Ragenovich and Mitchell, 2006). There is a gradual decline in BWA survival as temperature decreases, the developmental-hatching threshold is between

5 and 7°C (Amman, 1968), and BWA cannot survive temperatures below -30ºC (Balch, 1952). Therefore, the spread of BWA may be delayed by cold temperature. Regeneration has been good in many of the Fraser fir native stands, however, successive cycles of regeneration and mortality may result in decreasing populations over time (Ragenovich and Mitchell, 2006).

Life History of the Balsam Woolly Adelgid

Within the forests, BWA are inconspicuous insect pests (Mitchell, 1966). First instars, or neosistens, are typically 0.4 to 0.5 mm long and wingless adults are 0.6 to 1.3 mm in length (Havill and Foottit, 2007). The neosistens overwinter into the spring generation, hiemosistens, and are extremely prolific (Balch, 1952). Aestivosistens, the summer generation and offspring of the hiemosistentes, are less fecund than the hiemosistens, which often lay more than twice the number of eggs (Balch, 1952). In its native range, some adelgid species are winged and holocyclic with spruce (*Picea* spp.) as its primary host and *Abies* as its secondary host (Havill and Foottit, 2007). However, this species, as in Europe, has lost its ability to migrate to *Picea* species and is wingless and anholocyclic (Balch and Mitchell 1967, Havill and Foottit 2007). Therefore, the adelgid life cycle consists of generations from the apterous parthenogenetic females on *Abies* species (Balch and Mitchell 1967, Havill and Foottit 2007).

The crawlers are amber colored and are often observed moving rapidly over the bark, as they're the only life stage capable of self-directed movement (Ragenovich and Mitchell, 2006). When the crawler finds a suitable feeding site, she will insert her stylets, with an average length of 1.5 mms (Balch, 1934) into the tree, her body will turn deep purple or

black and she produces a thick mass of waxy wool-like material that covers the body and arises from pores on the dorsal side (Johnson and Wright, 1957). This wool-like material serves to protect the adult and her amber colored eggs, of which as many as 100-250 are laid (Ragenovich and Mitchell, 2006). When eggs are freshly laid, they have a pale shiny yellow appearance, and turn a dull amber color as development proceeds (Amman, 1968).

After the adelgid inserts her stylets into the tree, she becomes stationary and remains in that location for the remainder of her life (Balch and Carroll, 1956). The adelgid molts three times and has two additional instars between crawler and adult stages (Balch, 1934). At each molt, the adelgid becomes larger, more convex, and develops proportionately shorter legs and antennae than the crawlers (Johnson and Wright, 1957). Before each molt, the stylets are partially withdrawn: then the new stylets are drawn into the labial groove as the old stylets are pulled out (Balch, 1952). At this point, the new instar is capable of slow movement and is free to leave, but almost always reinserts her stylets into the same spot, or slightly in front of it (Balch, 1952).

Colder climates in the East and higher elevations in the West typically yield two generations per year while at lower elevations in the West and milder climates, there are three to four generations per year (Balch and Mitchell, 1967). In North Carolina, a partial third generation usually develops and in the lowland areas of Oregon and Washington, three generations with a partial fourth develops (Mitchell et al. 1970). The optimum humidity and temperature for longevity of crawlers is 75 percent relative humidity and 15°C. During periods of high humidity crawlers would probably have a shorter life and less chance of successfully attacking a tree (Amman, 1968).

Wingless BWA are passively dispersed primarily through wind and the eggs and nymphs are often carried far distances on air currents (Johnson and Wright, 1957). BWA can also travel to other trees over the ground or carried by humans, birds, squirrels, and other animals (Balch, 1934). As a tree receives more direct light and subsequent increase in temperature, an increase in BWA dropping from the tree occurs (Atkins and Hall, 1969). As a result, these crawlers are more apt to be carried off by daytime outflow of air compared to crawlers that are confined to a closed canopy and inner-stem space where they would disperse only short distances (Atkins and Hall, 1969).

There is a sparse fossil record for Adelgidae, but some have been found from the Pliocene and Pleistocene, and a fossilized adelgid gall was found in Japan from the Pleistocene (Havill and Foottit, 2007).

Feeding Behavior of the Balsam Woolly Adelgid

BWA feeding takes place in the cortical parenchyma within the outer 1.5 mm of bark but in young shoots the phloem is sometimes slightly penetrated (Balch, 1952). BWA stylets are inserted intercellularly, pass through the epidermis or phellem into the cortex or phelloderm, and seldom, if ever, penetrate the cell wall (Balch, 1952). After the stylets are inserted, a salivary substance is ejected from the tip of the maxillae which forms a sheath around the stylets that can be seen at the point of entrance and lining the path of the stylets (Balch, 1952). BWA feed by repeatedly withdrawing and reinserting their stylets in new directions, and they control the penetration, although it is not determined solely by the path of least resistance (Balch, 1952).

BWA are salivary-sheath feeders and produce at least two types of salivary secretions: watery, digestive enzyme saliva and lipoproteinaceous saliva which is secreted while the stylets penetrate the plant tissue (Backus, 1988). The latter saliva rapidly solidifies and forms a solid sheath around the stylets (Backus, 1988). Most insects in the order Hemiptera specialize on a certain range and region of host plants as well as on preferred feeding tissue within the plant (Backus, 1988). BWA inserts its stylets intercellularly and feeds in the cortical parenchyma of true firs (Balch et al. 1964). While feeding, BWA pumps a salivary substance into the tree that is toxic and causes an abnormal reaction in cambial cells (Mitchell, 1966). The saliva's function appears to be to stimulate growth and predigest food materials in the stimulated cells (Balch, 1952). The cells neighboring the tracks become enlarged and the nuclei and the cell walls thicken (Balch, 1952). This thickening is most noticeable in the walls adjacent to the tracts. The nuclei becomes attracted to the side of the cell that is in contact with the stylets and BWA appears to obtain its nutritive materials by dialysis through the cell wall (Backus, 1952). The saliva is thought to contain a chemical substance that is an irritant in small quantities and a toxin in larger quantities (Balch, 1952). It is possible that BWA saliva contains a substance similar to indole-acetic acid but it could also cause a concentration of auxin indirectly while the adelgid feeds, by stimulating its production within the cells or by attracting it from other parts of the tree (Balch, 1952). The trees' response to adelgid saliva is greater in more vigorous trees which suggests it produces abnormal activities of substances already in the tree by enzymatic or synergistic action (Balch et al. 1964).

In the bark, giant parenchyma cells develop, and the cambium is stimulated to produce an abnormal number of phloem and ray cells (Ragenovich and Mitchell, 2006). Adelgid populations in the outer portion of the tree's crown cause less damage than adelgid populations on the main stem and large branches (Mitchell, 1966). Swelling of the outer nodes and terminal buds are indicators of crown infestation (Mitchell, 1966). Simultaneously, an abnormally wide annual ring composed of cells with unusually thick walls is produced in the woody tissue (Ragenovich and Mitchell, 2006). In the stems of the trees that have heavy bole attacks, a reddish, irregular growth, referred to as rotholz, occurs (Ragenovich and Mitchell, 2006). Rotholz is similar to compression wood and disrupts water conduction to the crown of the tree and often results in tree death within two to three years after initial attack (Balch 1952, Ragenovich and Mitchell 2006).

Contorted swellings, commonly called gout, occur on twigs that have been attacked (Johnson and Wright, 1957). The cells surrounding the stylets' tracks experience hypertrophy and are followed closely by hyperplasia in the neighboring parenchyma, both of which contribute to the swelling of the cortex or bark (Balch, 1952). Hypertrophy and hyperplasia are common phenomena in gall formation by insects, and gout is considered a form of gall (Balch, 1952). These swellings may inhibit growth of new foliage to the point that the tree eventually dies (Johnson and Wright, 1957).

Ecological Importance

In the Black Mountains of North Carolina, there are approximately 7,200 acres of spruce-fir forests (Hollingsworth and Hain, 1991). Fraser fir can be found in six discrete areas of high altitude in the southern Appalachians that are considered glacial remnants from

the Pleistocene (Buell 1945, Hollingsworth and Hain 1991). True firs are one of the most admired trees in recreational areas that experience high-use (Mitchell et al. 1970) and BWA damage decreases their aesthetic value. Fraser fir is a high elevation sub-alpine species that can live for 150 years and is not only important for its scenic beauty, but also for providing a home for many wildlife species, such as the Carolina northern flying squirrel (*Glaucomys sabrinus* Shaw) and the spruce-fir moss spider (*Microhexura montivaga* Crosby and Bishop), now both endangered species due to habitat loss from BWA (Terwilliger and Tate 1977, USFWS 2000). Multiple other species have been affected by this habitat loss as well. For example, over one-half of avian species have declined by more than 50 percent over the past 25 years on Mount Collins (Rabenold et al. 1998).

In the past 50 years, over 95 percent of mature Fraser fir trees have been killed, leaving behind a trail of "ghosts" on the highest mountain peaks of the Southern Appalachians. In North Carolina, Fraser fir are the most popular Christmas tree and are shipped to every state in the U.S. as well as other locations all over the world (McKinley et al. 1996). The North Carolina Fraser fir was judged the nation's best through a contest sponsored by the National Christmas Tree Association, and has been chosen as the official White House Christmas tree nine times, more than any other species (NCCTA, 2005). Fraser fir Christmas tree sales are a multi-million dollar industry for our country. Fraser fir production alone represents 67 percent of North Carolina's total agricultural income, with 4,500-5,000 acres in production (Sidebottom, 2009). There are 50 million Fraser fir trees growing on over 25,000 acres in North Carolina (NCCTA, 2005), and North Carolina is the second largest Christmas tree producer in the U.S., which brings in over 100 million dollars

to the state annually (McKinley et al. 1996).

CONTROL AND MANAGEMENT OF THE BALSAM WOOLLY ADELGID

Heat on the surface of the bark due to direct sunlight naturally kills a large amount of BWA and also explains why most trees with trunks exposed to the sun are free of infestation on the south side (Balch, 1934). Only BWA below the snowline can survive temperatures of -34.4°C (Mitchell et al. 1970) and heavy rain can also cause mortality (Balch, 1934). Intensive cutting of infested stands to prevent dead trees from becoming unfit for salvage can minimize losses (Balch and Carroll, 1956). Overwintering larvae can develop and produce eggs on winter-cut logs so they should be barked, scorched with a blowtorch, or have the slash burned over them (Balch, 1952). However, complete eradication is unlikely (Balch and Mitchell, 1967). Other silvicultural treatments such as cutting and cleaning spot infestations, as well as switching to nonsusceptible tree species (Hall et al. 1971) have been tried, but have done little to control the spread of BWA.

In the forest, chemical control is impractical because each tree must be sprayed individually which is expensive and time consuming. Aerial spraying contact insecticides over large areas is also impractical because BWA often hide in protective niches in and below the crown and they're also protected by the wool-like mass that surrounds them (Mitchell et al. 1970). Individual trees can be sprayed and some insecticidal soaps and oils are capable of penetrating through the waxy coating of adult BWA. However, in order to avoid burning the foliage on the tree, the timing of application is important, generally in May through June and September through October (Ragenovich and Mitchell, 2006). Chemical

control will reduce populations below tree-killing levels, and some treated trees can remain free from adelgids for quite a few years (Ragenovich and Mitchell, 2006). In ornamentals, chemical control is very effective and trees can be treated any time of year (Sidebottom, 2009). Nearly all Fraser fir Christmas trees require treatment one or more times during a five to ten year rotation for BWA. Sight of a single infested tree is the threshold for treatment and the Christmas tree industry currently spends $1.5 million annually on BWA and the use of these pesticides minimizes the effectiveness of IPM strategies (Potter et al. 2005, Newton et al. 2011).

There are no known parasites for BWA (Ragenovich and Mitchell 2006, Johnson and Wright 1957) and a notable feature of the entire family Adelgidae is that they lack hymenopteran parasitoids, which has made biological control difficult to achieve (Havill and Foottit, 2007). It is unknown if this is the result of historical phylogenetic factors or the adelgids possess a unique defense against parasitoids (Havill and Foottit, 2007). Fungal species, specifically *Fusarium larvarum* (Smirnoff), have also been observed for potential use in biological control, but only combined with sublethal doses of chemical insecticides (Smirnoff, 1971). Several insect predators have been introduced to North America to remedy the lack of effective native predators (Mitchell et al. 1970). Six species from Europe have become established, three are Coleopterans: *Laricobius erichsonii* (Rosenhauer, Derodontidae), *Pullus impexus* (Mulsant, Coccinellidae), and *Aphidecta obliterata* (Linnaeus, Coccinellidae), and three are Dipterans: *Aphidoletes thompsoni* (Mohn, Cecidomyiidae), *Cremifania nigrocellulata* (Czerny, Chamaemyiidae), and *Leucopis obscura* (Haliday, Chamaemyiidae) (Mitchell et al. 1970). To date, none of these predators has achieved

detectable control because they appear to feed on stages of BWA that are unimportant in determining trends in BWA populations (Mitchell et al. 1970). Furthermore, BWA populations increase so rapidly during initial stages of infestation and because some trees are more susceptible to attack, the predators have little time to gain control before irreversible damage can be done (Ragenovich and Mitchell, 2006).

Another possible means of controlling BWA is through identifying and utilizing the chemicals associated with host plant defenses. In 1965, when Slama and Williams tried to rear the European bug, *Pyrrhocoris apterus* (Linnaeus), they noticed development was impaired and the insects would undergo one or more supernumerary molts and then die without becoming sexually mature (Slama and Williams 1965, Bowers 1966). After further research, they realized the insects somehow had access to an unknown juvenile hormone and found that the paper towels the insects were reared upon, which were made from balsam fir pulp, contained a "factor" that mimicked the juvenile hormone and they coined this phenomenon "the paper factor" (Slama and Williams, 1965). In 1966, Bowers first identified this "factor" by isolating the only active component from balsam firs and coined it juvabione (Bowers, 1966). Juvabione, a sesquiterpenoid found in fir wood, mimics the juvenile hormone in insects by inhibiting insect reproduction and growth (Barrero et al. 1989, Fowler et al. 2001). In more recent studies, juvabione levels in Fraser fir has been shown to increase with increasing BWA infestation (Fowler et al. 2001, Zhang 1994). Because Fraser fir produces juvabione in response to BWA infestation, this implies juvabione may contribute to host resistance of BWA (Puritch et al. 1974).

Synthetic Juvenile Hormones

Several compounds that occur in insects can be mirrored in fatty acid esters of different alcohols, especially those derived from glycerol (Canavoso et al. 2001, Wimmer et al. 2002). The insects' enzymatic system metabolizes these esters and has been the basis for the design and development of several hormonogenic fatty acid esters, which have been derived from several insect juvenile hormone bioanalogues (Slama and Roman 1976, Canavoso et al. 2001, Wimmer et al. 2002). Insect juvenile hormone bioanalogues are organic compounds that mimic the insect juvenile hormone and are thought to be released within the insect during the introductory step(s) of the metabolic process (Wimmer et al. 2002). Synthetic analogues of juvenile hormones, termed juvenoids, are commonly used agents for controlling noxious insects (Slama, 1999). Due to juvenoids' high biological activity and relatively low acute toxicity, practical use of juvenoid pesticides has been used on destructive insects of urban communities (Slama, 1999).

Wigglesworth first recognized and described the endocrie features of juvenile hormone on triatomid bugs, *Rhodnius prolixus* Stal (Wigglesworth 1936, Novak 1966, Slama 1999). Corpora allata, an insect's endocrine glands, are where the juvenile hormone is secreted. In the larval stages, the production of juvenile hormone is physiologically inhibited and suspends larval somatic growth (Slama, 1999). Juvenile hormones can also be used in the adult stage of several insect species for regulation of reproduction, especially during the cycles of somatic growth in reproducing females (Slama, 1999). When compared with an instant application of juvenoid itself, juvenogens in low concentration have the key advantage of being able to release the biologically active component (juvenoid) during longer

periods of time (Wimmer et al. 2002). Since the early 1970s when the first commercially available products, hydroprene and methoprene, were prepared, thousands of active juvenile hormone analogues (juvenoids) have been synthesized (Jedlicka et al. 2007). Because juvenogens are generally bulky molecules, it may be difficult or even impossible in topical screening tests to penetrate through the insect cuticle and are therefore intended for oral applications to insects. The implementation of a juvenogen based bait for the control of certain insidious insects provides a convenient and environmentally safe control method (Wimmer et al. 2002).

Current Research with the Balsam Woolly Adelgid

Considerable research has been done on chemical and biological control of BWA, however, recent work has shifted toward breeding fir species for host resistance. This was accomplished with the American chestnut in the 1970s to develop progeny that are resistant to chestnut blight, *Cryphonectria parasitica* (ACF, 2010). Unlike the chestnut situation, we hypothesize that there are BWA resistant or tolerant Fraser fir trees and so the approach will be a traditional within-species selection and breeding approach as opposed to the interspecific hybridization approach that has been taken in chestnuts.

HISTORY OF INSECT LABORATORY REARING

The successful development of rearing can be designed to prevent the adelgid from entering diapause and make it available for research needs. In order to test trees for resistance, a reliable supply of healthy BWA is needed, and the development of a rearing system in the laboratory will make this possible. This will also benefit other scientists who

require quality laboratory-reared insects to discover means of controlling this pest based on non-resistance technologies. Furthermore, the huge expenses incurred through rearing predators for biological control programs could be substantially reduced with a supply of abundant and healthy BWA as a food source.

The first successful attempt to rear an insect from egg to adult on an entirely artificial diet was accomplished in 1908 by Bogdanov, with blowflies, *Calliphora vomitoria* Linnaeus (Singh 1977, Cohen 2003). In 1915, Loeb reared *Drosophila* species on a simple medium for five generations, and other pioneering efforts at rearing were led by Guyenot in 1917 who reared *Drosophila*, Zabinski who reared cockroaches, and Fraenkel throughout the 1940s and 1950s who published several diets based on casein formulations (Singh 1977, Cohen 2003). Insect diets must fulfill sensory requirements, be nutritious, and reasonably stable so in essence, the diets must serve insects in the same way that human foods serve people (Cohen, 2003). One of the most significant breakthroughs in insect diet science was by Adkisson et al. (1960) by using wheat germ in their diet for pink bollworms, *Pectinophora gossypiella* Walsingham (Adkisson et al. 1960, Cohen 2003). Wheat germ has several nutritive properties that makes it an excellent food source for a broad spectrum of insects and thus, revolutionized diets for numerous other phytophagous insects (Cohen, 2003).

Three principles of nutrition discussed by House (1974) are still valid: the rule of sameness, the principle of nutrient proportionality, and the principle of complimentary supplements (House 1974, Cohen 2003). These principles add insight into how insect diets work or fail and indicates that all insects have more or less the same nutritional requirements but the proportions of essential nutrients in food is much more important in nutritional

quality than the absolute amount of nutrients present (House 1974, Cohen 2003).

ARTIFICIAL REARING INSECTS IN THE SUBORDER HOMOPTERA

All insects in the suborder Homoptera (aphids, adelgids, cicadas, leafhoppers, and scale insects) are stylet-sheath feeders (Miles 1987, Backus 1988). Sheath-feeders use two tactics of stylet movement during probing: 1) moving the entire stylet bundle through the plant tissues, with the maxillary stylets lagging slightly behind the mandibular stylets, and 2) bracing the mandibular stylets in the epidermal tissues and then probing more deeply with the maxillary stylets and is used by most members of the Homopteran infraorder Achenorrhyncha (Backus, 1988). In the infraorder Sternorrhyncha, all are sheath-feeders highly specialized to exploit primarily vascular tissues and they probe with the mandibular stylets ahead, intercellularly, following the plant cell walls (Backus, 1988). The sheath serves more than one function: 1) as a flange at the surface may steady the stylets for initial penetration, 2) directing the progress of the stylets through tissues of varying texture and hardness, 3) under turgor pressure, preventing the accidental loss of plant fluids, 4) preventing xylem sap or other unacceptable fluids from intercellular spaces from being sucked into the insect's food canal, and 5) preventing and sealing wounds made in the plant during penetration by the stylets (Miles, 1987). The nature and causation of the effects of some Homopterans on plants can be extremely difficult to research because the small size of the insects makes it difficult to determine what substances are injected into the plant, in what quantities, and precisely where (Miles, 1987).

All Homoptera are stylet-sheath feeders (Miles, 1987). Stylet-sheath feeding will occur when a complete stylet sheath is produced, and it is characteristic of the tapping of phloem or xylem, or of feeding successively on individual parenchyma cells (Miles, 1987). It is extremely important to understand an insect's feeding biology in order to develop and use a successful artificial diet (Cohen, 2003). These include mouthparts, factors that induce or sustain feeding responses, digestive enzymes, optimal gut residence time of suitable foods, absorption characteristics, nutritional requirements, and characteristics of excretion of waste products (Cohen, 2003). In general, insects are adapted to feed on either liquid diets, solid diets, or a mixture of liquid and solid foods (Cohen, 2003).

Host-acceptance behaviors have been better studied in insects in the order Homoptera but current studies indicate that basic recognition and acceptance sequence is fairly stereotypical from species to species (Sogawa 1973, Backus 1988). The insect will enact a series of behaviors to test the suitability of a plant using three steps (Backus, 1988). Plant surface exploration is the first step and during this phase the insect usually walks rapidly about the surface of the plant dabbing at it repeatedly with the tip of its labium or vigorously antennating it (Backus, 1988). The next step is the stylet probing stage, which occurs after the insect has been dabbing and/or antennating in one area for a while and can be subdivided into two more phases: test probing and exploratory probing (Sogawa 1973, Backus 1988). Test probing is done to test the host suitability and the stylets are inserted shallowly, into or just past the epidermal tissues (Backus, 1988). This continues into exploratory probing if the epidermal or mesophyll cues are acceptable where the insect then pushes its stylets deeper into the plant to locate its preferred feeding tissue (Backus, 1988). Finally, the insect will

ingest for a variable period of time before terminating the probe and withdrawing its stylets (Backus, 1988).

Evidence shows that Adelgidae, which feed on parenchyma, appear to secrete saliva more or less continuously (Astava, 1987). The role of saliva is extremely important because it aids during stylet penetration, is a carrier of enzymes and gustatory stimuli, and forms a stylet sheath that has various functions (Astava, 1987). The watery saliva of Homoptera is slightly alkaline, with a pH between 8 and 9, possibly due to the functioning of a cation pump (Miles, 1987). BWA saliva initiates changes in the tissue of the tree which conditions it for subsequent feeding (Balch et al. 1964).

Aphids as a Model Organism for Balsam Woolly Adelgid Feeding

Aphids can be used as a model in regards to the feeding behavior of BWA because they have similar mouthpart form and function, and they produce watery saliva that is excreted while feeding as well as creating a protective sheath around their mouthparts. While aphids have an alate form, many are wingless like BWA, making them parasites: winglessness is often regarded as an adaptation to parasitism (Klingauf, 1987). A hypothesis still stands that punctured plant cells seem to allow aphids to sense the high sugar content and slightly alkaline pH characteristic of sieve-tube sap via their stylet pathway in order to recognize the sieve-tube and feeding location (Auclair 1969, Hewer et al. 2010). Aphids appear to find their host plants by searching randomly. The aphids' small size, which lends to having little control over their direction of flight, is possibly a reason for this random search. For this reason, an aphids' ability to recognize a potential host plant at a distance provides minimal selective advantage for them. The same may be true for BWA, since they

are passively dispersed. Another factor contributing to this random search is the short period of time an aphid can survive off of their host plants (Dixon, 1987).

NUTRIENT REQUIREMENTS FOR INSECTS

Insect diets can be classified into three general types: holidic diets are fully defined chemically: these can be dry mixtures for cockroaches or aqueous solutions for plant-sucking insects such as aphids (Vanderzant 1974, Cohen 2003). A meridic diet has some ingredients that are defined chemically and some that are not. They contain substances such as plant tissue or yeast products (Vanderzant 1974, Cohen 2003). An oligidic diet has ingredients that are not chemically defined (Cohen, 2003). Plant-reared aphids are sometimes reluctant to settle and feed on a holidic diet during the first day of being confined to them so it is recommended that they be temporarily adapted to a holidic diet before attempting to raise them on oligidic diets and a simple sugar solution may serve this purpose (Mittler and Koski, 1976).

For most insects, a nutritionally complete diet must contain all or most of the following: protein or amino acids, carbohydrates, fatty acids, cholesterol, choline, inositol, pantothenic acid, nicotinamide, thiamine, riboflavin, folic acid, pyridoxine, biotin, vitamin B_{12}, β-carotene or vitamin A, α-tocopherol, ascorbic acid, several minerals, and water (Vanderzant, 1974). The nutrients listed can sometimes be substituted by compounds of related structure (Vanderzant, 1974). Insects that interact with symbionts involve the supplementation of essential or other key amino acids by the microbial guests (Cohen, 2003). Most other insects use whole proteins as their principal source of nitrogen (Cohen, 2003).

Soybeans contain good quality protein but they must be heat treated because raw soybeans can contain toxic substances (Vanderzant, 1974).

Lipids in insect diets are important and their main functions are as building-blocks of cell membranes (including sterols and phospholipids), hormones, nutrient transporters, sources of energy, and as structural material for building other molecules (Cohen, 2003). In most phytophagous insects, ascorbic acid is an essential nutrient, however, it is very unstable and losses can occur at room temperature (Dadd et al. 1967). Therefore, freezing diets at -20°C will greatly reduce the loss of ascorbic acid for up to three weeks (Dadd et al. 1967). Thiamin, riboflavin, nicotinic acid, pyridoxin, folic acid, pantothenate, biotin, and the lipogenic factors, choline and inositol, are the nine water-soluble vitamins commonly needed by insects (Dadd et al. 1967). The first six are generally considered indispensable for all insects except those that harbor microorganisms with synthetic abilities which allows them to provide the dietary dispensable vitamins (Dadd et al. 1967).

Carbohydrates are used by insects as building materials and as fuel (Cohen, 2003). The cuticle of insects contains chitin, a polysaccharide made of amino sugars (Cohen, 2003). Most insects cannot digest or utilize some carbohydrates (cellulose) but they may be useful as fillers (bulk) in diets that help promote intestinal mobility (Cohen, 2003). Phytophagous insects in particular fail to thrive on diets low in carbohydrates, each specific insect must have the type of carbohydrate fitted to them (Cohen, 2003). Sugars that are usable by some insects cannot be used by other insects (Cohen, 2003). A majority of ingredients already contain some minerals or salts so the overall mineral composition of a diet is not identical to the salt mixtures that are added to the diet and if an insect requires a mineral, that mineral

must be present in the diet in adequate amounts and appropriate form because minerals cannot be biosynthesized (Cohen, 2003). The mineral nutrition in insects is still a poorly understood aspect of insect nutrition as a whole (Cohen, 2003).

The pH of the gut is influential in terms of absorption kinetics and as a modifier of digestive enzyme activity (Cohen, 2003). pH can influence the diet's palatability, stability, activity of preservatives, solubility of nutrients, and probably several other factors (Cohen, 2003). Antifungal agents generally only work at a lower acidic pH but even without antibiotics, bacterial growth is suppressed at a lower pH (Cohen, 2003). In most insects reared in the laboratory, microbial growth is controlled in diets by the addition of chemical inhibitors, fumigants, and adjustment of pH (Vanderzant, 1974). These procedures have moderate success if the ingredients aren't initially contaminated, the equipment and environment are clean, and the insect eggs are washed (Vanderzant, 1974). Antimicrobial agents are sometimes toxic to insects but the degree of toxicity depends on the insect species, the kind and concentration of the agent, and the diet (Vanderzant, 1974). For example, Mittler found that antibiotics inhibited growth, development, and survival of the green peach aphid, *Myzus persicae*, by suppressing the growth of its symbionts (Vanderzant, 1974).

It is important to change artificial diets in a timely manner to avoid deterioration of the diet but this can adversely affect it in other ways (Dadd et al. 1967). For instance, the longevity and larviposition of *M. persicae* was severely reduced when they were handled daily as opposed to every other or couple of days (Dadd et al. 1967). In aphids, if the diets cannot sustain aphid cultures beyond two or three generations, the diet probably doesn't

contain all the essential nutrients or contains some in inadequate quantities (Mittler and Koski, 1976).

Water is the most fundamental nutrient and without the appropriate amount of water, all life processes fail (Cohen, 2003). In general, the normal amount of water present in an insect's natural food is required in an artificial diet (Cohen, 2003). For example, leaf feeders are adapted to food that is about 90 percent water (Cohen, 2003). An insect can become water stressed even with the right percentage of water if the nitrogen content is too high (Cohen, 2003). A diet like this could cause the insect to excrete extra waste nitrogen which would force it to excrete an inordinate amount of water to rid itself of toxic nitrogenous wastes (Cohen, 2003).

Stimulants

Even if an insect is presented with the most nutritious diet, they will not thrive on it unless it is eaten (Vanderzant, 1974). The majority of diets contain special components that stimulate normal feeding responses and are called token stimuli (no nutritional value) or phagostimulants (contain nutritional value) (Cohen, 2003). Phagostimulants are a feeding stimulant or substance that elicits a feeding response in a target species (Cohen, 2003). Several nutrients including sugars, some amino acids, lipids, ascorbic acid, and minerals double as feeding stimuli (Cohen, 2003). Token stimuli stimulate the feeding process such as biting, chewing, and swallowing (Cohen, 2003). Wheat germ contains substances that stimulate a feeding response in insects, but can be a feeding deterrent for some insects (Vanderzant, 1974). Ascorbic acid was also found to be a feeding stimulant as well as an essential nutrient for the growth of locusts (Vanderzant, 1974) and several other

phytophagous insects (Cohen, 2003).

For decades, the essentiality of vitamins, A, C, and E among insects has been known but the potential function of these vitamins as antioxidants in insects and in the diets themselves has been only recently recognized (Cohen, 2003). In phytophagous insects, vitamin C is essential and serves as a phagostimulant, antioxidant, and possibly as other defensive reactions (Cohen, 2003). Water-soluble vitamins generally have a relatively short half-life in insects because they are readily excreted and lost from the insect's metabolic pool because of their solubility (Cohen, 2003). The use of fillers such as cellulose and gelling agents such as agar can be used to modify diet texture to create a desirable texture for the insect (Cohen, 2003).

Color plays a large part in recognition and acceptance of foods by insects (Cohen, 2003). Insect diets often have natural colors that are associated with various nutrients such as plant pigments (chlorophyll, carotenes) and not only add color to the foods, but also serve as antioxidants (Cohen, 2003). As a rule of thumb, the brighter the color of the food, the higher the concentration of some type of antioxidant (Cohen, 2003). For example, white grapes contain lower concentrations of antioxidants than red grapes (Cohen, 2003).

Microbial Symbionts

Adelgids contain primary endosymbiotic bacteria that are housed in special cells called bacteriocyctes, and secondary endosymbionts that are generally found outside of bacteriocytes (Buchner 1965, Havill and Foottit 2007). Endosymbiotic bacteria in adelgids has not been studied in as much detail as in aphids but primary endosymbionts in aphids synthesize essential amino acids that the insects cannot produce themselves and are necessary

for survival, and secondary endosymbionts have been implicated in defense from parasitoids and pathogens (Havill and Foottit, 2007). The specific role of adelgid endosymbionts are still unknown but treatment with antibiotics on an insect in the same family as BWA, the hemlock woolly adelgid, *Adelges tsugae*, killed their endosymbionts and resulted in death, which suggests that endosymbionts play a critical role in nutrition (Havill and Foottit, 2007).

Microbes play a major role in insect rearing in three ways: The microbes may be symbionts in or on the body that are engaged in either a neutral or mutually beneficial relationship with the insect, microbes may occur as pathogens or parasites of the insects, or microbes may occur as contaminants of the diets or other rearing materials in cultures (Cohen, 2003). Termites and their microbes as well as most Homopterans and their microbes are great examples of mutually beneficial relationships (Cohen, 2003). In aphids, the addition of yeast extract at two percent to a holidic diet appears to provide the insect-symbiote complex with nutrients in adequate amounts to sustain aphid growth at 60 to 80 percent of that on plants (Mittler and Koski, 1976). Riboflavin has been found to be detrimental to the growth of some aphid species (Mittler and Koski, 1976) because an excess amount results from production by microbial symbionts, but it is probably essential to most other insects (Cohen, 2003).

Insects that do not have associations with microorganisms have a dietary requirement for sterols for normal growth, metamorphosis, and reproduction (Mittler 1970, Robbins et al. 1971). Sterols serve as structural components of cells and tissues and as precursors for essential steroid metabolites and regulators such as hormones (Robbins et al. 1971). Insects must obtain essential cholesterol from either their diet or from a dietary sterol that can be

readily converted to cholesterol because they lack the capacity for the *de novo* biosynthesis of the steroid nucleus (Robbins et al. 1971). The requirement of insects for dietary sterols is the only proved unique difference between insect and mammalian nutrient requirements (Vanderzant, 1974). Phytophagous and omnivorous insects use C_{28} and C_{29} plant sterols instead of cholesterol and the conversion of the phytosterols to cholesterol may be a prerequisite to the utilization of these compounds in certain species (Robbins et al. 1971).

THESIS RESEARCH OBJECTIVES

The present studies focus on understanding the necessary requirements to successfully develop artificial rearing techniques for BWA. Five objectives were investigated in order to better understand these requirements, to: 1) Produce laboratory reared BWA on a continuous basis from Fraser fir logs or seedlings to determine the best substrate (logs or seedlings) and optimum environmental conditions for BWA development and reproduction. 2) Analyze the bark of infested and un-infested trees for texture and bark hardness and to develop a membrane that simulates characteristics of the bark that favor BWA infestation. 3) Analyze chemical properties of Fraser fir bark and wood, BWA guts, and honeydew to identify proteins and carbohydrates present. 4) Rear the green peach aphid on an artificial diet as a model for the efficacy of ingesting a host defense chemical for the BWA. And finally, 5) Evaluate semi-artificial and artificial diets for BWA.

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CHAPTER II

Assessment of Essential Environmental Conditions and Preference for Rearing the Balsam Woolly Adelgid, *Adelges piceae*, on Natural Host Material

ABSTRACT

The balsam woolly adelgid (BWA; *Adelges piceae* Ratzeburg) is an exotic pest that has killed thousands of acres of Fraser fir, *Abies fraseri* (Pursh) Poir, an important natural resource and prominent Christmas tree species. This study aimed to determine the optimum environmental conditions for rearing BWA on natural host material (logs or seedlings) by varying temperature, photoperiod, and humidity. The availability of laboratory-reared BWA throughout the year would be useful for research on host plant resistance, biological control, and other approaches to control this insidious pest. Five different locations were chosen to test for optimum environmental conditions: three incubators of which we controlled the temperature and relative humidity (RH) at 17°C, 20°C, and 25°C with an RH of 75 percent. The insectary temperature was maintained at 21.1°C and 72 percent RH and the greenhouse had varying temperatures and RH. The 20°C temperature appeared to be the most successful for BWA development. The 17°C and 20°C incubators varied drastically, and did not show equal success. Overall, the least favorable environment was the 25°C incubator and full development was reached on logs in all five environments. The buds were the most successful position for BWA development. In reference to long term rearing in the laboratory, excised branches might not serve as good surrogates for whole trees. Future rearing of BWA in the lab should be done with logs or seedlings in a large enough area to permit adequate airflow, set at 20°C and 75 percent RH.

INTRODUCTION

Since its introduction to the U.S, the balsam woolly adelgid (BWA; *Adelges piceae*) has swept through the southern Appalachians, killing thousands of acres of Fraser fir, an important natural resource and prominent Christmas tree. Fraser fir, *Abies fraseri*, occurs naturally only in the southern Appalachians and before the devastation of BWA, was the dominant tree at the highest elevations (Dull et al. 1987). Fraser fir is a high elevation sub-alpine species that can live for 150 years and is not only important for its scenic beauty, but also for providing a home for many wildlife species, such as the Carolina northern flying squirrel (*Glaucomys sabrinus* Shaw) and the spruce-fir moss spider (*Microhexura montivaga* Crosby and Bishop), now both endangered species due to habitat loss from BWA (Cooper et al. 1977, Terwilliger and Tate 1994, USFWS 2000).

North Carolina Fraser firs are the most popular Christmas tree in North America and are shipped to every state in the U.S. as well as other locations all over the world (McKinley et al. 1996). Fraser fir Christmas tree sales are a multi-million dollar industry. North Carolina is the second largest Christmas tree producer in the U.S., which brings in over 100 million dollars to the state annually (McKinley et al. 1996). To date, control of this exotic pest has proven to be extremely difficult because of its feeding behavior. It is assumed that BWA saliva injects a substance into the bark while feeding, causing atypical development in bark and newly formed wood (Mitchell 1966, Dull et al. 1988). Unknown compounds present in the saliva stimulate cell growth, cause changes in the composition and anatomy of the bark, and affect a premature transition of sapwood into heartwood (Timell, 1986). Abnormal annual rings are produced in the woody tissue that disrupts water conduction to the

crown, often resulting in tree death within two to three years (Balch 1952, Dull et al. 1988).

The availability of laboratory-reared BWA throughout the year would be useful for research on host plant resistance, biological control, and other approaches to control this insidious pest. The research on host resistance/tolerance to BWA has been promising and suggests that there is potential for a screening and breeding program for BWA resistance/tolerance. Although the majority of mature Fraser fir trees have been killed within their native range, populations remain that have survived multiple decades of adelgid attack. Current techniques for screening for resistance involve collecting adelgids from naturally infested host material but the quality of adelgids collected in the field can be highly variable. Therefore, in order to carry out these studies, it would be advantageous to have a steady source of adelgids produced of similar quality.

In the rearing process, insects are taken out of their natural setting and have stresses inflicted upon them that they never face in nature (Cohen, 2003). One can try to mitigate these issues by presenting the insect with optimal conditions such as temperature, humidity, geo- and phototaxic preference, and nutrition based on previous studies (Bell et al. 1981). However, current research on optimal conditions and rearing for BWA is lacking. Therefore, our first objective in this study was to determine the optimum environmental conditions for rearing BWA on natural host material (logs or seedlings) by varying temperature, photoperiod, and humidity. Previous studies have shown that rearing BWA on their natural host material is effective in producing BWA infestations when the infested material is changed regularly and the material is checked for infestation at set intervals (Newton et al. 2011). Our second objective was to evaluate un-infested Fraser fir bark discs to further

determine texture and geotaxic preference as well as the percentage of crawlers that actually insert into their host material. Several researchers have found that orientation of crawlers shows predominantly photonegative behavior although some crawlers do orient toward the light (Balch 1952, Atkins and Hall 1969, Havill and Foottit 2007). Since BWA are passively dispersed and are easily affected by environmental conditions, we hypothesize that the percent of BWA that actually insert into the host material is less than 20 percent.

MATERIALS AND METHODS

We chose five locations to test for optimum environmental conditions: three incubators of which we controlled the temperature and relative humidity (RH) at 17°C, 20°C, and 25°C with an RH of 75 percent (16:8 L:D). The insectary temperature was maintained at 21.1°C and 72 percent RH (14:10 L:D) and the greenhouse had varying temperatures and RH. Amman (1968) found that any temperature between 11 and 20°C coupled with any relative humidity between 75-98 percent gives approximately equal hatching success for BWA. Atkins and Hall (1969) found that crawlers dropping to the forest floor and vigor declines as temperatures increase and warm periods may reduce crawler populations considerably due to the dropping of low-vigor crawlers. Therefore, we hypothesize that the 17°C and 20°C incubators will exhibit equal success if maintained at an RH of 75-98 percent and BWA development in the 25°C incubator would have the lowest numbers of crawlers.

Seedlings had been stored in the greenhouse prior to this study, so we placed the seedlings in the other four environments in the beginning of February 2012, one month before the study started to give them time to acclimate to the environment. The un-infested

cuttings, seedlings, and logs, and infested bolts were added in March 2012. Infested and un-infested Fraser fir logs were collected from trees in an abandoned Christmas tree farm in Avery County in March of 2012 and brought back to North Carolina State University. Excised branches from five different un-infested trees were also collected to be used as cuttings in all environments, which would allow comparison among the same five genotypes within each environment. The infested Fraser fir bolts were set up using the suspended bolt method, an effective technique for artificially infesting fir trees with BWA, which mimics natural infestation, and is more economical on time than attaching bark (Newton et al. 2011). Seedlings, cuttings, and logs in all five locations were watered twice a week and temperature and RH were recorded at this time.

In the incubators and insectary, the infestation level was determined for *A. piceae* development 13 weeks after infestation by counting the number of crawlers, settled immatures, adults, and eggs present. For the greenhouse, the infestation level was determined after 11 weeks. The location that the crawlers settled on each substrate (top, middle, bottom, needle cushion, branch, bud, needle, crevice) was also noted. The condition (health) of each seedling, cutting, and log was assessed and recorded throughout the study. This study included a total of 14 un-infested logs, 37 seedlings, and a total of 45 excised cuttings from the five trees (nine cuttings per tree) collected in the field distributed throughout the five environments, and 12 excised cuttings corresponding to each of the 12 seedlings in the incubators (four seedlings in each incubator). All the excised branches (cuttings) were placed individually in 50 ml centrifuge tubes filled with wet sand. Both infested and un-infested logs had a blue, absorbent paper towel wrapped around each end of

the log that were kept moist to slow down dehydration. Analyses of variance (ANOVA) for cuttings, seedlings, and logs were done with log transformations to account for scale effects.

Incubators: Three incubators were held at a constant temperature of 17°C, 20°C, and 25°C with a RH of 75 percent. Humidity frequently observed in the field is 75 percent (Amman, 1968). We tried to maintain this humidity in the incubators by using saturated NaCl (Wexler and Hasegawa, 1954) placed in trays in the bottom of the incubators. Fluorescent grow lights with a timer installed were placed in each incubator to provide light for the seedlings and cuttings (16:8 L:D). Amman (1968) also observed crawlers on logs in direct sun move directly into shaded crevices or to the underside of the log. Therefore, the fluorescent grow light was placed directly above the infested suspended bolt to direct the crawlers toward the bottom of the bolt to allow them to "rain down" onto the un-infested material below. In each incubator, one infested log (60.96 cm long) was suspended horizontally over an un-infested log (30.48 cm long) placed perpendicular to the infested log, four Fraser fir seedlings (4-5 years old) potted in 25.4 cm high containers, excised branches from each tree, propped up in the tree that the cutting was taken from, and five excised branches (ten in 20°C) collected from the five un-infested trees collected for the logs (Figure 2.1).

Insectary: The insectary was held at a constant temperature of 21.1°C, and 72 percent RH (14:10 L:D). Four racks were set up in the insectary and two infested logs (121.92 cm long) were suspended from each rack, totaling eight infested logs. Below the infested bolts in each rack were two un-infested logs (60.96 cm long), one of which was perpendicular to the infested pieces and one was vertical, for a total of four vertical un-infested logs and four

horizontal un-infested logs (Figure 2.2). Four Fraser fir seedlings (4-5 years old) potted in 25.4 cm high containers were placed in each rack along with excised branches from the five un-infested trees collected for the logs. Two seedlings were added to a side bench as a control.

Greenhouse: The greenhouse had a natural photoperiod which permitted temperature and RH to fluctuate. Two infested logs (121.92 cm long) were suspended above three un-infested logs (60.96 cm long), two of which were perpendicular to the infested pieces and one that was vertical. Seven Fraser fir seedlings (4-5 years old) potted in 25.4 cm high containers were placed next to the logs along with cuttings from excised branches from the five un-infested trees collected for the logs.

Paired Samples: One excised branch from each of the 12 seedlings in the incubators was taken to see if there was a significant difference of total adelgids per centimeter found in a cutting compared to the seedling it came from. This was done to determine if cuttings could be used as surrogates for whole trees (Newton et al. 2011).

Sample Observations: At the end of the study period, cuttings were examined on all sides to count the number of crawlers, settled nymphs, adults, and eggs present under the microscope. A dissecting scope was used for these examinations. The location of the adelgid was also noted, including the needle, needle cushion, stem, or bud. A subsampling scheme developed by Newton et al. (2011) found that a subsample of the bushiest branch from the second whorl from the top was indicative of overall seedling infestation. Therefore, we took a subsample for each seedling and counted it as we did with cuttings. On each side of the log, three spots were chosen (top, middle, bottom), with a diameter of 4.5 cm and total

area of 15.90 cm^2 per spot, to make a total of 12 spots and a total area of 190.8 cm^2 for each log observed. The number of crawlers, settled nymphs, adults, and eggs present were recorded at each spot. If there were branches on the log that had visible woolly masses, it was noted or recorded if chosen as an observed spot, but most of the logs did not have branches.

Percent Inserted: Un-infested Fraser fir bark discs were cut with a cork corer (3 cm in diameter) and under the microscope (20X) texture was determined (high= a lot of roughness, medium= medium roughness broken up with flat/smooth areas, and none= smooth and completely flat). A total of 15 discs were set up in two incubators, held at a constant temperature of 17°C and 20°C with an RH of 75 percent. Four discs standing vertically (leaned up against the petri dish) and four horizontally were placed in the 17°C incubator and three discs standing vertically and four horizontally were placed in the 20°C incubator. Petri dishes were filled with sand and shop cloth was placed on top. Bark discs were placed on top of the shop cloth and the shop cloth was watered and kept moist to keep the bark discs on top hydrated. BWA crawlers and eggs were placed by hand in the center of each disc (Table 2.11), and were monitored daily. Observations included the number of hatched and unhatched eggs, stationary or mobile crawlers and their location on the wood disc (eg. bottom left in a crevice). Change in BWA appearance was also noted once the crawlers inserted and started to feed (presence of wax, change in color, etc.). Once there were no more crawlers, and/or eggs that remained were unhatched and appeared damaged, the study was ended and the final number and location of crawlers that inserted into the bark was counted.

RESULTS

Cuttings: There was a significant environmental effect as well as a significant interaction between sample and environment on the log transformed data (Table 2.1). The infestation of all adelgids per centimeter length was highest in the insectary and lowest in the 25°C incubator (Table 2.2). Among all environments, the most abundant life stage observed was second instars (Figure 2.3) and overall appeared highest in the 20°C incubator and lowest in the 25°C incubator (Table 2.3). Complete development and egg production in cuttings was only seen in the insectary. Position preference on the cuttings showed 35 percent of all adelgids preferred to insert into the buds (Figure 2.4), which was also the most successful position for adelgids to reach full development (Figure 2.5).

Seedlings: No significant differences were found among seedlings. There was no significant environmental effect for the log transformed data (Table 2.4). The infestation of all adelgids per centimeter length was highest in the 20°C incubator and lowest in the 25°C incubator (Table 2.5). Among all environments, the most abundant life stage observed were second instars (Figure 2.6) and overall appeared highest in the 20°C incubator and lowest in the 25°C incubator (Table 2.6). Full development was seen in the insectary and greenhouse and eggs were observed in the 17°C and 20°C incubators, but no adults. Position preference on the seedlings showed 49 percent of all adelgids preferred to insert into the buds (Figure 2.7), which was also the most successful position for adelgids to reach full development (Figures 2.8 and 2.9).

Logs: Among all environments, the most abundant life stage observed were adults and overall adelgids appeared highest in the insectary and lowest in both the greenhouse and

the 25°C incubator (Table 2.7). Full development was reached in all five environments but eggs were not. Because the woolly masses are easy to observe, looking outside of the chosen sample locations showed there were other adelgids, but the infestation level was still very low. ANOVA for logs was not done because the logs were barely infested across all environments and we concluded further analysis wasn't necessary.

Paired Samples: There was a significant environmental effect as well as a significant effect between sample types (Table 2.8). The infestation of all adelgids among cuttings and seedlings was highest in the 17°C and 20°C incubators and lowest in the 25°C incubator (Table 2.9) and seedlings were five times more infested with adelgids than cuttings (Table 2.10).

Environment Conditions: The mean temperature and relative humidity (RH) was recorded for each environment and was found to be 17.4°C (51.2%) in the 17°C incubator (Figure 2.11), 20.1°C (65.4%) in the 20°C incubator (Figure 2.12), 26.2°C (80.6%) in the 25°C incubator (Figure 2.13), 21.3°C (65.2%) in the insectary (Figure 2.14), and 24.2°C (56.1%) in the greenhouse (Figure 2.15). The mean temperature in each environment was fairly close for all the set temperatures but the RH varied a lot. An RH of 75 percent was set for all of the environments except the greenhouse, but no mean RH reached 75 percent. Most locations were under 75 percent except the 25°C incubator, which was above at 80.6 percent.

Mortality: All of the cuttings died during the study and had the shortest longevity in the greenhouse (44 days) and longest in the insectary (73 days) (Figure 2.16). Some seedlings were still alive by the end of the study (Figure 2.17): two in the 17°C incubator, one in the 20°C incubator, and 10 in the insectary. All of the seedlings died in the

greenhouse and 25°C incubator. Across all environments among the seedlings that did die, the shortest longevity was in the three incubators (76 days) and longest in the insectary (111 days). Overall seedlings lasted longer than cuttings across all environments (Figure 2.18).

Percent Inserted: Discs with medium texture had about twice the amount of inserted crawlers compared to discs with high texture (Table 2.12) and all adelgids that inserted into the bark developed into second instars. No crawlers inserted into the discs with no texture. The 17°C incubator with discs standing vertically and 20°C incubator with horizontal discs had the highest percentage of inserted crawlers in each temperature (Table 2.12). Overall, the vertical discs in the 17°C incubator had the highest percentage of inserted crawlers (Figure 2.10) across both temperatures and disc positions (Table 2.13).

DISCUSSION

Since BWA are also passively dispersed, they might find host plants by searching randomly. However, once on a new host, the aphid or adelgid needs to recognize the host-plant. Host-plant recognition refers to the insect's choice to feed and/or oviposit on host plants and to leave nonhost plants (Visser, 1988). In the event an adelgid lands on a nonhost plant, she will most likely die because she lacks the means of easily dispersing to another host. Texture clearly plays a role in BWA preference. A smooth/flat surface as well as too much roughness was unfavorable, while a medium texture with flat areas separating crevices and buds was most favorable. This is possibly because it provides protection for BWA or provides better access to parenchyma cells.

Chemical cues from the plant surface also play a role in host plant selection and acceptance. Recent studies have shown primary metabolites on the surface of the plant can be detected by insects after making contact with the plant (Lombarkia and Derridj, 2008). Therefore, another possible explanation is the texture (crevices and buds) provides a chemical cue for crawlers to feed and in the absence of texture, the crawlers will leave in search of another food source.

The bottom side of the vertical bark discs, and on or around buds were the most favorable insertion sites for BWA. This suggests BWA show positive geotaxis preference. The optimum temperature appeared to be 17°C. The discs were a quick way to determine environmental preference but because of their small size, are not realistic for mass rearing adelgids. However, they could be used to infest artificial diets or as a control to compare against artificial diets. Less than 20 percent of crawlers inserted in host material, suggesting that artificial diets will need to be infested with a very large number of adelgids to get them to feed. This can also explain why rearing on artificial diets thus far has proven difficult.

Between the five different environments no significant differences were found because there was a lot of variation throughout all the environments. This is probably due to a combination of environmental factors: RH was very hard to control in all of the environments, the light intensity in the incubators was different than in the greenhouse, and there was little airflow in the incubators. Therefore, it is hard to say if temperature and RH were direct factors in the results. However, due to the similarity of set up, the three incubators can probably best be compared. Amman (1968) stated that at intermediate temperatures, hatching success was best and crawler life was longest. He also found that

humidity greatly effected incubation periods, hatching success, and survival of crawlers. When comparing among incubators in our study, the 20°C appeared to be the most successful temperature for BWA development. The 17°C and 20°C incubators varied drastically, and did not show equal success. Overall, the least favorable environment was the 25°C incubator. This could be due to a number of factors including the combined temperature and RH being too high for the adelgids and very low air flow in the incubators. The space is much more confined and the low airflow could encourage more contaminants, both of which could lead to reduced success among rearing environments. Between cuttings and seedlings, the two worst environments were consistent: 17°C and 25°C incubators. The insectary's open space allows for more airflow and more material to be tested and observed in one environment. This was also the only environment where adelgids were able to develop into adults on every substrate. Among the seedlings, eggs were present but adults were not observed in the 17°C and 20°C incubators. This is likely from adelgids getting accidentally knocked off while moving the seedlings and or possibly getting knocked off when everything was watered twice a week.

In every environment, the logs had adelgids develop into adults but no eggs were observed and infestation levels were very low. This is possibly because the length of the study didn't provide enough time to reach full development or development was slowed due to log stress. The sampling technique could have also been inadequate: there may be more hiding places making the adelgids harder to see, the spots observed could have been less infested by chance, or the size of the logs compared to the seedlings and cuttings requires more time to reach a high infestation rate. Another reason is possibly because the logs were

cut and dried out and was no longer making suitable metabolites BWA needs in order to insert and therefore didn't appear favorable to BWA once they landed on it. Specific volatiles from the plant usually aid in host recognition (Visser, 1988). Fir trees, like other conifers, release volatile compounds such as monoterpenes, sesquiterpenes, and terpenoids, from the foliage and other tissues, giving the tree a distinctive aroma (Rudloff, 1966). Therefore, the volatiles released from needles are possibly needed for BWA to identify its host material. The un-infested logs we used had most of the branches and needles removed so BWA possibly couldn't identify their host. Future rearing in the lab should include logs that contain branches with a lot of needles to see if that increases the infestation rate and provides an optimal rearing substrate for BWA. Future studies should also be done at longer time intervals to allow the logs more time to become infested.

The highest life stage observed in the cuttings and seedlings was usually second instars. In cuttings, the only environment that went past second instar was the insectary. This is possibly due to the high infestation level on a small surface area where BWA competed for nutrients. Once adequate nutrients were taken up, it inhibited further development and resulted in death of the cuttings and seedlings. The reason the adelgids on the cuttings didn't progress could also be due to cutting death. Host mortality can play a role in BWA success. All of the cuttings died before the duration of the experiment was finished. Several seedlings also died but 10 of the 18 in the insectary did not die. However, the two control seedlings placed in the insectary (neither became infested) both died. Again, this can be due to a combination of environmental factors. Overall, seedlings lasted longest in the insectary.

Between cuttings and seedlings, BWA were fairly consistent among the position they chose. Buds were the most successful position to reach development for BWA, possibly because it contains more nutrients. This was evident in both seedlings and cuttings. Excised branches are under more physiological stress than seedlings as well as lack roots, which alters normal active growth (Newton et al. 2011). The cuttings also died quicker than seedlings and more adelgids overall seemed to insert into seedlings. This is possibly because infestation levels became too high in the cuttings, forcing adelgids to compete for nutrients and thus using up the cuttings nutrient supply whereas the seedlings still had roots and weren't as stressed. In this study we found paired samples of cuttings were not indicative of equal success as seedlings because cutting longevity was compromised. In terms of future research involving host resistance or rearing in the laboratory, excised branches might not be adequate substitutes for whole trees. This study aimed to determine the optimum environmental conditions for rearing BWA on natural host material. Future rearing of BWA in the lab should be done with seedlings in a large enough area to permit adequate airflow, set at 20°C and 75 percent RH, and infested material should be checked at closer intervals than was done in this study to confirm BWA development. Infested Fraser fir bolts continue to produce thousands of crawlers after being hung for a long period of time and shouldn't need to be changed often.

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Table 2.1 Results of analyses of variance for cuttings (prob > F)*.

Source	Environment Effect: Cuttings
Sample #	0.2461
Environment	0.0451
Sample* Environment	0.6446

*Tukey-Kramer log transformed value.

Table 2.2 The number (per centimeter) of all adelgid life stages across all environments for cuttings*.

Environment	All Adelgids
Insectary	4.20 (2.767)^{A}
Greenhouse	3.04 (2.767)^{AB}
20 C Incubator	2.89 (1.956)^{AB}
17 C Incubator	0.16 (2.767)^{AB}
25 C Incubator	0.07 (2.767)^{B}

*Nontransformed means in the same column followed by the same letter are not significant according to a=0.05, according to Tukey Kramer HSD procedure. Mean (Std Error).

Table 2.3 The number (per centimeter) of different adelgid life stages across all environments for cuttings*.

Environment	Stationary crawlers	2nd Instar	3rd Instar	Adults	Eggs
17 C Incubator	0.02 (0.022)	0.13 (0.042)	0	0	0
20 C incubator	0.76 (0.102)	5.02 (3.591)	0	0	0
25 C Incubator	0.07 (0.102)	0	0	0	0
Greenhouse	0.58 (0.496)	2.47 (2.411)	0	0	0
Insectary	0.93 (0.139)	2.64 (1.037)	0.07 (0.044)	0.42 (0.422)	0.13 (0.133)
All	0.47 (0.122)	2.05 (0.896)	0.01 (0.010)	0.08 (0.084)	0.03 (0.027)

*Mean (Std Error).

Table 2.4 Results of analyses of variance for seedlings (prob > F)*.

Source	Environment Effect: Seedlings
Environment	0.0731

*Tukey-Kramer log transformed value.

Table 2.5 The number (per centimeter) of all adelgid life stages across all environments for seedlings*.

Environment	All Adelgids
20 C incubator	1.72 (0.411)^{A}
Greenhouse	1.58 (0.347)^{A}
Insectary	1.26 (0.217)^{A}
17 C Incubator	0.22 (0.751)^{A}
25 C Incubator	0.12 (0.919)^{A}

*Nontransformed means in the same column followed by the same letter are not significant according to a=0.05, according to Tukey Kramer HSD procedure. Mean (Std Error).

Table 2.6 The number (per centimeter) of different adelgid life stages across all environments for seedlings*.

Environment	Stationary crawlers	2nd Instar	3rd Instar	Adults	Eggs
17 C Incubator	0.19 (0.074)	0.04 (0.036)	0.05 (0.045)	0	0.05 (0.045)
20 C incubator	0.80 (0.237)	7.78 (4.105)	0	0	0.04 (0.038)
25 C Incubator	0.12 (0.068)	0	0	0	0
Greenhouse	0	6.22 (1.795)	0	0.09 (0.058)	0
Insectary	0	4.82 (1.038)	0.10 (0.075)	0.13 (0.089)	0
All	0.12 (0.048)	4.37 (0.819)	0.05 (0.037)	0.08 (0.045)	0.01 (0.006)

*Mean (Std Error).

Table 2.7 The number (190.8 cm^2 per log) of different adelgid life stages across all environments on logs*.

Environment	Stationary crawlers	2nd Instar	3rd Instar	Adults	Eggs	All BWA
17 C Incubator	0.07	0	0	0.1	0	0.16
20 C incubator	0	0	0	0.13	0	0.13
25 C Incubator	0	0	0	0.07	0	0.07
Greenhouse	0	0.02 (0.025)	0	0.03 (0.016)	0	0.06 (0.041)
Insectary	0	0.05 (0.0311)	0	0.21 (0.066)	0	0.27 (0.086)

*Mean (Std Error).

Table 2.8 Results of analyses of variance from paired samples (prob > F)*.

Source	Environment Effect
Sample Type	0.0126
Environment	0.0006

*Tukey-Kramer log transformed value.

Table 2.9 The number (per centimeter) of all adelgid life stages across all environments in paired cuttings and seedlings 1-12*.

Environment	All Adelgids
20	12.96 (5.071)^{A}
17	14.55 (5.071)^{A}
25	0.27 (5.071)^{B}

*Nontransformed means in the same column followed by the same letter are not significant according to a=0.05, according to Tukey Kramer HSD procedure. Mean (Std Error).

Table 2.10 The number (per centimeter) of all adelgid life stages between paired cuttings and seedlings 1-12*.

Sample Type	All Adelgids
Cutting	3.02 (4.141)
Seedling	15.51 (4.141)

*Mean (Std Error).

Table 2.11 The percent (%) of settled crawlers that inserted by environment x position*.

Source	Start # Eggs	Start # Crawlers	Unhatched Eggs	# 2nd instars inserted	% Inserted
20C,V	20	15	1	1	2.9
20C,V	20	15	1	2	5.7
20C,V	20	0	1	0	0
20C,H	10	10	0	3	15.0
20C,H	10	10	1	4	21.0
20C,H	5	15	0	4	20.0
20C,H	20	0	1	0	0
17C,V	20	0	2	1	5.5
17C,V	15	15	1	9	31.0
17C,V	20	20	2	6	15.8
17C,V	5	15	1	2	10.5
17C,H	15	15	0	4	13.3
17C,H	20	0	0	0	0
17C,H	15	15	3	5	18.5
17C,H	5	15	0	2	10.0

*Unhatched eggs were deleted from the total count and not factored into the percent inserted. V= vertical and H= horizontal.

Table 2.12 The number of settled crawlers that preferred texture*.

Topography	2nd Instar
High	1.3 (0.142)
Medium	2.3 (0.620)
None	0 (0)

*Mean (Std Error).

Table 2.13 The number of settled crawlers and overall percent (%) of BWA that inserted by environment x position*.

Source	2nd Instar	% 2nd Instars
17C,H	1.6 (0.450)	11.3
17C,V	1.6 (0.360)	17.3
20C,H	1.4 (0.421)	14.1
20C,V	0.8 (0.596)	3.4

*V= vertical and H= horizontal. Mean (Std Error).

Figure 2.1 Environment set up in the incubator.

Figure 2.2 Environment set up in the insectary.

Figure 2.3 Heavily infested cutting with a majority of BWA around the needle cushions.

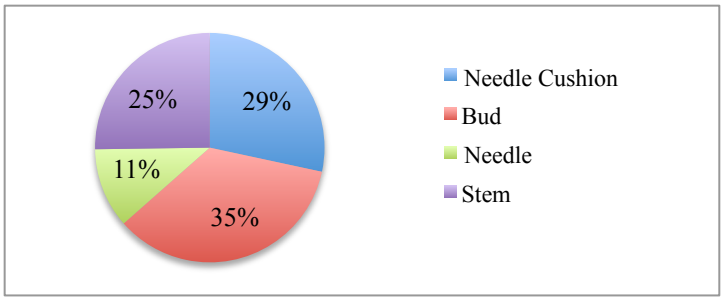

Figure 2.4 The percentage of all adelgids across position in cuttings.

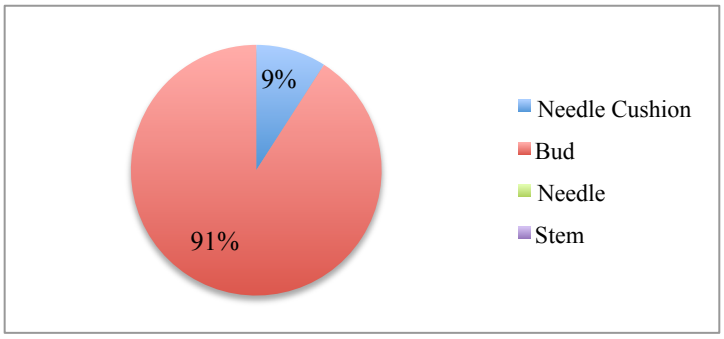

Figure 2.5 The percentage of adelgids that reached full development across position in cuttings.


Figure 2.6 Heavily infested seedling with a majority of BWA around the needle cushions.

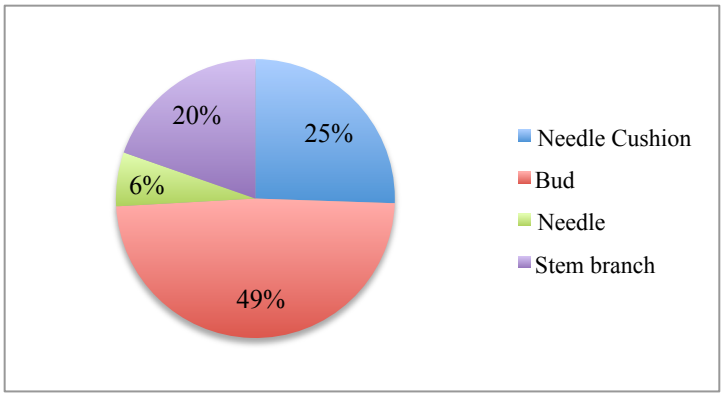

Figure 2.7 The percentage of all adelgids across position in seedlings.

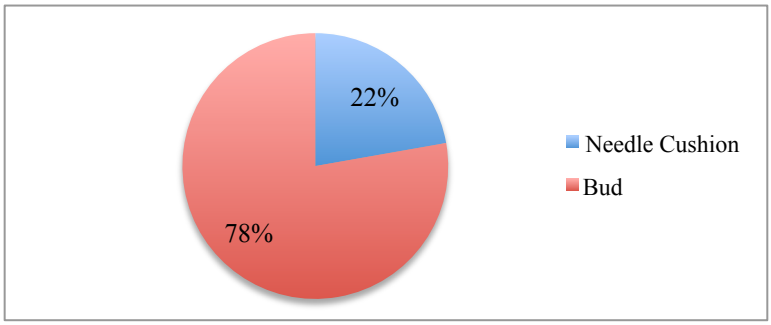

Figure 2.8 The percentage of adelgids that reached full development across position in seedlings.

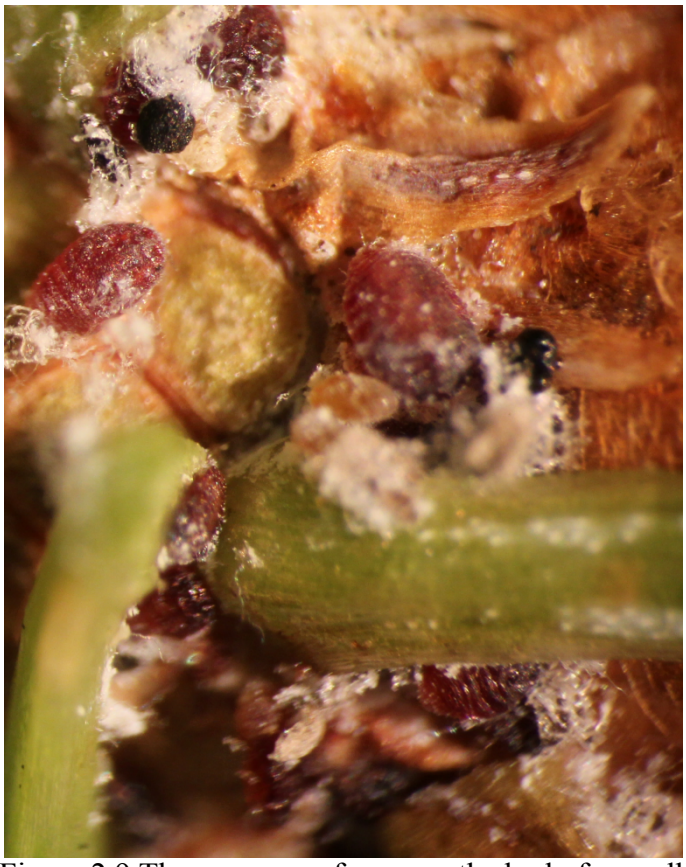

Figure 2.9 The presence of eggs on the bud of a seedling in the insectary.

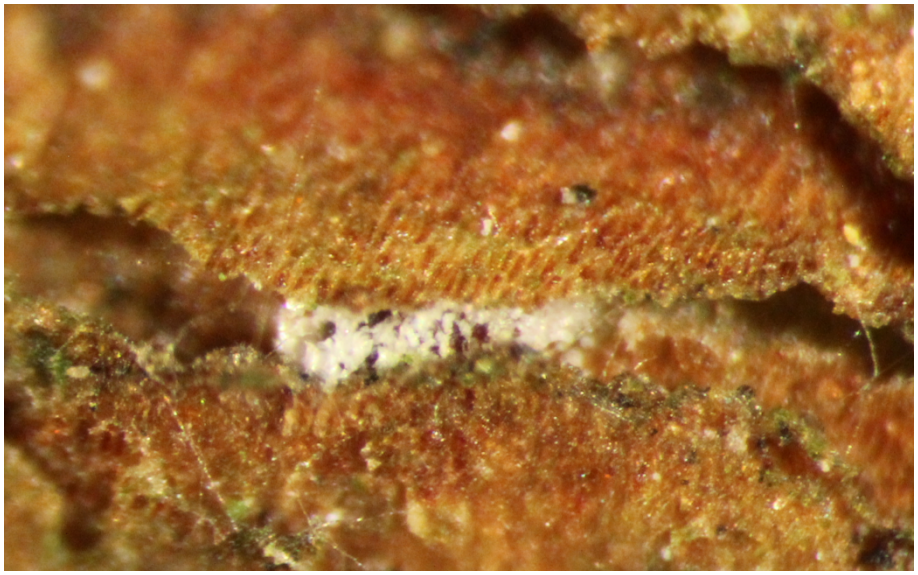

Figure 2.10 BWA developing in a crevice in cut discs, magnified 50x.

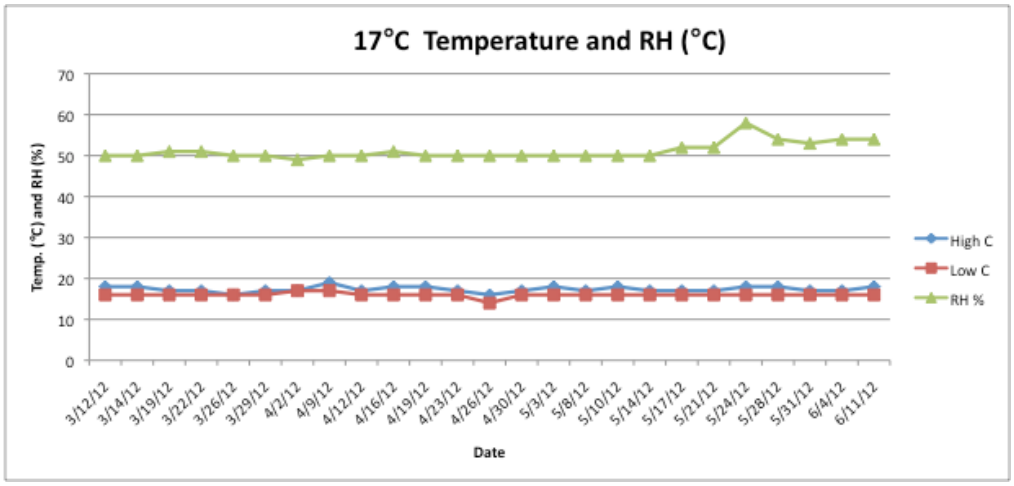

Figure 2.11 The mean temperature (RH) in the 17°C incubator: 17.4°C (51.2%).

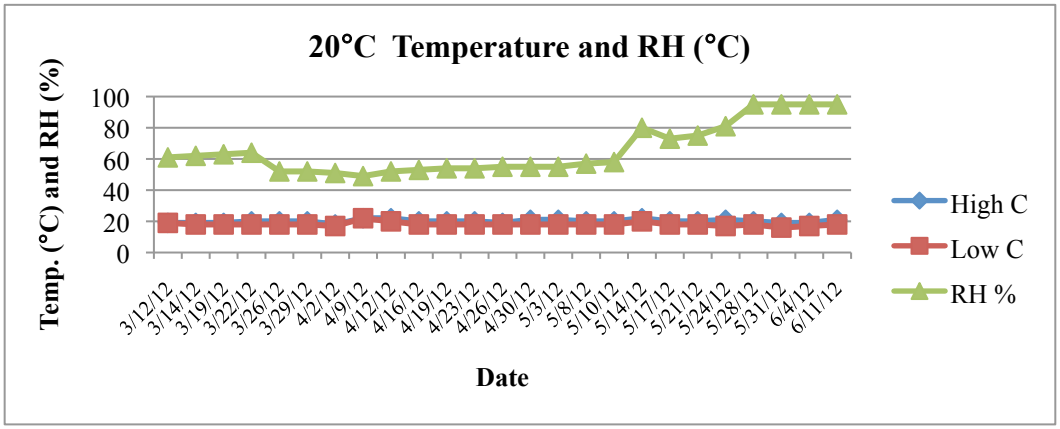

Figure 2.12 The mean temperature (RH) in the 20°C incubator: 20.1°C (65.4%).

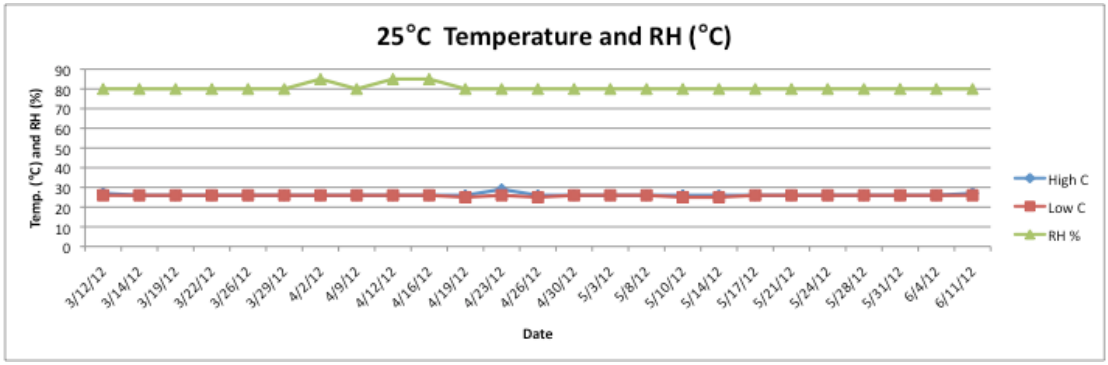

Figure 2.13 The mean temperature (RH) in the 25°C incubator: 26.2°C (80.6%).

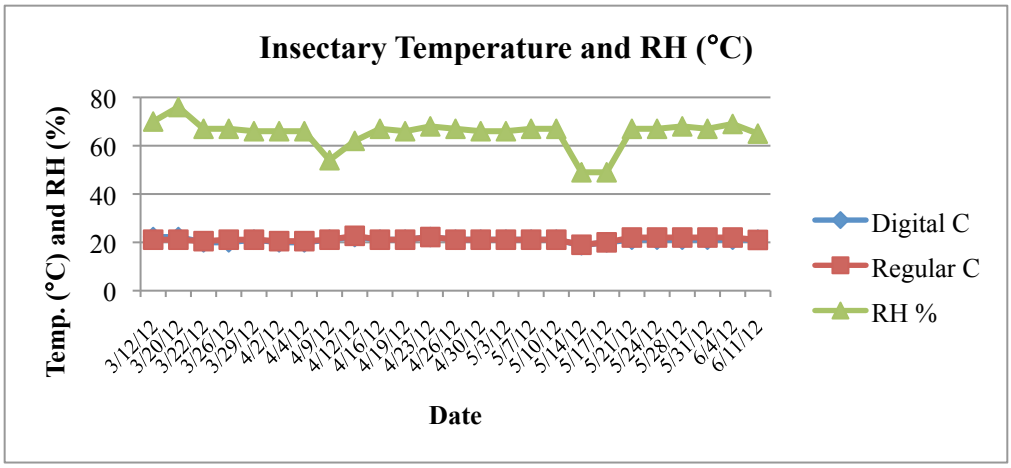

Figure 2.14 The mean temperature (RH) in the insectary: 21.3°C (65.2%).

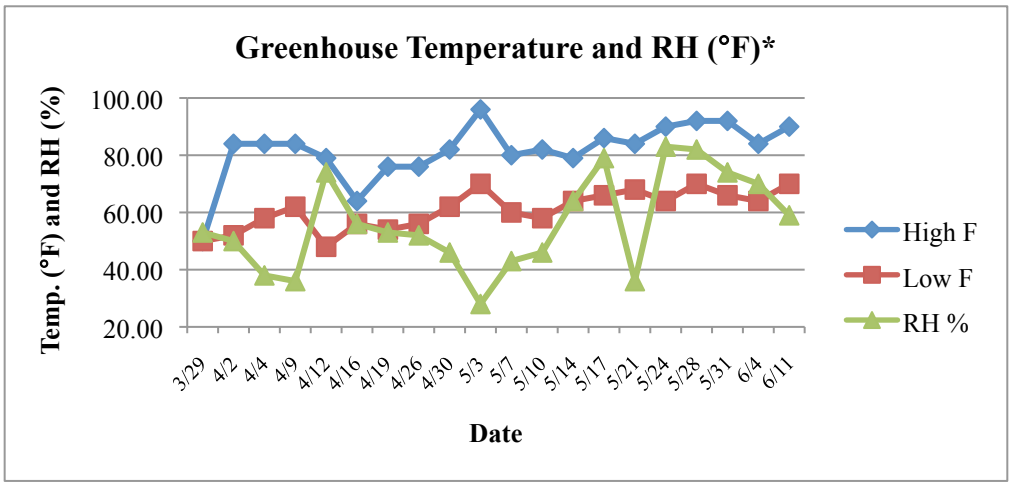

Figure 2.15 The mean temperature (RH) in the greenhouse: 24.2°C (56.1%).
*Recorded high/low in °F by greenhouse staff.

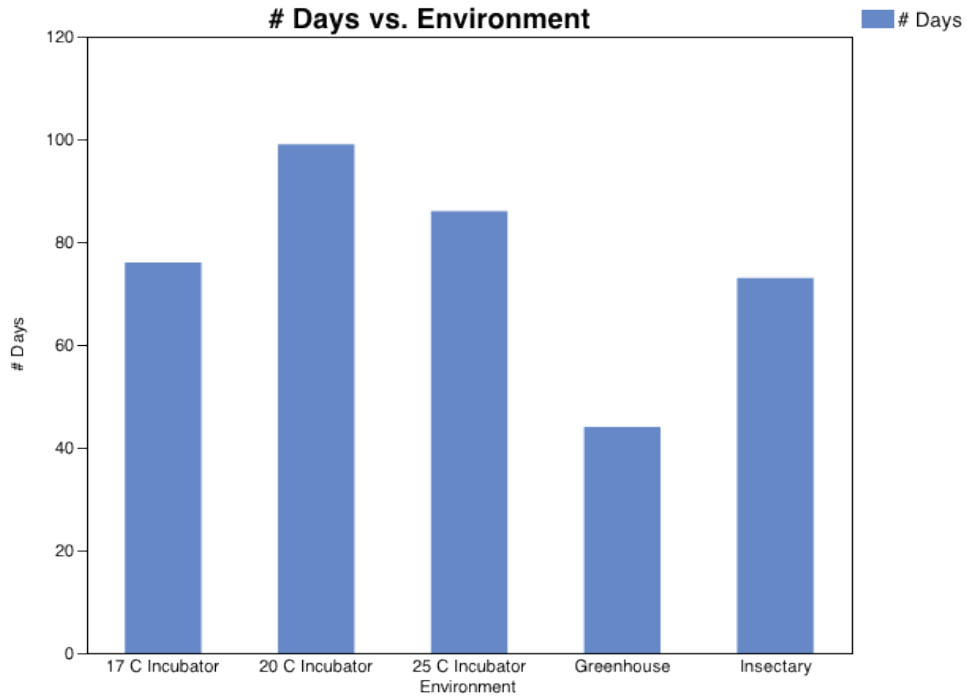

Figure 2.16 The number of days for cuttings to reach 100% mortality in each environment.

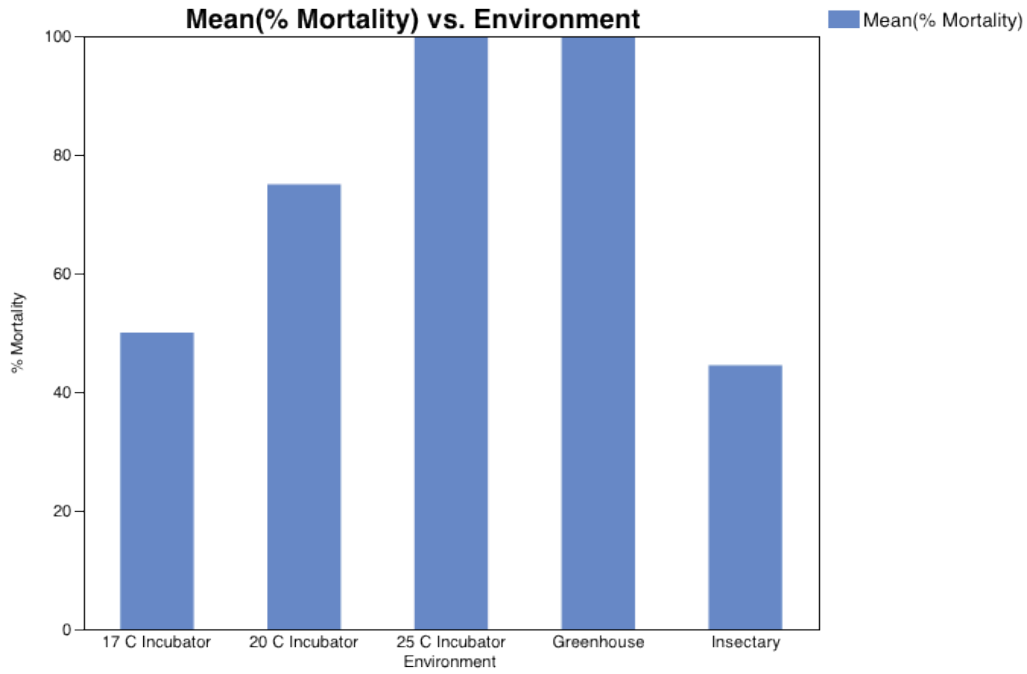

Figure 2.17 Mortality rate (%) at day 111 in seedlings in each environment.

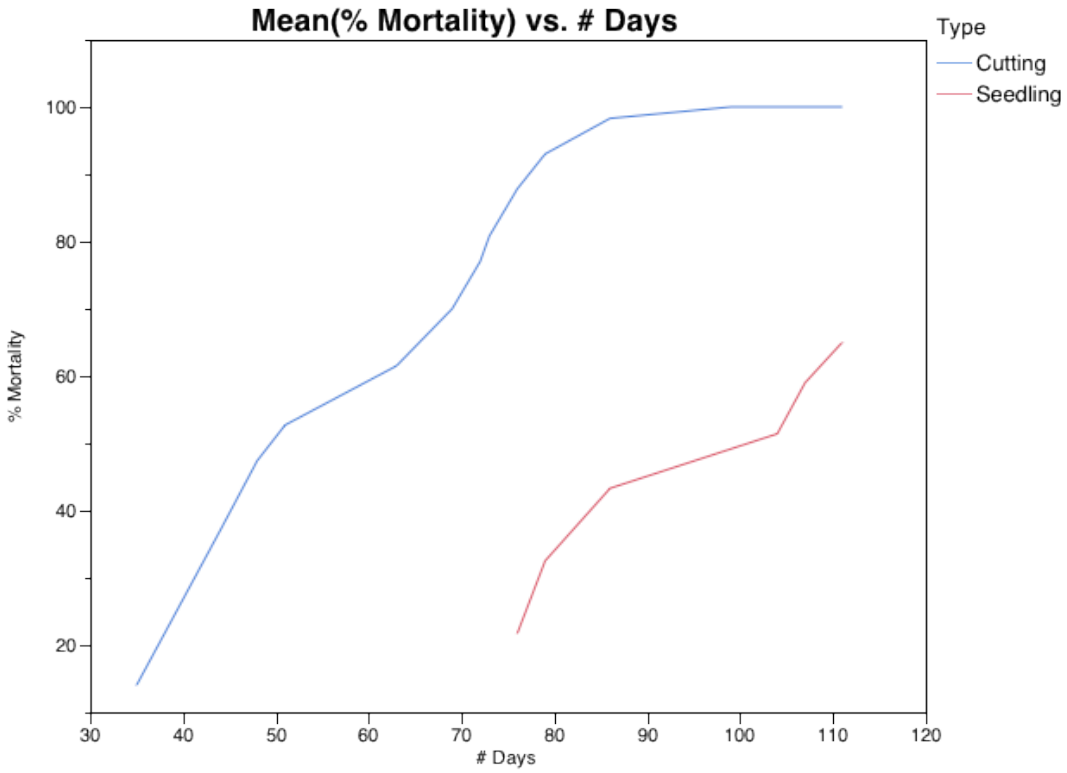

Figure 2.18 Mortality rate between cuttings and seedlings across all environments.

CHAPTER III

Novel Artificial Rearing Discoveries for the Balsam Woolly Adelgid, *Adelges piceae*

ABSTRACT

The balsam woolly adelgid (*Adelges piceae*, BWA) is a tiny, piercing-sucking insect and is a major pest in Fraser fir (*Abies fraseri*) Christmas tree plantations. We developed and analyzed artificial bio-based membranes that mimicked infested Fraser fir bark that were to be used to cover artificial diets for rearing BWA. Films were compared to favorable feeding characteristics of the bark and tested for antimicrobial properties. Finally, nutrients present in Fraser fir wood were analyzed as a starting point for BWA diet development.

Infested bark was found to be twice as hard as un-infested bark, suggesting an insect-induced response and possibly a tree defense mechanism against adelgid attack. The hardness of films, unblended hardwood-beeswax (0.4 mJ) and blended hardwood-beeswax (0.35 mJ), were between the un-infested and infested bark hardness levels and are suggested as candidates for further testing of BWA film preferences. Blended hardwood was most resistant to microbial growth. Beeswax samples were more resistant to microbial growth than paraffin samples, and Fraser fir discs became contaminated quicker compared to the films. Ion chromatography (IC) results demonstrated that glucose levels of infested bark was 3.5 x higher than un-infested bark. Thus, it is hypothesized that BWA modifies the bark causing a response in the tree to generates more glucose increasing nutrient availability for the adelgids. This study has presented a novel approach in rearing an insect species by developing an artificial bark substrate as well as forming alliances with a multi-disciplinary team of specialists. Further IC analysis and mechanical testing (Instron) data should also be

done with Turkish fir, *Abies bornmuelleriana*, and Veitch fir, *Abies veitchii,* both tolerant and resistant to BWA attack, respectively, to examine how their sugar levels correspond to Fraser fir as well as bark hardness.

INTRODUCTION

The balsam woolly adelgid (*Adelges piceae* Ratzeburg, BWA) is a major pest in Fraser fir, *Abies fraseri* (Pursh) Poir, Christmas tree plantations. BWA is a tiny, piercing-sucking insect, specific to the genus *Abies* that is wingless and entirely female, reproducing through parthenogenesis (Annand 1928, Balch 1952). There are generally two to three generations per year in the southern Appalachians (Arthur and Hain, 1984) and development includes five life stages: egg, three larval instars, and adults. The only motile stage is the first instar, crawlers. Eggs and crawlers are passively dispersed from tree to tree primarily by wind but also on animals. Shortly after hatching, BWA inserts its mouthparts intercellularly into the bark and finds a feeding site in the cortical parenchyma (Balch, 1952). Thereafter, the developing adelgid spends the rest of its life permanently attached to that site (Balch and Carroll, 1956). Therefore, it is possible that the surface BWA spends its life upon is just as important, if not more important, then the actual diet itself.

We developed artificial membranes that mimicked infested Fraser fir bark and were used to cover semi-artificial or artificial diets to rear adelgids in the laboratory. The development of an artificial bark substrate is a novel approach in BWA rearing. We formed alliances with a multi-disciplinary team of specialists from the departments of entomology, food science (to measure and test mechanical properties of bark and synthetic materials), and

forest biomaterials (film production and IC analysis) to conduct this research.

The analysis of the insect's natural food, such as individual amino acids, fatty acids, sterols, simple sugars, and minerals, can be a useful tool in better understanding an insects nutrient requirements (Cohen, 2003). Several researchers have hypothesized that knowledge of a complete nutrient profile of natural foods allows for artificial diets to be simulated by the addition of a few complementary compounds to an otherwise inexpensive foodstuff (Cohen, 2003). This leads to the insect's use of chemical cues from the plant surface in identifying host plant selection and acceptance. Once the insect makes contact on the plant, it can detect primary metabolites on the surface of the plant (Lombarkia and Derridj, 2008).

For example, soluble carbohydrates such as glucose, fructose, sucrose, and sugar alcohols present on apple leaves and fruits act as signals for site acceptance and egg-laying stimulation for the codling moth, *Cydia pomonella* Linnaeus (Derridj et al.1999, Lombarkia and Derridj 2002, 2008). The addition of materials from the insect's natural host can be included in an artificial diet for several reasons: chemicals present in the natural materials may serve as token stimuli, which may be mandatory for insects to feed on the artificial diet, and cryptic nutrients or suitable proportions of nutrients may make the diet desirable for specialized insects that feed on certain plant species (Blossey et al. 2000, Cohen 2003). Finally, several different artificial diets using the various films as a substrate (Appendix A) were tested for BWA development. Insects that are reared on artificial diets are used in many programs including the rearing of biological control agents, sterile insect technologies, and bioreactors for production of pharmaceuticals (Cohen, 2003).

The successful eradication of the screwworm, *Cochliomyia hominivorax* (Coquerel)**,** in the southern U.S. is due to the development of an artificial diet. This has also proved successful with the gypsy moth, *Lymantria dispar* (Linnaeus), a destructive forest defoliator in the northeastern U.S., in which rearing permitted the field-testing of biological and autocidal control methods (Bell et al. 1981). The development of an efficient rearing prototype for BWA will greatly benefit other scientists who require quality laboratory-reared insects to discover means of controlling this pest based on host plant resistance, biological control, chemical control, or other novel approaches. To conduct laboratory and field experiments, it is important that the insects used as test populations are healthy, vigorous, and capable of performing in a reasonably uniform manner (Bell et al. 1981). The techniques employed for successful rearing of BWA could also be applied to another forest pest within the same genus, *Adelges*, the hemlock woolly adelgid (*Adelges tsugae* Annand, HWA), an exotic pest that infests and kills hemlock trees. HWA poses a very serious threat to the ecology of the Appalachian Mountains, including the Great Smoky Mountains National Park (GSMNP, 2010). Hemlocks play an important role in forest ecosystems by providing deep shade along creeks, and maintaining cool micro-climates critical to survival of trout and other cold water species (GSMNP, 2010). HWA is likely to kill most of the hemlock trees in the national park if a successful intervention isn't applied.

There were two main objectives in this study: 1) analyze the bark of infested and un-infested trees and films for texture and hardness, and 2) analyze nutrients present in Fraser fir wood and BWA honeydew as a starting point for BWA diet development.

MATERIALS AND METHODS

Films: Artificial films containing pulps from different hardwoods, softwoods, and infested and un-infested Fraser fir bark were developed in the forest biomaterials lab at North Carolina State University (NCSU) using a chemical method described by Biermann (1996). Film sheets were produced with a standard pulp (hardwoods, softwoods, or infested and un-infested Fraser fir bark) consistency of 0.3 percent (30g/1000ml water). Two variations of films for each pulp were produced: pulp was either blended to make smooth films or the pulp was unblended, providing a very textured surface. The texture created in the unblended films may provide a favorable environment that mimics Fraser fir bark and encourages BWA insertion for feeding.

A total of eight films were made: blended-hardwood mixture (maple/oak/hickory) (B-HW) (Figure 3.1), unblended-hardwood mixture (un-HW) (Figure 3.1), blended-Fraser fir bark-un-infested (B-FF-un), unblended-Fraser fir bark-un-infested (un-FF-un), blended-Fraser fir bark-infested (B-FF-in), unblended-Fraser fir bark-infested (un-FF-in), blended-softwood mixture (no lignin) (B-SW), and unblended-softwood mixture (un-SW). After the films were formed, they were air dried and then dipped into melted wax to make them waterproof. Beeswax and paraffin wax were tested to determine how they affect the hardness of the films and their resistance to water. The two waxes were also chosen to see if they acted as a stimulant or deterrent in feeding and BWA preference.

Instron: An Instron electromechanical system evaluates the mechanical properties of materials and components using tension, compression, flexure, fatigue, impact, torsion and hardness tests. An Instron was used to compare bark texture and hardness of infested and un-

infested Fraser fir to Parafilm™ and various pulp films (Figure 3.2). Infested and un-infested bark samples were taken from several Fraser fir trees (2.5 x 2 cm squares) and analyzed to determine the hardness of the bark, the time it takes for the needle tip to penetrate into the parenchyma cells, and the energy expended with insertion (top, middle, bottom of the tree was noted). A needle was made for the Instron to be inserted into the tree to try to mimic the adelgids stylet, which has an average length of 1.5 mm (Balch, 1934). It was not possible to construct a needle the exact width of a stylet, as they are 3.5 μm for most of the length of the stylet bundle and 2.5 μm towards its apex (Forbes and Mullick, 1970). Therefore, the needle was made from a minutien pin: 0.15 mm in diameter, 18 μm tip size, and 6 mm in length, attached to a collet which could be attached to the Instron. The Instron was set up for the needle to travel 1.5 mm into the sample at a time interval of 1.5 minutes per test. Measurements were in millijoules (mJ), a unit of energy equal to the energy expended when force is applied.

For each sample, the needle was randomly inserted into the center of the sample in two different locations, and inserted once on the left side of the sample and once on the right side of the sample (four values per sample). Un-stretched Parafilm, a Parafilm square stretched four times its normal size, a blue, absorbent paper towel, Sigma optical grade polycarbonate rubber (0.3 cm thick), and the eight films (1.5 cm in diameter) were also evaluated to compare their thickness and hardness to Fraser fir bark. Three variations of each film were tested: film encased with a paraffin-based wax, film encased with beeswax, and the film un-waxed. A total of 113 values for Fraser fir bark were recorded (N=68 infested bark and N=45 un-infested bark) and four values for each film variety as well as four values for

Parafilm, shop cloth, and rubber.

Antimicrobial Plates: The antimicrobial properties of films as well as Fraser fir bark discs were evaluated after exposure to three known contaminations: the wall inside a 20°C incubator, the top rack inside a 20°C incubator, and plates left open 30 minutes in a 20°C incubator as an air contaminant. Three variations of each film with a diameter ranging from 1-1.5 cm were evaluated: film encased with a paraffin-based wax, film encased with beeswax, and film with no wax. Glass plates were sterilized in an autoclave for 15 minutes at 121°C and films were surface-sterilized under a laminar flow hood before placing them on agar by spraying and completely saturating them with 70 percent ethanol. The films were allowed to dry and then they were flipped over and the other side was sprayed and completely saturated. Difco nutrient agar, which cultivates a wide variety of microorganisms, was either swabbed with sterile tips or left out in the environment for 30 minutes. Then the eight films were placed on top of the agar (Figure 3.3), the lid was placed on top of each plate and placed (right side up) in a 37.5°C incubator (Figure 3.4). Fraser fir was collected from an abandoned Christmas tree farm in Avery County and two variations of both infested and un-infested discs were made: Fraser fir bark discs with wood removed and bark plus wood discs. Half of the Fraser fir discs were also sterilized like the films, and the plates were prepared and incubated exactly like the films. All of the films were observed daily for the first sign of antimicrobial growth and whether it was growing around or under the film. A total of eight replications of each film and Fraser fir discs were tested in each contaminant.

IC: Samples were analyzed in the forest biomaterials lab at NCSU according to Min et al. (2012) methods using a Dionex-3000 ion chromatography system (ICS), a type of High Performance Liquid Chromatography (HPLC), that allows for an efficient separation process and identifies sugars from non-sugars. In June of 2012, infested and un-infested Fraser fir wood was collected from an abandoned Christmas tree farm in Avery County and brought back to NCSU. As soon as discs were cut in the field, they were immediately put on dry ice to preserve the Fraser fir cells and nutrients inside. Upon arrival to NCSU, the samples were removed from dry ice and placed in an -80°C freezer. The following day, radial pieces of the bark, wood, and bark plus wood were cut (about 1.0 x 1.5 cm in size) and ten pieces of each sample were added to 10 ml of distilled water and left for 24 hours. Since BWA feed on ray parenchyma cells (Balch, 1952), radial pieces were cut because it provides the largest surface area of the ray parenchyma cells. After 24 hours, the samples were removed and the liquid was used for sugar analysis. We thought water extraction was a good starting point for analyzing Fraser fir sugars because most homopterans are liquid feeders (Cohen, 2003) and that's possibly what BWA are feeding on. Furthermore, a previous study by Balakshin et al. (2005) already analyzed Fraser fir sugars from whole wood samples. However, Cohen et al. (2008) has showed that hemlock woolly adelgid's may use extra-oral digestion when feeding, where the insects' salivary enzymes pre-digest solid components of cells, then the insect ingests a concentrated nutrient "broth" (Cohen et al. 2008). Therefore, this may also apply to BWA and water soluble sugars might not be fully indicative of BWA feeding.

Hydrolysis breaks down the glycosidic bonds in carbohydrates by converting the linked polysaccharide to its monosaccharide sub-units. This process can be accelerated with

acids so the liquid samples were hydrolyzed by taking 0.26 ml of 72 percent sulfuric acid and 7.74 ml of distilled water (resulting in an acid concentration of three percent), and it was added to each 2 ml sample. The mixture was autoclaved for 1.5 hours at 123°C. After acid hydrolysis, 0.1 ml of internal standard (IS), fucose, was added to the 2 ml samples, vortexed, then filtered (0.20 μm) into small vials to run under IC. Two vials containing ten drops of BWA honeydew each, was dissolved into 2 ml distilled water and filtered with a 0.45 μm syringe filter to remove any molten or woolly mass that might have also been collected. One vial was acid hydrolyzed in the same manner as the Fraser fir samples, and one vial was left as is. Analysis of variance (ANOVA) including slice analysis to investigate interaction effects between variables was done to analyze the data.

RESULTS

Texture Analysis: Infested bark (0.6 mJ) is twice as hard as un-infested bark (0.3 mJ) and samples taken from higher up an un-infested tree had softer bark whereas samples taken toward the base of the tree were hardest (Table 3.1). Infested trees also had softer bark higher up the tree but the hardest bark was found in the middle of the tree, not the bottom. The Parafilm and shop cloth hardness values were extremely small compared to the bark samples (Table 3.2). There were significant differences in the hardness among the films and the types of wax and the wax x film interaction was significant. Averaged across all films, paraffin was harder than beeswax (Table 3.3). Unblended softwood film with paraffin wax was the hardest film (2.4 mJ), about four times harder than infested bark samples (Table 3.2). Slice analysis was done and there was a significant difference among the films for

paraffin and beeswax, but not for un-waxed films (Table 3.4).

Antimicrobial Plates: There was a significant difference among the films in the number of days it took to see microbial growth under them and blended hardwood took the longest compared to the other films (Table 3.5, Figures 3.5 and 3.6). There was no significant difference in the types of wax used, but there was a significant difference in the contaminant effects between the two waxes (Table 3.6). Beeswax was more resistant to microbial growth than paraffin waxed films (except 20°C rack). Samples were analyzed using a full model with wax, contaminant, and sample including all two- and three-way interactions but the three-way interactions was dropped because it wasn't significant for either trait. Reduced models were run for each wax (slice analysis) and there was a significant difference among contaminants for beeswax but not for paraffin in the days it took to see microbial growth around the films (Table 3.7).

The Fraser fir discs showed no significant differences or interactions for resistance to microbial growth (Table 3.8) and sterilizing the discs didn't seem to have an effect in preventing microbial growth. There were also no significant differences or interactions among un-waxed films for the number of days it took to see microbial growth (Table 3.9). Overall, bark took one day to get microbial growth under the films, unwaxed films took about one to two days, and the waxed films took one to three days.

Sugar Analysis: The infested bark plus wood samples had higher quantities of arabinose, rhamnose, galactose, and xylose than un-infested bark plus wood samples, while in the infested bark samples, five of the six sugars: arabinose, galactose, glucose, mannose, and xylose were higher than the un-infested bark samples (Table 3.10). Furthermore,

infested bark glucose levels were about 3.5 times higher than un-infested bark. The wood sample yielded only rhamnose in the infested sample. The BWA honeydew did not show any peaks and no sugars were identified.

DISCUSSION

Texture Tests: Host resistance mechanisms include rapid accumulation of monoterpene and juvabione-related compounds, the production of secondary periderm (Mullick 1975, Hain et al. 1991), and a thick layer of outer bark at the wound site. Infested bark was found to be twice as hard as un-infested bark, suggesting an induced response and possibly the trees defense mechanism against adelgid attack. Hardness comparisons should also be done with Turkish fir (*Abies bornmuelleriana* Mattfeld) and Veitch fir (*Abies veitchii* Lindle) both tolerant and resistant to BWA attack, respectively, to see if hardness acts as a defense mechanism. Native firs in North America (balsam fir, Fraser fir) are highly susceptible whereas firs native to central Europe (silver fir) tolerate infestation (Varty 1956, Newton 2013). However, some Asian species, such as *A. veitchii* appear to be immune to attack, at least in North America (Hall et al. 1971, Newton 2013).

Infested bark was found to be hardest in the middle of the tree, which supports Mitchell's (1966) findings that adelgid populations in the outer portion of the tree's crown cause less damage than adelgid populations on the main stem and large branches. While BWA could easily insert into Parafilm, a harder substrate may mimic their natural host more. The un-infested and infested Fraser fir bark ranged from 0.30 mJ to 0.60 mJ, respectively. BWA seem to favor trees that are slightly infested because the initial induced response in the

tree modifies the bark and increases fertility and survival of adelgids (Amman, 1970). Therefore, a value in between the infested and un-infested bark (0.40 mJ) was chosen as optimum for the films. Disregarding the unwaxed films because they aren't water proof and won't be used in artificial diets, the two films: unblended hardwood-beeswax (0.40 mJ) and blended hardwood-beeswax (0.35 mJ), are closest to this value and should be further tested to see if BWA shows preference for these films. The remaining films should still be tested, but based on their values, may be too hard for BWA to insert into or create an unfavorable environment. A lot of the un-waxed films were within the optimum 0.40 mJ value. Perhaps other waxes should be explored that might allow use of these films as well. Rubber was around the chosen value at 0.45 mJ, which may make it a possible substrate to cover artificial diets as BWA inserted in it several times in previous diet trials (Appendix A). The unblended softwood-paraffin is four times harder than infested bark, which is most likely too hard and will be an unfavorable surface for BWA.

Antimicrobial Plates: Blended hardwood took the longest to obtain microbial growth (most resistant) and it took beeswax samples longer to foster microbial growth than paraffin samples, which could possibly be because they have natural inhibitors. Guillard et al. (2009) tested edible coatings to reduce the total amount of preservatives added in food and found that beeswax coatings free of sorbic acid were able to inhibit *Saccharomyces cerevisiae* (Meyen ex -Hansen) growth due to their high barrier properties. This supports the findings that beeswax is more resistant. Furthermore, Beck (1960) found that beeswax might contribute to nutritional suitability, though it was not determined whether the beeswax contributed required nutrients or contained chemo-sensory feeding stimulants. This further

suggests that beeswax is not only better suited to inhibit microbial growth than paraffin, but can also act as a stimulant to encourage feeding.

The Fraser fir discs became contaminated quicker compared to the films, regardless of whether they were sanitized or not, which was expected to happen because bark is exposed to the elements and a fair amount of dirt/contamination is expected to cover it. Because several films still experienced contamination, a possible avenue to explore is developing different sterile techniques and running this experiment again to see if contamination is delayed or omitted. While the films are still vulnerable to microbial contamination, they can possibly retain artificial diets for a longer period of time (Reference Appendix A). Furthermore, the texture created from the unblended films may provide a favorable environment for the adelgid and encourage feeding.

Sugar Tests: The values were varied throughout all the samples, possibly because sections of wood or bark are not uniform and lack sugars or nutrients seen in other samples, or because there weren't enough samples tested. Several other samples should be tested again to verify similar results. The wood and BWA honeydew samples were either too dilute and quantities so low as not to be identified by the IC, or sugars weren't present in the samples chosen. Furthermore, the chemicals used to derivatize the honeydew could have been too strong and destroyed the sample or another IS should have been used. At this time it isn't possible to test other sugars that may be present in Fraser fir because the IC column and program we used is designed for seven sugars (fucose, arabinose, rhaminose, galactose, glucose, xylose and mannose). To check more sugars, a suitable column and program are needed for those sugars. Rotholz contains higher amounts of ray tissue and thicker cell walls

compared to that of un-infested wood (Hain, 1988). Balakshin et al. (2005) analyzed the composition of infested Fraser fir wood (rotholz) and found more than five-fold the amount of galactans but lower amounts of mannose and glucose than that of un-infested wood. Our galactose levels were also higher in the infested samples, which supports this, however, we saw higher levels of glucose in the infested bark compared to un-infested bark. Sucrose, a disaccharide comprised of glucose and fructose, appears to be a phagostimulant to the green peach aphid, *Myzus persicae* Sulzer (Dadd and Mittler, 1965), which could also hold true for BWA.

Amman (1970) found that the result of adelgids modifying the bark increased fertility and survival of adelgids per unit area of bark. Based on the IC results of glucose levels of infested bark being 3.5 times higher than in un-infested bark, it is hypothesized that BWA modifies the bark, causing an induced response in the tree, forcing the tree to generate more glucose. This allows BWA to survive and thrive because they (or the symbionts in their gut) use the high glucose levels as an energy source in glycolsis. Endosymbiotic bacteria present in adelgids haven't been studied in nearly as much detail as in aphids. In aphids, primary endosymbionts, housed in special cells called bacteriocytes, are necessary for survival because they synthesize essential amino acids that insects can't produce themselves (Buchner 1965, Havill and Foottit 2007). Therefore, it is also possible that these endosymbionts require the large glucose levels to synthesize for the adelgids. Another possible conclusion is that BWA possibly ingest higher sugar levels than initially thought, resulting in artificial diets to this point being insufficient because it was thought BWA didn't consume as much sugar as aphids.

Furthermore, it is also possible that BWA use glucose as an immediate energy source, as well as act as an energy reserve. Several glycolytic enzymes used to generate ATP from glucose are present in high abundance of the guts/digestive tract of BWA: enolase, fructose 1,6-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, and triosephosphate isomerase (Appendix B). Digestive enzymes are essential because sugars can only pass through the gut wall as monosaccharides (Boeve and Wackers 2003, Weil 1978). In insects, the metabolism of sugars is based on the monosaccharide glucose from which trehalose and glycogen are synthesized (Boeve and Wackers 2003, Mullins 1985). Therefore, glucose functions both as an instant source of energy, through direct glycolysis, and as a means of energy storage (Boeve and Wackers 2003, Candy 1985). Further research is needed to explore the glucose relationship in BWA and could provide a basis for diet development. IC comparisons should also be done with *A. bornmuelleriana* and *A. veitchii* to see how their sugar levels correspond to Fraser fir. Finally, future artificial diets for BWA should contain higher levels of sugars comparable to levels used for aphids to see how they fare.

Initial feeding by BWA on *Abies* causes a rapidly induced response that increases adelgid survival corresponding to increased protein content of the bark (Havill and Foottit, 2007). Subsequent generations experience a delayed induced response that negatively affects them by bark resinosis, deformation of xylem, depletion of nutrients, death of phloem cells, disruption of water flow, rapid decline in adelgid survival, and often death of the tree (Amman 1970, Havill and Foottit 2007). This study exhibited several of these induced responses and they should be explored further.

A multidisciplinary approach has allowed a slightly larger look at the big picture of integrated pest management (IPM) strategies for BWA. Further IC analysis should be explored that includes BWA honeydew and gut samples to compare to sugars found in Fraser fir. This could possibly aid in the successful development of an artificial diet containing the correct percentage of nutrients to sustain BWA populations. Once a method for rearing BWA has been established, the potential BWA management strategies include rearing BWA predators on the BWA for biological control, and/or screening host material with the BWA for host resistance.

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Table 3.1 The average hardness of infested vs. un-infested Fraser fir bark*.

Sample	Tree Position	Work of Penetration (mJ)
Infested		0.60 (0.042)
Un-infested		0.30 (0.023)
Infested	Top	0.52 (0.065)
	Middle	0.72 (0.086)
	Bottom	0.62 (0.067)
Un-infested	Top	0.23 (0.030)
	Middle	0.26 (0.031)
	Bottom	0.35 (0.029)

* Mean (Std Error).

Table 3.2 The average work of penetration (mJ) for all substances*.

Substance	Work of penetration (mJ)
Bark	0.48 (0.030)
Parafilm stretched 2x	0.01 (0.001)
Shop Cloth	0.07 (0.010)
Infested bark	0.60 (0.041)
Un-infested bark	0.32 (0.023)
unSW,Para	2.4 (0.147)
BSW,Para	1.3 (0.147)
unFFun,Para	1.2 (0.147)
un-FF-in,Bee	1.1 (0.147)
unHW,Para	0.93 (0.147)
BFFun,Bee	0.90 (0.147)
unFFun,Bee	0.82 (0.147)
unSW,Bee	0.72 (0.147)
BFFun,Para	0.64 (0.147)
BHW,Para	0.58 (0.147)
BSW,Bee	0.55 (0.147)
un-FF-in,Para	0.52 (0.147)
BFFin,Para	0.52 (0.147)
BFFin,Unwaxed	0.44 (0.147)
un-FF-in,Unwaxed	0.40 (0.147)
unHW,Bee	0.40 (0.147)
unSW,Unwaxed	0.39 (0.147)
unFFun,Unwaxed	0.36 (0.147)
BHW,Bee	0.35 (0.147)
BSW,Unwaxed	0.31 (0.147)
BFFun,Unwaxed	0.28 (0.147)
BFFin,Bee	0.23 (0.147)
BHW,Unwaxed	0.21 (0.147)
unHW,Unwaxed	0.11 (0.147)
Rubber	0.46 (0.249)

*Hardwood blended (B-HW) (Figure 3.1), hardwood unblended (un-HW), Fraser fir bark-un-infested-blended (B-FF-un), Fraser fir bark-un-infested-unblended (un-FF-un), Fraser fir bark-infested-blended (B-FF-in), Fraser fir bark-infested-unblended (un-FF-in), softwood blended (B-SW), and softwood unblended (un-SW). Mean (Std Error).

Table 3.3 The average hardness among waxes across all films*.

Wax	Work of Penetration (mJ)
Paraffin	1.0^{A} (0.052)
Beeswax	0.6^{B} (0.052)
Unwaxed	0.3^{C} (0.052)

* Mean (Std Error).

Table 3.4 Significance of film differences in hardness sliced by wax.

Wax	Prob>F
Beeswax	0.0007
Paraffin	<.0001
Unwaxed	0.7740

Table 3.5 The number of days until microbial growth observed under films*.

Film	Days under
Blended hardwood	3.28 (0.198)[A]
Blended softwood	1.84(0.183)[B]
Blended Fraser fir-uninfested	1.57 (0.184)[B]
Unblended softwood	1.57 (0.184)[B]
Unblended Fraser fir-uninfested	1.56 (0.177)[B]
Blended Fraser fir-infested	1.31(0.177)[B]
Unblended Fraser fir-infested	1.21 (0.177)[B]
Unblended hardwood	1.17 (0.180)[B]

* Mean (Std Error).

Table 3.6 Interaction between type of wax and contaminants*.

Wax	20C Air	20C Rack	20C Wall
Beeswax	1.60 (0.136)	1.77 (0.246)	1.88 (0.206)
Paraffin	1.35 (0.136)	1.82 (0.246)	1.70 (0.205)

* Mean (Std Error).

Table 3.7 Interaction between wax and microbial growth seen around films: Slice analysis.

Wax x Around	Prob>F
Slice Wax=Beeswax	0.0052
Slice Wax=Paraffin	0.6384

Table 3.8 The number of days for microbial growth on Fraser fir discs*.

Sample	Days under
Bark-Infested	1.15 (0.043)
Bark- Uninfested	1.11 (0.040)
Wood- Infested	1.14 (0.043)
Wood- Uninfested	1.06 (0.040)

* Mean (Std Error).

Table 3.9 The number of days for microbial growth on unwaxed films*.

Unwaxed films	Days under
Blended Fraser fir-infested	2.33 (0.207)
Blended Fraser fir-uninfested	2.53 (0.204)
Blended hardwood	2.28 (0.197)
Blended softwood	1.87 (0.197)
Unblended Fraser fir-infested	2.02 (0.204)
Unblended Fraser fir-uninfested	2.14 (0.207)
Unblended hardwood	2.02 (0.199)
Unblended softwood	1.85 (0.197)

* Mean (Std Error).

Table 3.10 IC-Sugar concentration (ug/ml) of infested and un-infested Fraser fir.

Sample	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose
Un-infested Bark and Wood	37	21	37	104	0	22
Infested Bark and Wood	38	61	129	11	24	0
Un-infested Bark	0	30	22	35	0	0
Infested Bark	55	19	32	124	10	19
Un-infested Wood	0	0	0	0	0	0
Infested Wood	0	26	0	0	0	0

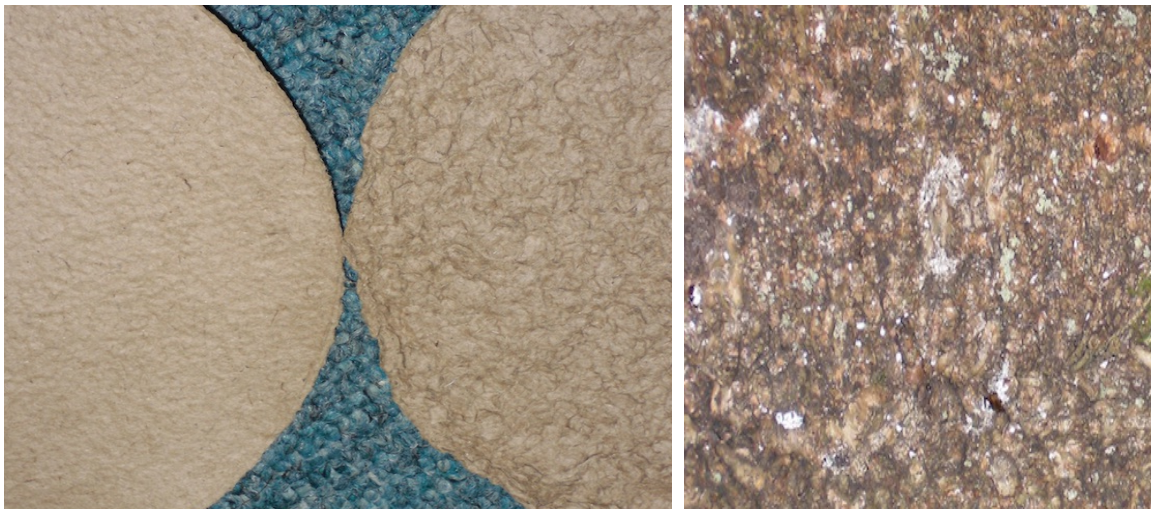

Figure 3.1 Hardwood films unblended (left) and blended (right) compared to Fraser fir bark.

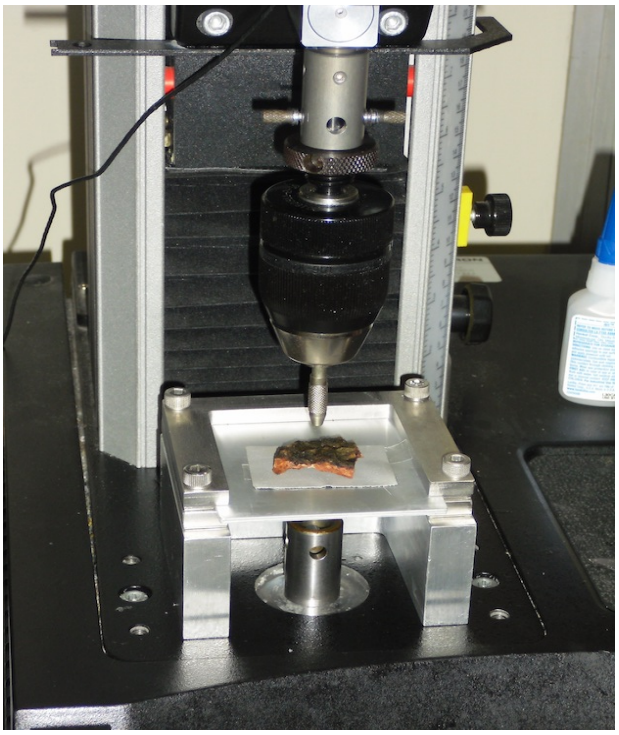

Figure 3.2 Instron electromechanical system.

Figure 3.3 Eight unwaxed films placed on Difco nutrient agar.

Figure 3.4 Plates in the 37.5°C incubator.

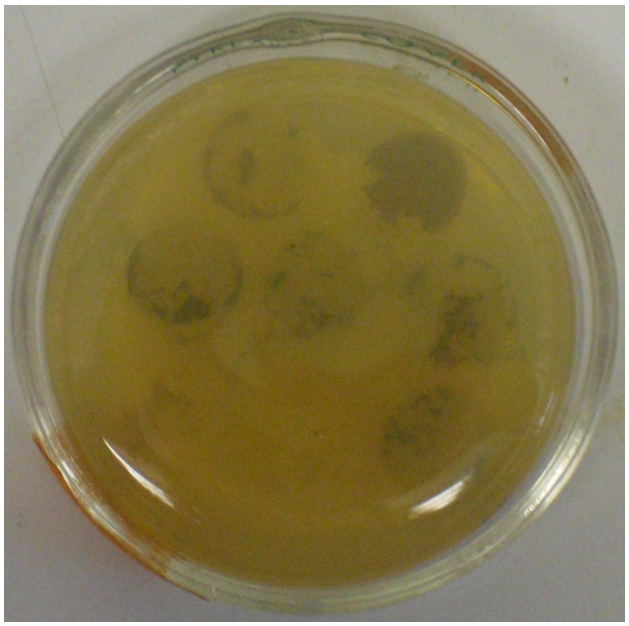

Figure 3.5 Beeswax films: Growth around and under all films except blended hardwood (top right), which is only ¼ under and around.

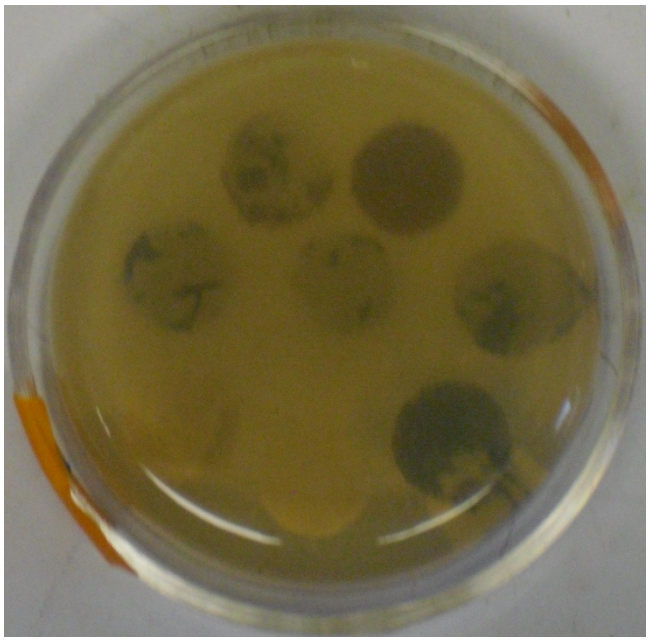

Figure 3.6 Beeswax films: No growth under blended hardwood (top right) and minimum growth under Fraser fir bark-infested-unblended (bottom right).

CHAPTER IV

Use of the Green Peach Aphid, *Myzus persicae*, as a Model Organism for the Efficacy of Ingesting a Host Defense Chemical

ABSTRACT

Juvabione and other juvenoids mimic the juvenile hormone in insects by inhibiting insect reproduction and growth and are effective control measures against several insect pests. The balsam woolly adelgid, *Adelges piceae* Ratzeburg (BWA, Hemiptera: Adelgidae), is an exotic, aphid-like insect that threatens Fraser fir trees, an important natural resource and a prominent Christmas tree species. Juvabione levels in Fraser fir have been shown to increase with increasing BWA infestation. The objective of this study was to test the effect of petroleum ether soluble compounds extracted from Fraser fir bark and whole wood of infested and un-infested trees on the development and fecundity of the green peach aphid, *Myzus persicae* (Sulzer), as a surrogate for BWA since manipulating the adelgid is too difficult to allow for direct observation. The aphids fed readily on the infested wood diet, suggesting that infested wood induces a chemical response that encourages feeding. Results indicate that juvabione or other compounds present in the Fraser fir petroleum ether extract delayed reproduction, which can be a form of resistance and a possible population control method. Juvabione levels in Fraser fir trees should be the focus of further research, including their contribution to host resistance to BWA, and its use as a convenient and environmentally safe control method for BWA.

INTRODUCTION

Juvabione, a sesquiterpenoid found in fir wood, mimics the juvenile hormone in insects by inhibiting insect reproduction and growth (Barrero et al. 1989, Fowler et al. 2001). In the larval stages, when the production of juvenile hormone is physiologically inhibited, larval somatic growth is suspended whereas in the adult stage of several insect species, juvenile hormones can also be used to regulate reproduction, especially during the cycles of somatic growth in reproducing females (Slama, 1999). Since the early 1970s when the first commercially available products, hydroprene and methoprene, were prepared, thousands of active juvenile hormone analogues (juvenoids) have been synthesized to be used as control measures for insect pests (Jedlicka et al. 2007). The use of juvenoids as a control measure against insidious insects provides a convenient and environmentally safe control method.

The balsam woolly adelgid, *Adelges piceae*, is an exotic, aphid-like insect that was introduced to the U.S. from central Europe in the early 1900s. Since its introduction, the balsam woolly adelgid (BWA) has swept through the Southern Appalachians killing thousands of acres of Fraser fir, an important natural resource and a prominent Christmas tree species. Fraser fir, *Abies fraseri* (Pursh) Poir, occurs naturally only in the Southern Appalachians, and is the dominant tree at the highest elevations (Dull et al. 1988). To date, control of this exotic pest has proven to be extremely difficult.

Previous studies have shown that topical applications of juvabione has an effect on several insects, including the European bug, *Pyrrhocoris apterus* (Linnaeus), and mealworms, *Tenebrio molitor* (Linnaeus): the insects undergo one or more supernumerary

molts and then die without becoming sexually mature (Slama and Williams 1965, Bowers 1966, Levinson and Zlotkin 1971). Mealworms have been used as a surrogate in juvabione bioassays because they can be easily manipulated in terms of diet and environment and they are known to be susceptible to juvabione and its analogs and derivatives (Levinson and Zlotkin, 1971). However, mealworms may be too genetically and physically different to extrapolate juvabione effects onto BWA. Because aphids have similar mouthpart form and function and produce saliva that is secreted while feeding to create a protective sheath around their mouthparts (Pollard 1973, Miles 1987), they can be used as a model in regards to the feeding behavior of BWA. Other studies have evaluated the impact of juvabione when topically applied (Slama 1979, Fowler 1999, 2001). However, Jedlicka et al. (2007) evaluated the juvenilizing effect on the pea aphid, *Acyrthosiphon pisum* (Harris), of ingesting derivatives of juvabione (natural juvabiol and juvabiol acetate) in diet assays. Because manipulating the adelgid is too difficult to allow for direct observation, the green peach aphid, *Myzus persicae*, was used in this study to act as a surrogate and be reared in the lab on artificial diets containing Fraser fir extracts, to determine if growth is inhibited when extracts are ingested.

Previous studies have shown juvabione levels in Fraser fir increases with increasing BWA infestation (Zhang 1994, Fowler et al. 2001). Because Fraser fir produces juvabione in response to BWA infestation, this secondary plant chemical may contribute to host resistance to BWA (Puritch et al. 1974). Our objective was to evaluate the development and fecundity of *M. persicae* after ingesting artificial diets containing petroleum ether soluble compounds extracted from Fraser fir bark and from whole wood of infested and un-infested trees. We

hypothesize that artificial diets containing disruption factors from Fraser fir trees (juvabione or other possible juvenizing factors) will affect the growth and development of phloem feeding insects and decrease their fecundity. These disruption factors were tested on green peach aphids feeding on artificial diets. Understanding the effects juvabione plays on *M. persicae* potentially can be extrapolated to potential BWA control measures.

MATERIALS AND METHODS

A yeast extract diet was used based upon preliminary observations (Appendix C) as well as from a previous study in which *M. persicae* was reared on an oligidic diet containing yeast extract that appeared to provide the insect-symbiont complex with nutrients in adequate amounts to sustain aphid growth at 60 to 80 percent of that on plants (Mittler and Koski, 1976). Because we were only observing the effect of plant extracts on aphid development, the use of an oligidic diet was of value because precise dietary composition was not a primary concern and allowed diets to be rapidly prepared by using less expensive, undefined ingredients (Mittler, 1976).

Petroleum ether soluble compounds were extracted from whole wood and bark preparations of infested and un-infested Fraser fir using a Soxhlet apparatus following modified methods described by Puritch and Nijholt (1974). Briefly, 3 g of bark or 8 g of wood were ground to 5 mm pieces and placed in 25 mm x 80 mm single thickness cellulose extraction thimbles. A Soxhlet apparatus containing 150 mL petroleum ether to extract soluble compounds for 12 hours was used and then the petroleum ether was allowed to evaporate for 48 hours under a reduced pressure. Yellow oil extracts were dissolved in 20

mL acetone for transfer to pre-weighed 40 mL vials with teflon screw caps. Acetone was evaporated for 48 hours under reduced pressure and dried in a gravity convection incubator (45ºC) with anhydrous calcium sulfate (Drierite) for 48 hours before weighing.

Un-infested whole wood weighed 0.257 g, un-infested bark 0.252 g, infested whole wood 0.370 g, and infested bark 0.171 g. The crude extract was re-dissolved in acetone and analyzed with a gas chromatograph (HP 6899) with mass spectrometer (HP 5973) (GCMS). The GCMS oven was programmed to run from 80-200°C at 5°C·min^{-1}, 200-230°C at 1°C·min^{-1}, and 230-300°C at 30°C·min^{-1}. Temperature was taken down to 80°C at 30°C·min^{-1} between samples and held for four minutes before running the next sample. One μL of sample was injected into the GCMS using an autosampler with helium carrier gas over a 30 m column (HP-5MS, I.D. 25 mm).

Quantitative assessment of juvabione and other petroleum ether extractable material were performed using an external calibration, using five known concentrations of a pure juvabione standard ranging from 15.01 ng·μL^{-1} to 120.08 ng·μL^{-1} (Figure 4.1). The calibration standards and samples were run in duplicate and we injected 200 ng·μL^{-1} of infested whole wood extract, 200 ng·μL^{-1} of un-infested whole wood extract, 7,000 ng·μL^{-1} infested bark, and 10,000 ng·μL^{-1} un-infested bark. The relative amount of juvabione in each sample was quantified by using the standard curve for peak area, after integration of the peaks. Infested whole wood extract contained 330 ng·μL^{-1} juvabione and un-infested whole wood extract contained 508 ng·μL^{-1} juvabione, but neither infested nor un-infested bark samples contained a peak for juvabione. Peaks were compared to ion signatures using WileyNIST library version 7. Five μg of the crude extract were dissolved in 0.5 μL of

acetone (Bower et al. 1966) for use in the artificial diet treatments. Green peach aphid colonies reared on black pearl pepper plants, *Capsicum annuum* (Linnaeus), were used to test juvabione treatments in the artificial diets.

Cages were constructed by cutting 50 mL centrifuge tubes into 2.25-3.00 cm long cylinders. One end had the cap, which could be opened to place aphids into the cages and for daily observations. Syngenta water sensitive paper was taped inside the cap to observe when honeydew was first seen. Mesh fabric was placed over the open end of the cage, and the diet sachets where placed on top of the mesh, on the outside of the cage. Cages were cleaned and sterilized by being washed in warm soapy water, allowed to air dry under a laminar flow hood, and then dipped in a 70 percent ethanol solution and allowed to air dry. The cages were then placed vertically with the diet at the top. Aphids were kept in an incubator with a 16:8 photoperiod, using an Utilitech T8 fluorescent plant grow light that hung 77.5 cm above the aphids, at a temperature of 25°C, and 75 percent relative humidity **(**DeLoach, 1974).

The yeast extract diet (Table 4.1) was the base for seven treatments. Green peach aphids were given: 1) the basic artificial diet, 2) the basic diet with methoprene (1.2%), 3) the basic diet plus petroleum ether extract from infested Fraser fir whole wood, 4) the basic diet plus petroleum ether extract from infested Fraser fir bark, 5) the basic diet plus petroleum ether extract from un-infested Fraser fir whole wood, 6) the basic diet plus petroleum ether extract from un-infested Fraser fir bark, or 7) the basic diet with acetone. Each treatment was replicated in five different cages with groups of 10 green peach aphids (1^{st}-3^{rd} instars) placed in each cage, totaling 50 aphids per treatment. Diet sachets were made by using a sterilized single square of Parafilm, stretched out to twice its width and length, with half of it spread

over the mesh opening of the cage and 200 μL of diet was placed on top of the opening. For each treatment, 0.5 μL (5μg) of extract was added and mixed into the diet and the other half of the Parafilm was pulled over top of the diet to seal the sachet. The aphids were observed daily and diet sachets were changed every other day.

The number of aphids present in the artificial diet cage, the number of aphids feeding on the artificial diet, the length of time it took for honeydew to be present, the length of time it took for them to reproduce, and the number of times they reproduced per treatment was recorded daily for as long as the aphids stayed alive (12 to 17 days). Analysis of variance (ANOVA) and Tukey-Kramer HSD was done to analyze the data.

RESULTS

Comparison of ion signatures from whole wood extracts showed high (49-90) quality match scores between the library reference for juvabione and our unknown extracts (WileyNIST version 7). Our chromatogram showed several other peaks of interest eluting between 24 and 29 minutes including a peak for dehydro-juvabione which appeared to occur at a higher relative amount in the healthy wood compared to the infested (Figure 4.2). The bark contained three compounds known to be defensive against insects: 1) diterpene resin acid, shown to be defensive against insects and microbial pathogens by Kersten et al (2006), 2) 18-Norabieta-8,11,13-triene diterpenoid resin, another defense against insects (Hanson, 1991), and 3) 1,5- dimethylcyclohexane- 5-carboxaldehyde, a possible precursor to monoterpenes (Zulak and Bohlmann, 2010), as well as several other compounds, but no juvabione was present within the bark.

The aphids were able to feed (ingestion was evident by the presence of honeydew) and reproduce in all seven treatments. Significantly more aphids fed on the diets containing infested wood and un-infested bark than all other diets (Tables 4.2 and 4.3). The mean number of aphids seen feeding in the infested wood diet (6.0) was over three times greater than in the un-infested wood diet (1.6) (Table 4.3). There were no significant differences in the number of days it took for the aphids to produce honeydew. It took the aphids more than four times longer to reproduce on the un-infested bark diet (17.3 days) compared to the un-infested wood diet (3.7 days) (Table 4.3). Reproduction was also delayed in the infested wood diet (14.5 days), compared to the other treatments. While the analysis of variance detected significant differences among diets for the number of times reproduction occurred, the Tukey-Kramer HSD procedure did not.

DISCUSSION

The number of aphids that fed varied from 16 to 60 percent across the various treatments. The mean number of aphids seen feeding on the infested wood was over three times greater than the un-infested wood diet, suggesting that BWA infestation may induce trees to produce chemical factors that stimulate feeding, possibly acting as a phagostimulant. A phagostimulant is a feeding stimulate or substance that elicits a feeding response in a target species. While these don't have direct nutritional function, they stimulate normal feeding responses (Cohen, 2003). After Soxhlet extraction, the infested and un-infested whole wood samples contained juvabione (petroleum ether extracts) and only varied in juvabione concentrations. When the extracts were prepared, the concentration of juvabione was

controlled when dissolved in acetone by changing the volumes to reflect the different juvabione concentrations to yield a 5 μg solution. Therefore, it appears that the petroleum ether extracts from infested wood (86% juvabione) increased feeding compared to the other samples. There was a lot of variability in both the number of days it took for the aphids to produce honeydew as well as reproduce on the different diets.

Aphid fecundity, and longevity are less for aphids reared on artificial diets compared to plants (Wille and Hartman, 2008), which was evident in our study. Under optimal conditions, *M. persicae* development can take 10 to 12 days for a complete generation and females give birth to offspring six to 17 days after birth with an average age of 10.8 days (Horsfall, 1924). We did not see a delay in reproduction on the unaltered artificial diet, but there was a significant difference in the number of days it took the aphids to reproduce on diets containing un-infested bark, infested bark, and infested wood treatments. All three of these treatments had a reproduction time about four times that of un-infested wood. Through GCMS analysis, there was no juvabione detected in Fraser fir bark however there were many other compounds present in the petroleum ether extract. This could suggest there is a compound beside juvabione that can possibly delay reproduction. Bauernfeind and Chapman (1984) showed that concentrations around 0.1% of methoprene, hydroprene, and kinoprene eliminated or reduced progeny of green peach aphids exposed in their first, second, or third instars. While reproduction did seem slightly delayed in the methoprene treatment, once they reproduced, the aphids seemed unaffected by the treatment. However, the previous study (Bauernfeind and Chapman, 1984) topically applied these chemicals, whereas we fed them to the aphids. The low number of aphids seen feeding prior to delayed reproduction could be

indicative of the methoprene changing the taste of the diet, thus, making it distasteful and reducing feeding activity.

Wimmer et al. (2002) stated that insect juvenile hormone bioanalogues (JHAs, juvenoids) are generally bulky molecules which can make it difficult or even impossible to penetrate through the insect cuticle in topical screening tests. Therefore, they believe it is better intended for oral screening tests (Wimmer et al. 2002). While this may be true, results from our study suggested that many aphids didn't even feed, thus not being affected by juvabione or other petroleum ether extracts. Feeny (1970) stated that defense compounds present in large amounts, termed quantitative defense compounds, aren't necessarily potent metabolic poisons but rather act by disrupting the feeding process or digestive interference (Cohen, 2003). Quantitative examples include terpenoids and phenolic compounds, such as those found in GCMS analysis of Fraser fir bark in this study. Therefore, these compounds could have contributed to the aphids not feeding as well as disrupting their development.

Plant secondary compounds can provide chemical feeding stimuli that helps an insect recognize its host and a suitable food source. While many of these chemicals can elicit feeding, they also act as feeding deterrents which cue some insects to bypass the toxic effects (Fraenkel 1959, Cohen 2003). Therefore, the aphids in this study could have possibly avoided the treatments by selecting the more nutritious, non-toxic materials available. Even if an insect is presented with the most nutritious diet, they will not thrive on it unless it is eaten (Vanderzant, 1974). The same is true in trying to see the effects of a treatment on aphid fecundity. Adding juvabione or other chemicals to artificial diets may make the diets distasteful and therefore inedible to the aphids or they can avoid the chemicals all together

and never ingest them. Furthermore, adding chemicals systemically to plants for insects feeding on their natural host can also yield variable results as the abilities of individual plant roots can limit take up and translocation of compounds through the vascular system (Jedlicka et al. 2007). Therefore, while several plants may have been given the same amount of chemicals, some plants may not spread them throughout and insects may feed without ingesting the chemicals (Jedlicka et al. 2007).

Jedlicka et al. (2007) tested a series of juvenoid alcohols and their glycosidic derivatives on the pea aphid, *A. pisum*, to evaluate their systemic juvenizing activity when delivered through the root system of broad bean plants (Jedlicka et al. 2007). They also evaluated derivatives of juvabione (natural juvabiol and juvabiol acetate) in their assays based on research on insect hormones in plants reported by Slama (1979) and Fowler (1999, 2001). However, the authors saw no activity when juvabiol acetate was offered to *A. pisum*. Their results indicated that compared to the European bug, *P. apterus*, a low rate of active juvenoid is released from the juvenogen in the digestive tract of aphids (Jedlicka et al. 2007). Based on these results, it is possible that juvabione and methoprene didn't appear to prevent reproduction in our study, because the aphids simply don't liberate these compounds during the metabolic process. Therefore, they are not as affected by juvabione concentrations as are some other insects.

Our results did show that juvabione or other compounds present in the Fraser fir petroleum ether extract delayed reproduction. We believe this can be seen as a form of resistance and a possible population control method. When reproduction is delayed, adults are exposed to the elements and predators longer (in their natural range), both of which can

kill them. This delay also gives the tree or plant time to recoup and mend itself, possibly preventing its death. The implementation of juvabione or a juvenogen is effective against several insects as a control method. Further research is needed to see if these chemicals could provide a convenient and environmentally safe control method for aphids and the balsam woolly adelgid.

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Table 4.1 Basic Aphid Diet.

Ingredients	Concentration
Sigma Aldrich Yeast extract	18 g
Sucrose	3 g
Treatment	0.5 ml (per 200 µl diet sachet)
Ingredients dissolved in 100 ml cholesterol water (100 mg in 1,000 ml DI water) then sterilized with a Millipore Steriflip® filter unit (0.45 µm).	

Table 4.2 Results of analyses of variance (prob > F).

Source	# Feeding	# Days to produce honeydew	# Days to reproduce	# Reproductions
Days*	0.0197	-----	-----	-----
Diet	<0.0001	0.1847	0.0101	0.0381

*Days from study initiation included as a co-variable for feeding.

Table 4.3 Number of aphids feeding, number of days to honeydew production, number of days to reproduction, and minimum and maximum days until honeydew and reproduction was seen.

Treatment	# Feeding	# Days to produce honeydew	# Days to reproduce	# Reproductions	Honeydew Min--Max	Reproduced Min--Max
Yeast Extract	1.7^{B} (0.597)*	1.6^{A} (2.150)	6.0^{AB} (2.750)	0.4^{A} (0.421)	1--2	6--6
Acetone	2.6^{B} (0.525)	1.5^{A} (2.403)	9.0^{AB} (2.750)	0.6^{A} (0.421)	1--2	5--7
Methoprene	1.6^{B} (0.562)	1.4^{A} (2.150)	8.0^{AB} (2.750)	0.4^{A} (0.421)	1--3	8--8
Infested Bark	2.5^{B} (0.550)	5.7^{A} (2.775)	15.0^{AB} (3.890)	0.6^{A} (0.421)	1--13	2--8
Un-infested Bark	4.8^{A} (0.508)	9.0^{A} (2.150)	17.3^{A} (1.944)	2.0^{A} (0.421)	1--12	5--9
Infested Whole Wood	6.0^{A} (0.507)	3.8^{A} (2.150)	14.5^{A} (1.944)	1.8^{A} (0.421)	1--12	5--8
Un-infested Whole Wood	1.6^{B} (0.596)	2.8^{A} (2.150)	3.7^{B} (2.246)	0.6^{A} (0.421)	2--3	2--7

* Means in the same column followed by the same letter are not significant (α=0.05) according to according to Tukey-Kramer HSD procedure. Least squares means (Std Error).

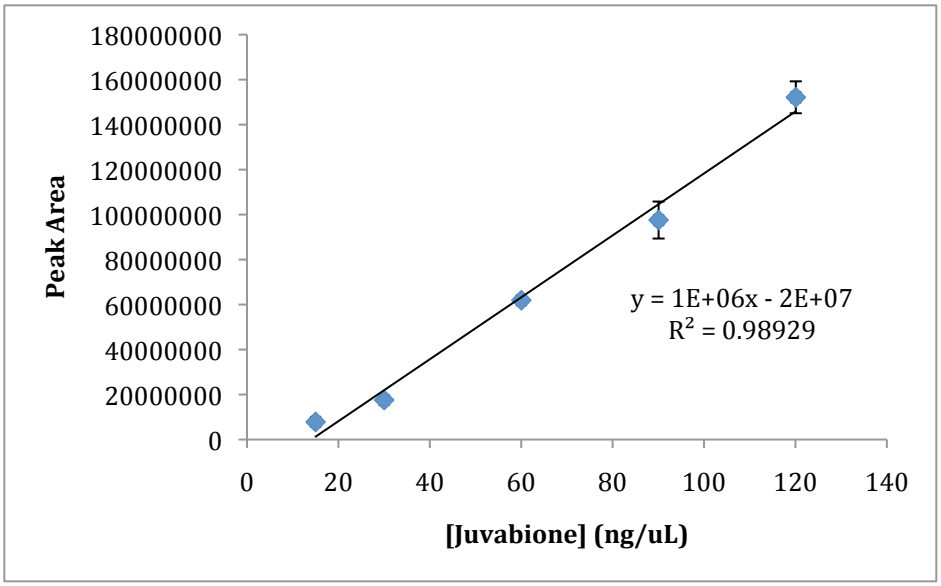

Figure 4.1 Calibration curve for peak area using external juvabione standards at concentrations: 15.01 ng·µL^{-1}, 30.02 ng·µL^{-1}, 60.04 ng·µL^{-1}, 90.06 ng·µL^{-1}, and 120.08 ng·µL^{-1}.

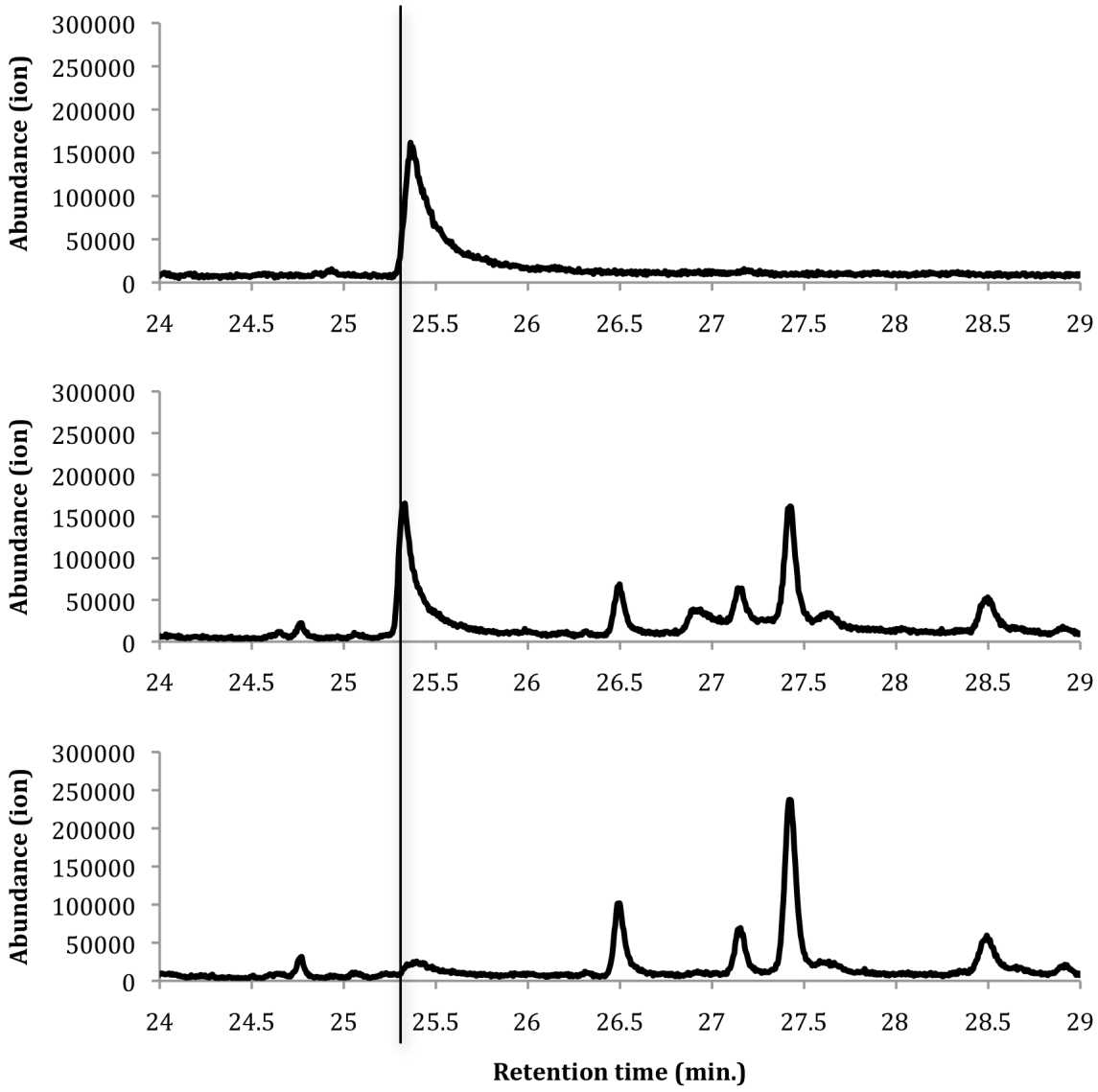

Figure 4.2 Representative chromatograms showing peaks for a 30ng/μL juvabione standard (top), infested whole wood (middle), and healthy whole wood (bottom), between 24 and 29 minutes. Peaks for juvabione eluted at 25.3 minutes.

APPENDICES

Appendix A

Evaluation of artificial diets and films on the balsam woolly adelgid, *Adelges piceae*

The balsam woolly adelgid, *Adelges piceae*, is an exotic, aphid-like insect that was introduced to the U.S. from central Europe in the early 1900s. Several artificial diets were tested to try and rear BWA in the laboratory. The success of rearing the BWA will be a major breakthrough in insect rearing since to-date no one has been able to successfully develop a diet for an insect with intractable feeding habits. The development of an efficient rearing prototype for BWA will greatly benefit other scientists who require quality laboratory-reared insects to discover means of controlling this pest based on host plant resistance, biological control, chemical control, or other novel approaches. Furthermore, the huge expenses incurred through rearing predators of BWA for biological control programs could be substantially reduced with a supply of abundant and healthy BWA as a food source.

All diet trials took place in incubators in the forest entomology lab at North Carolina State University. Fluorescent grow lights with a timer installed were placed in each incubator to provide light for the adelgids (16:8 L:D) and we tried to maintain a 75 percent humidity in the incubators by using saturated NaCl (Wexler and Hasegawa, 1954) placed in trays in the bottom of the incubators. Ground Fraser fir bark was added to diet trial one to see if it stimulated feeding. The addition of an insect's natural host can be included to an artificial diet for several reasons: chemicals present in the natural materials may serve as token stimuli, which may be mandatory in order for insects to feed on the artificial diet, and cryptic nutrients or suitable proportions of nutrients may make the diet desirable and it

reinforces the insects specialized feeding habits that steers it to certain plant species (Blossey et al. 2000, Cohen 2003).

Diet Trial 1

Five diets were made on a weight by weight basis:

1) Cannellini: 2 g sucrose (added after autoclave to prevent caramelizing), 1 g Vanderzant vitamins, 10 g cannellini bean meal, 12 g wheat germ, 75 ml water.

2) Soy: 2 g sucrose (added after autoclave), 1 g Vanderzant vitamins, 10 g Arrowhead soy re-fattened, 12 g wheat germ, 75 ml water.

3) Torula: 2 g sucrose (added after autoclave), 1 g Vanderzant vitamins, 22 g torula yeast, 75 ml water.

4) Starch: 1 g sucrose, 4 g ACS reagent soluble starch, 20 g wheat germ, 75 ml water.

5) Alfalfa: 1 g sucrose, 2 g starch, 10 g alfalfa bean meal, 12 g wheat germ, 75 ml water.

Each diet was divided in half (except the starch diet); half was placed into a small petri dish (5-6 cm in diameter) and the other half was mixed well with 1 g infested Fraser fir bark, which included BWA life stages, and then placed into another petri dish. One square of Parafilm was stretched and tightly placed over the petri dishes to cover the diets. Vacuum grease was placed around the edges of each petri dish to keep BWA contained. BWA eggs and crawlers were hand placed on top of each diet and varied from 3-15 eggs to 1-15 crawlers. These numbers are so variable because they depended on the availability in the infested materials we had (the infested Fraser fir wood had been in the lab for several months). The petri dishes were incubated at 15°C and monitored daily for the number of days it took to see mold growth on the diets and the number of adelgids that inserted into the

diet. The torula diets never got mold on them during the duration of the diet trial (12 days) and crawlers only inserted into three of the diets: cannellini with infested wood, alfalfa, and torula with infested wood (Table A.1). All of the inserted crawlers died shortly after and did not mature any further.

Diet Trial 2

The petri dishes were increased to a diameter of 9 cm (Figure A.1). The same five diets from trial one were used (without infested wood) but the cannellini diet had methyl paraben added (0.05% of diet) to see if it delayed the mold growth and if it affects BWA. The torula diet was also made in two forms: liquid and semi-solid to see if BWA showed a preference. Instead of placing crawlers and eggs on the diets, a cage was constructed with infested bark discs suspended above the diets to allow BWA to rain down on diets (Figure A.2). The petri dishes were incubated at 15°C and monitored daily for the number of days it took to see mold growth on the diets and the number of adelgids that inserted into the diet. All of the diets got mold growth with torula taking the longest (14 days) to get growth and alfalfa the shortest (one day). The diet containing methyl paraben was one of the diets to get mold growth the quickest (two days), perhaps there wasn't enough used in the diet to help preserve it. Only one crawler inserted into the torula-liquid diet and died without maturing (Table A.2).

Diet Trials 3 and 4

In order to make the area and the diets as sterile as possible, a clean room was constructed and a HEPA filter was placed in the room to help with air contaminates. The diets were prepared under a laminar flow hood to try and keep them sterile. Instead of Parafilm to cover the diets, four different films were made and tested as a substrate to cover

the diets: blended hardwood mixture (maple/oak/hickory) (BHW), unblended hardwood mixture (unHW), blended Fraser fir bark (BFF), and unblended Fraser fir bark (unFF) (all Fraser fir films made from un-infested wood). Two variations of films for each pulp were produced: pulp was either blended to make smooth films or the pulp was left unblended, providing a very textured surface. The texture created in the unblended films may provide favorable texture that mimics Fraser fir bark and encourages BWA insertion for feeding. All films were encased in a paraffin based wax. There were a total of six diets combined with each film: soy liquid diet with each of the four films, torula liquid diet with each of the four films, solid soy diet with each of the four films and one with Parafilm, solid torula diet with each of the four films and one with Parafilm, water diet with each of the four films, and no diet with each of the four films. We were low on some ingredients so not all diets had equal amounts of nutrients.

1) Torula liquid: 22 g torula yeast, 0.6 g Vanderzant vitamins, 2 g sucrose, 100 ml DI water.

2) Torula solid: 22 g torula yeast, 1 g Vanderzant vitamins, 2 g sucrose, 75 ml DI water.

3) Soy liquid: - 6 g Arrowhead soy re-fattened, 12 g wheat germ, 2 g sucrose, 100 ml DI water.

4) Soy solid: 10 g Arrowhead soy re-fattened, 15 g wheat germ, 1 g Vanderzant vitamins, 2 g sucrose, 75 ml DI water.

A Sigma optical grade polycarbonate rubber slab (0.3 cm thick), with five cut-out circles (2 cm in diameter) provided wells for the diets and was placed on top of a microscope slide. The slides, rubber slabs, and films were washed with warm soapy water and sanitized in a five percent Clorox solution for five seconds then dipped in deionized water for five

seconds and allowed to air dry under the laminar flow hood. A small amount of grease was placed on back of the rubber slabs and mounted onto a slide. The diets were placed into the wells and a different film was placed on top of each diet (Figures A.3 and A.4). Each slide had the same diet placed in each well with the five different films placed on top (except liquid diets didn't have Parafilm because of difficulty in sealing). Each slide had films set up in the same way:

BHW	unHW	unFF	BFF	Parafilm

The microscope assays were placed under infested wood logs to allow BWA to rain down on the diets. There were several problems with this set up: first, it's not a choice assay because the size of the slides are so large compared to the adelgids, that they may never walk around to know there are other choices, they may just stay at the first spot they land on. Second, adelgids were allowed to move between sites and it wasn't monitored if there was a pattern to their wandering, if they chose between locations they visited, or if they didn't travel far at all. We originally thought a positive to this set up was the rubber slabs could be separated from the slides to refill the diets. However, it was difficult to change out the diets because it was hard to not touch the side with crawlers while changing the underside. Therefore, several crawlers on the slides at the time of diet change could have been thrown off, smashed, or inserted crawlers could have been thrown off and thus, not accounted for.

For diet trial 3, there were three repetitions of each film and diet combination for the soy and torula liquid and solid diets, and two repetitions for the water and no diet slides. For diet trial 4, there were two repetitions of each film and diet combination except the water diet and the soy liquid diet because it got contaminated and was thrown out. The slides were

incubated at 20°C and monitored daily for the location of crawlers, if they inserted, and the number of days it took to see mold growth on the diets.

Among the films, the highest number of crawlers inserted into the unblended un-infested Fraser fir and none inserted into the blended hardwood or Parafilm (Table A.3). Among the diets, the torula yeast and soy solid diets appeared to be the most favorable and none inserted into the water diet, but did in the no diet films (Table A.4). Many of the diets with film substrates never got mold growth on them (Tables A.5 and A.6), indicating the films had antimicrobial properties (see chapter III). The soy liquid diet with hardwood unblended film appeared to be most susceptible to mold growth, but it took an average of 13.6 days until mold was visible, which means this could still be a viable diet if an easy and noninvasive method is developed to change out the diets regularly. In trials 3 and 4 the diets covered in Parafilm got mold growth in every repetition. Mold growth was also visible quicker in the diets covered in Parafilm, varying from four to 11 days, whereas most of the diets covered in films never got mold and if they did, it varied from five to 15 days.

What was interesting was that overall crawlers inserted into the rubber (0.10) more than the films (0.08) (Table A.4). This could suggest that BWA possibly feed like another adelgid species, the hemlock woolly adelgid (*Adelges tsugae*), which inserts into hemlock trees in one location but its stylets actually travels to feed in another location (Young et al. 1995). Furthermore, across all the diets the solid soy diet had the highest insertion rate in rubber (0.24), which was also twice as high as the insertion rate in the films (0.12). Further research is needed to better understand the feeding mechanisms of BWA feeding. Rubber may also be very porous allowing for easy insertion for BWA and thus provide a more

favorable substrate then the films and should be used as a cover for the diets in future trials.

ACKNOWLEDGEMENTS

Thank you to John Strider for his help in preparing the incubators, constructing the diet cages, and for all his help in traveling up to Avery County to collect samples and transport them back to North Carolina State University.

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Table A.1 The number of crawlers that inserted into the diets in trial one.

Diet	# Inserted	# Days mold
Alfalfa	1	12
Alfalfa-IN	0	5
Cann	0	7
Cann-IN	2	7
Soy	0	5
Soy-IN	0	5
Starch-IN	0	6
Torula	0	0
Torula-IN	1	0

Table A.2 The number of crawlers that inserted into the diets in trial two.

Diet	# Inserted	# Days mold
Alfalfa	0	1
Cann	0	2
Cann/ methyl paraben	0	2
Soy	0	2
Starch	0	3
Torula	0	14
Torula-Liquid	1	11

Table A.3 The number of crawlers that inserted into the films across all diets*.

Films	# Inserted
Fraser fir-blended	0.19 (0.079)
Fraser fir-unblended	0.23 (0.084)
Hardwood-blended	0
Hardwood-unblended	0.04 (0.038)
Parafilm	0

*Mean (Std Error).

Table A.4 The number of crawlers that inserted into the diets and rubber slab around the diets in trials 3 and 4*.

Diet	# Rubber	# Inserted
No Diet	0.06 (0.063)	0.06 (0.063)
Soy	0.24 (0.213)	0.12 (0.058)
Soy-Liquid	0	0.08 (0.083)
Torula	0.16 (0.111)	0.20 (0.082)
Torula-Liquid	0.15 (0.109)	0.05 (0.05)
Water	0	0
All	0.10 (0.083)	0.08 (0.056)

*Mean (Std Error).

Table A.5 The number of days it took to see mold growth between the diet and film combinations in trial 3.*

Films	Diets	Rep 1	Rep 2	Rep 3
Fraser fir-blended	No Diet	----	----	
	Soy	----	----	----
	Soy-Liquid	----	----	----
	Torula	----	----	----
	Torula-Liquid	----	----	----
	Water	----	----	
Fraser fir-unblended	No Diet	----	----	
	Soy	----	----	----
	Soy-Liquid	----	----	----
	Torula	----	----	----
	Torula-Liquid	13	13	----
	Water	----	----	
Hardwood-blended	No Diet	----	----	
	Soy	12	----	15
	Soy-Liquid	----	----	----
	Torula	----	----	----
	Torula-Liquid	----	----	----
	Water	----	----	
Hardwood-unblended	No Diet	----	----	
	Soy	15	----	----
	Soy-Liquid	13	13	15
	Torula	11	----	----
	Torula-Liquid	----	13	----
	Water	----	----	
Parafilm	Soy	4	11	4
	Torula	7	11	11

* Lines (----) indicate the diet never got mold growth and blank spots indicates that repetition wasn't done.

Table A.6 The number of days it took to see mold growth between the diet and film combinations in trial 4.*

Films	Diets	Rep 1	Rep 2
Fraser fir-blended	No Diet	----	----
	Soy	----	7
	Torula	----	----
	Torula-Liquid	----	----
Fraser fir-unblended	No Diet	----	----
	Soy	----	7
	Torula	----	----
	Torula-Liquid	----	----
Hardwood-blended	No Diet	----	----
	Soy	----	7
	Torula	----	5
	Torula-Liquid	----	----
Hardwood-unblended	No Diet	----	----
	Soy	----	----
	Torula	----	----
	Torula-Liquid	----	----
Parafilm	Soy	5	5
	Torula	5	5

* Lines (----) indicate the diet never got mold growth and blank spots indicates that repetition wasn't done.

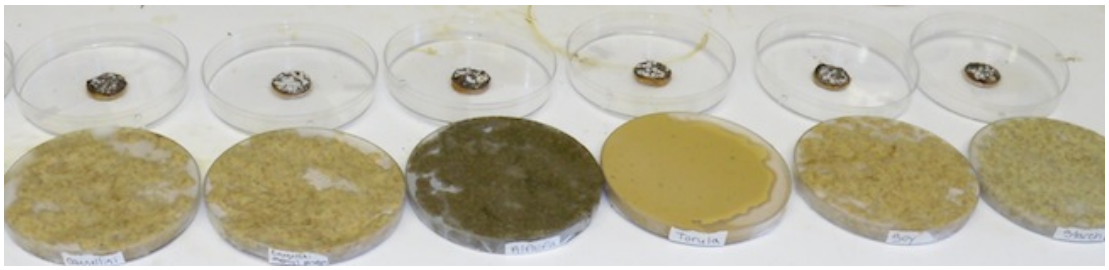

Figure A.1 Diets for trial two with Parafilm sealing them. The tops with infested Fraser fir discs to allow BWA to rain down on the diets.

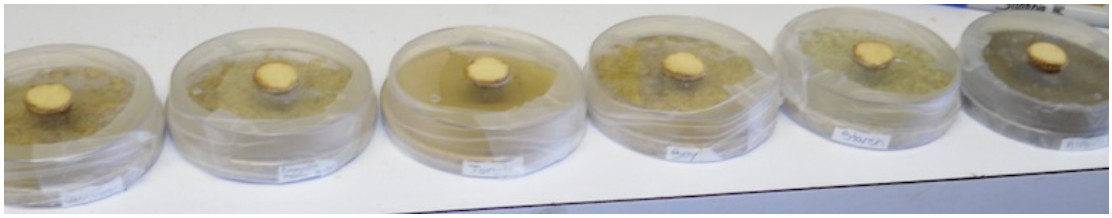

Figure A.2 Diets for trial two set up and sealed.

Figure A.3 Diets being filled in the wells.

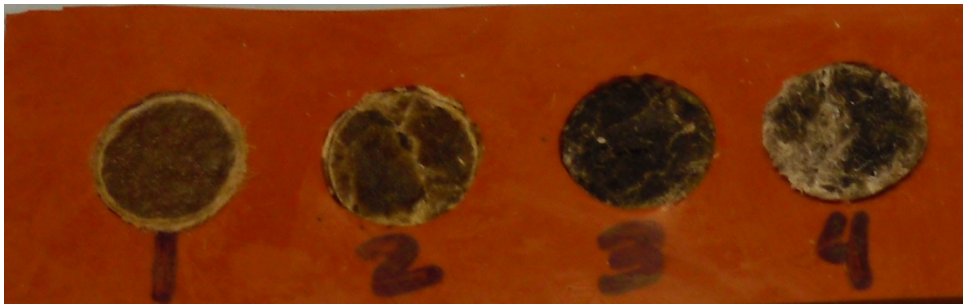

Figure A.4 Four different films placed on top of the diets. From left to right: blended hardwood, unblended hardwood, unblended Fraser fir, and blended Fraser fir.

Appendix B

Protein Analysis of The Balsam Woolly Adelgid, *Adelges piceae*

The analysis of individual amino acids, fatty acids, sterols, simple sugars, and minerals of insect diets can be an excellent method to obtain a nutrient profile, which can act as a model for new diets (Cohen, 2003). In order to identify proteinaceous components in the gut of the balsam woolly adelgid, *Adelges piceae* (BWA), the guts of adult BWA were dissected, digested with trypsin, and analyzed using liquid chromatography-tandem mass spectrometry (LC/MS/MS) to identify proteins present. This analysis would capture data on BWA gut protein components as well as those components ingested from the host. Amino acids are obtained by most insects from their foods by ingesting proteins (Nation, 2002). The steps of how the samples were analyzed are briefly described below:

1. Eleven BWA guts were dissected and the isolated guts were solubilized in 50 μL of 50 mM ammonium bicarbonate (NH_4HCO_3) buffer (pH 8.5) containing 1M Guanidine HCl.
2. Next, a solution of endoproteinase Lys-C was prepared at 1μg/μL in 50mM NH_4HCO_3, pH 8.5.
3. Two microliters (μL) of Lys-C solution was added to the 100 μL of solubilized adelgid guts.
4. The sample was incubated overnight for proteolytic digestion in a 37^{o}C water bath.
5. The following day the sample was completely dried in a Speed Vac system.
6. The dried, digested sample was resolubilized in 20 μL of 0.1% formic acid.
7. The sample was analyzed by LC/MS/MS using a NanoAcquity ultra-pressure liquid chromatograph (UPLC) coupled to a Q-Tof Premier quadrupole time-of-flight mass

spectrometer using a top-8 data-dependent acquisition mode.

Chromatography: 75 μm inner diameter (id) x 25 cm analytical column packed with 1.7 μm Bridged Ethyl Hybrid (BEH) reversed-phase particles; Mobile Phase A = 0.1% formic acid in H_20, Mobile Phase B = 0.1% formic acid in acetonitrile (CH_3CN); flow rate = 300 nL/min. Trapping column: 180 μm id x 5 mm Symmetry C18, 5 μm reversed-phase particles. Sample (20 μl injection) was loaded onto the trap column at a flow rate of 10 μL/min for 5 minutes for desalting. After switching the analytical column in-line, a 60 minute linear gradient from 7-40% B was used to elute peptides into the electrospray ion source of the Q-Tof Premier MS.

Mass Spectrometry: tandem mass spectra were acquired using data-dependent LC/MS/MS with 1.3 second MS survey scans, and up to 8 product ion spectra per MS/MS cycle. Product ion scans were 2 seconds each, with a maximum of 2 MS/MS scans per precursor ion.

Data Analysis: to generate peak list (.pkl) files for database searching, raw MS data files were processed using ProteinLynx Global Server 2.4 software. Peak list (.pkl) files were searched against the following databases using Mascot 2.4 software: NCBI nonredundant, SwissProt, NCBI *Pinus taeda* (loblolly pine), NCBI *Myzus persicae* (green peach aphid), and NCBI *Adelges piceae* (balsam woolly adelgid). Peptide matches were manually reviewed for assessing match quality.

LC/MS/MS analysis of the digested adelgid gut sample indicated a large number of high quality MS/MS spectra of high abundance, suggesting a large number of component proteins (Figure B.1) present in the gut. However, due to the very limited number of protein

entries specific to BWA in any of the available protein sequence databases, most of the resulting MS/MS spectra could not be matched and assigned to any protein. No doubt many of these unassigned spectra could be matched if the genome for BWA were fully sequenced and the corresponding protein database available. For the most part, searches of the aphid or tree databases were devoid of any matches. However, searches of the more comprehensive SwissProt and NCBI nonredundant protein databases enabled identification of several proteins from other insect species, including four enzymes involved in the glycolysis/glucose metabolism pathway (Figure B.2). Glycolysis is the process by which an insect starts to metabolize glucose (Nation, 2002).

The proteins identified that are involved in the glycolysis/glucose metabolism pathway include enolase (Figure B.3, Tables B.1 and B.2), fructose 1,6-bisphosphate aldolase (Figure B.4 and Table B.2), glyceraldehyde 3-phosphate dehydrogenase (Figure B.5 and Table B.1), and triosephosphate isomerase (Figure B.6 and Table B.1). All of these enzymes generate ATP from D-glucose in the glycolysis pathway and since they were identified, they must be present in high abundance in the gut, which makes sense if this is a major route of energy production. Further research in this field is needed to complete the BWA genome in order to identify the proteins present and gain insight into the nutritional requirements of BWA.

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Table B.1 Proteins identified in Swiss Prot protein database that are involved in glycolysis. Threshold Score >36 ($p<0.05$).

Protein	Sequence	Mascot Score	# Peptides
Protein #2: Enolase	GATSFTEAMK	42	4
	DGQYDLDFK	28	
	FGLDATAVGDEGGFAPNI	61	
	LAMQEFMILPTGATSFTE	29	
Protein #3: Glyceraldehyde-3-phosphate dehydrogenase			
	LISWYDNEFGYSNRVID LIK	15	1
Protein #4: Triosephosphate isomerase	DWSKVVLAYEPVWAIG TGK	14	1

Table B.2 Proteins identified in NCBI nonredundant protein database that are involved in glycolysis. Threshold Score >52 ($p<0.05$).

Protein	Sequence	Mascot Score	# Peptides
Protein # 1: Fructose 1,6-bisphosphate aldolase	IAQAIVAPGK	39	2
	KIAQAIVAPGK	22	
Protein # 2: Enolase	DGKYDLDFK	28	2
	ITANTSIQIVGDDLTVTNI	28	

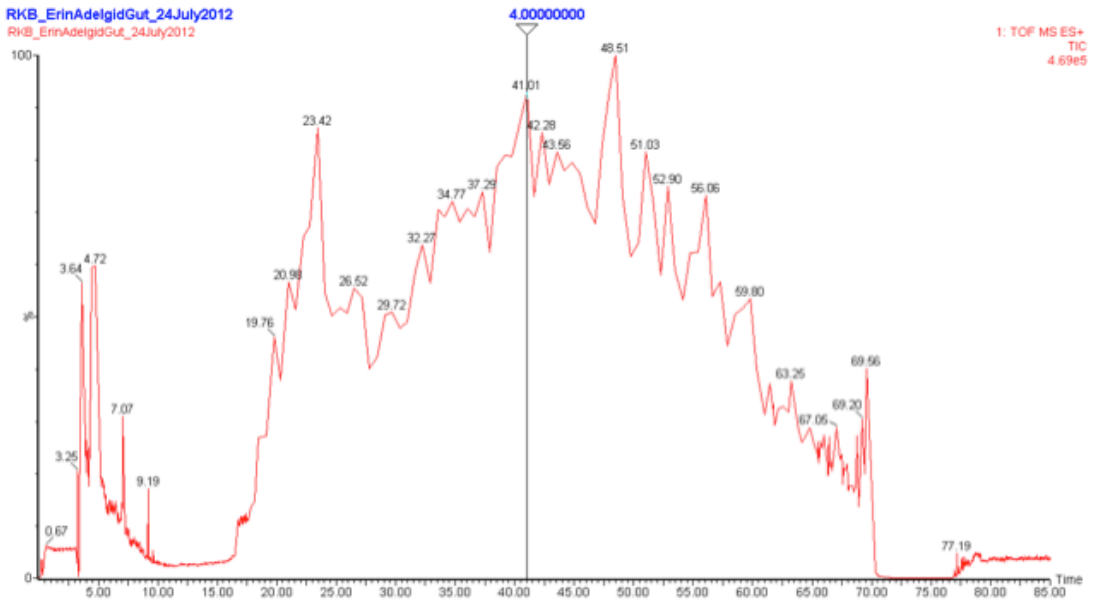

Figure B.1 Total Ion Chromatogram: LC/MS/MS analysis of the digested adelgid gut sample.

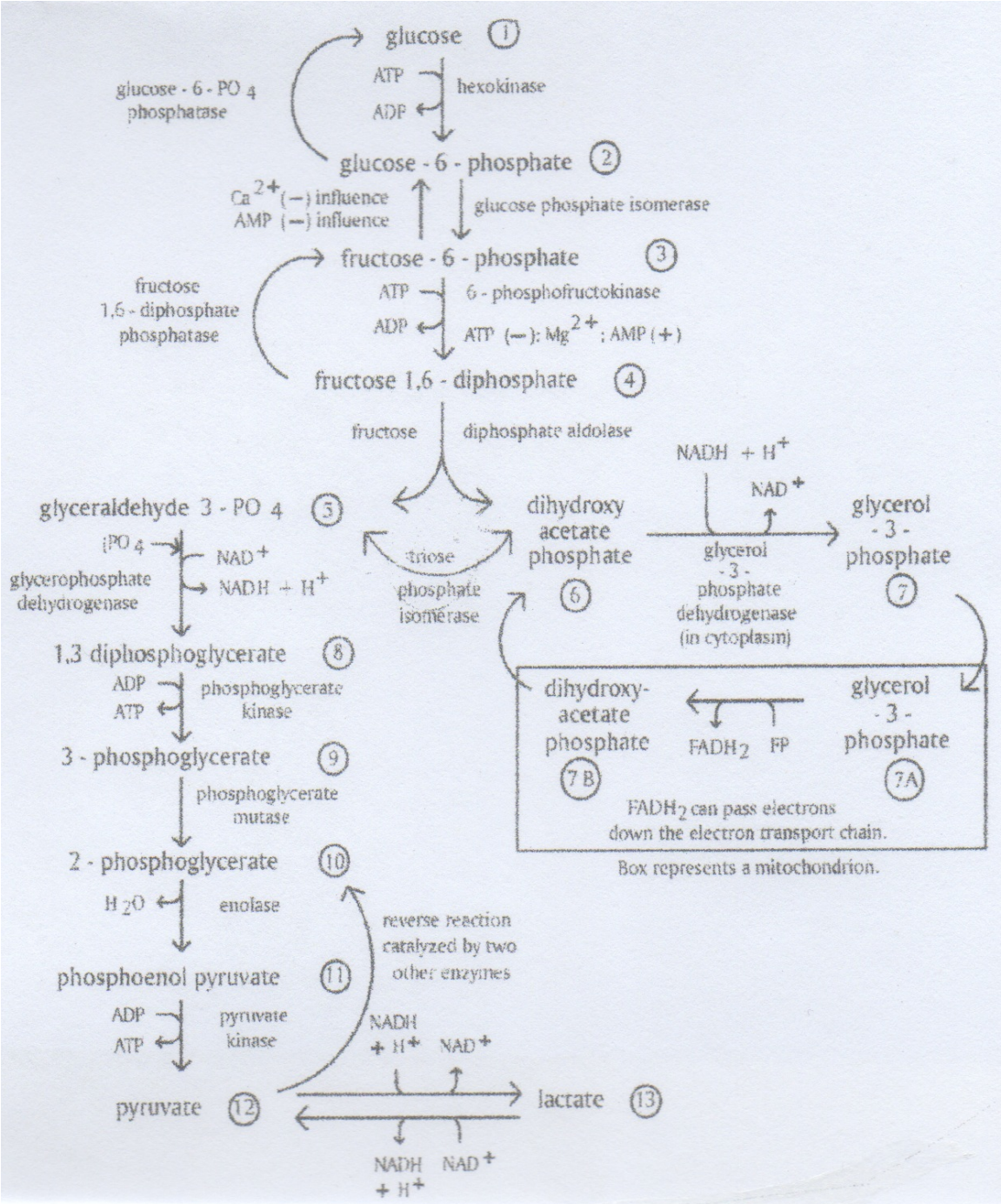

Figure B.2 The glycolytic pathway for metabolism of glucose in insects. Figure taken from Nation (2002).

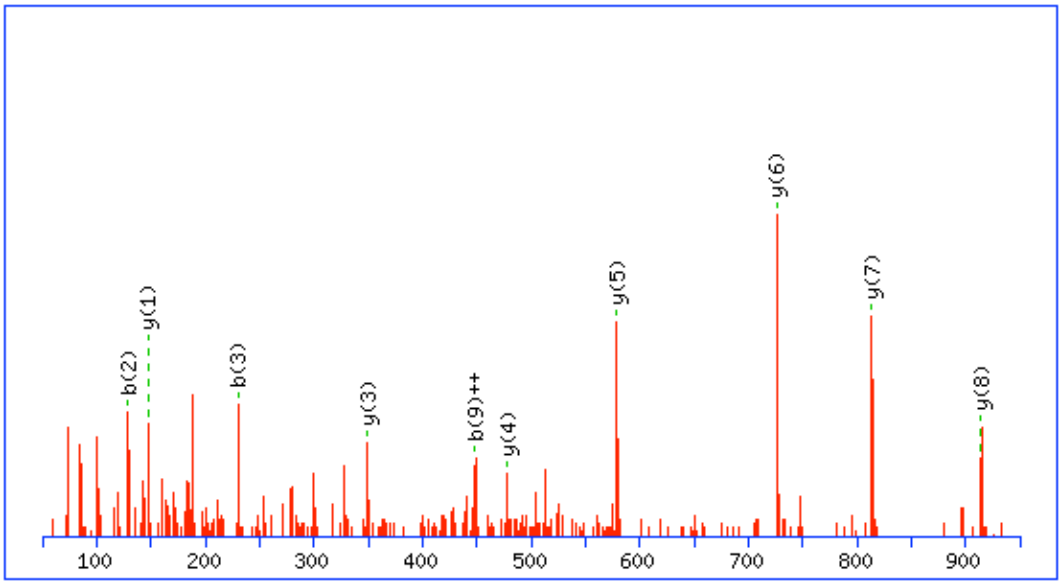

Figure B.3 Tandem MS spectrum match to enolase peptide GATSFTEAMK.

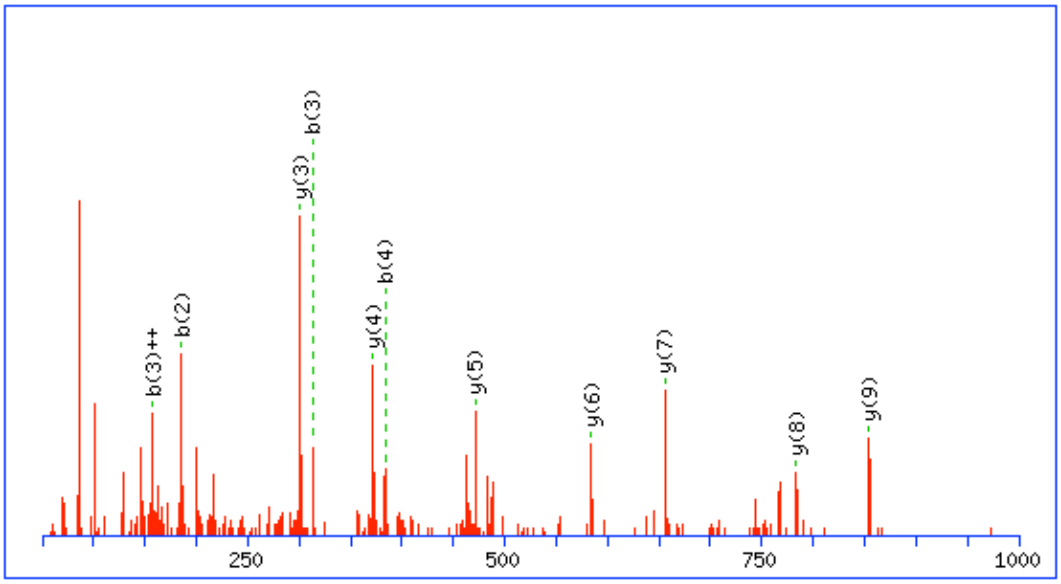

Figure B.4 Tandem MS spectrum match to fructose 1,6-bisphosphate aldolase peptide IAQAIVAPGK.

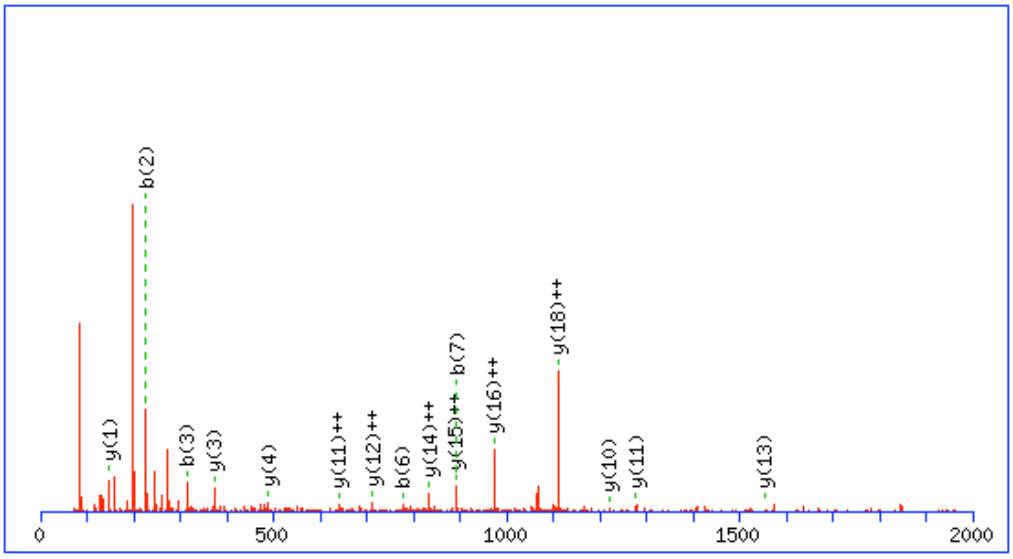

Figure B.5 Tandem MS spectrum match to glyceraldehyde-3-phosphate dehydrogenase peptide LISWYDNEFGYSNRVIDLIK.

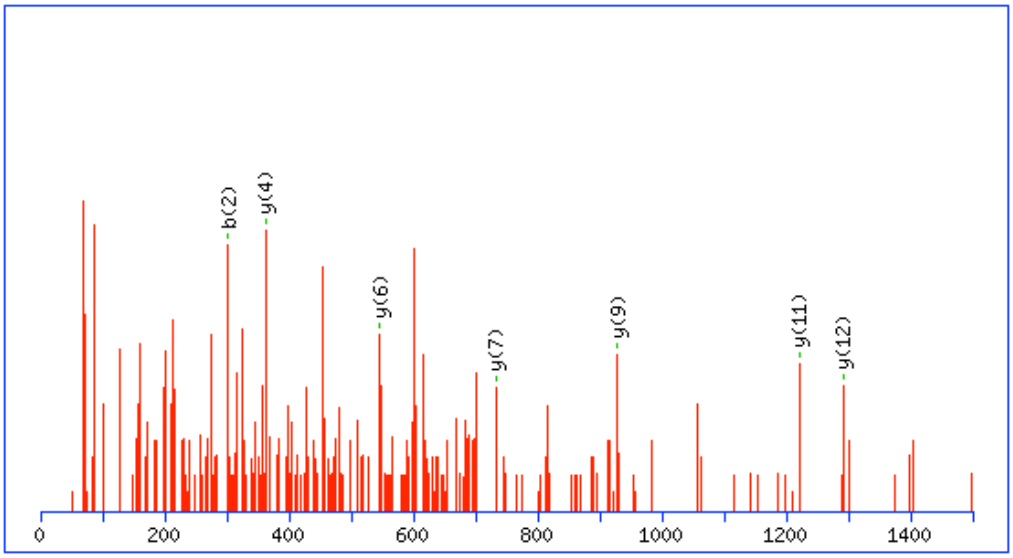

Figure B.6 Tandem MS spectrum match to triosephosphate isomerase B peptide DWSKVVLAYEPVWAIGTGK.

Appendix C

Evaluating Artificial Diets for Rearing the Green Peach Aphid, *Myzus persicae*

The summer of 2011 was spent rearing the green peach aphid, *Myzus persicae*, to gain experience in insect rearing. Aphids are often used as a model for the feeding behavior of insects because they have similar mouthpart form and function, and produce watery saliva while feeding that creates a protective sheath around the mouthparts. The insects were fed as described in chapter IV of this volume with the diets contained within a sachet. Five diets were set up to measure the efficacy and length of time *M. persicae* could survive: a water diet, sucrose and water diet, yeast extract and sugar diet, a known aphid diet (Mittler and Dadd, 1962), and no diet (empty sachet).

Since color plays a large part in recognition and acceptance of foods by insects (Cohen, 2003), half of the diets (except the no-diet) had green food coloring added to them to see if the color acted as an attractant for the aphids. The green was compared against the natural color of the diets (clear for the water and sugar diets, and yellow for the known aphid diet and yeast extract). To observe if the aphids showed a preference in their feeding environment, mesh fabric was attached to half the diet sachets to see how the aphids responded to the texture, compared to a surface that was just the diet sachet (Parafilm), which was smooth and provided no texture. There were a total of 18 diet treatments and the test was replicated three times. The diets were observed daily and the number of aphids seen feeding, the date honeydew was first present, and if they reproduced was recorded. *M. persicae* were maintained in a 18°C incubator with a 16:8 photoperiod, and humidity was not controlled (Mittler and Koski,1976).

The diets were evaluated to see which was most successful in order to be used as the main diet in the aphid juvabione study. The water diet, sucrose diet, and no-diet were dropped from the analysis since none of the aphids were able to survive (Table C.1). The yeast extract diet with the mesh surface was selected based upon the preliminary observations from this rearing experience (Tables C.2 and C.3). Precise dietary composition was not a primary concern because only the effect of a treatment was being observed in the juvabione study, not their dietary needs. This allowed diets to be rapidly prepared by using less expensive, undefined ingredients (Mittler and Koski,1976).

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Mittler, T.E., and P. Koski. 1976. Development of meridic and oligidic diets for rearing the aphid Myzus persicae. Journal of Insect Physiology 22: 1135-1141.

Table C.1 Number of aphids seen feeding daily for all diets across trials 1-3*.

Diet	# Feeding
No diet (Non Estimable)[A]	-2.20E-16
Sucrose	4.0 (0.421)[A]
Sucrose-Food coloring	2.2 (0.420)[AB]
Sucrose-Food coloring-Mesh	0.3 (0.596)[B]
Sucrose-Mesh	0.5 (0.596)[AB]
Water	0.6 (0.596)[B]
Water-Food coloring	0.3 (0.596)[B]
Water-Food coloring-Mesh	0 (0.596)[B]
Water-Mesh	0 (0.610)[B]

* Means in the same column followed by the same letter are not significant according to α=0.05, according to Tukey Kramer HSD procedure. Mean (Std Error).

Table C.2 Number of aphids seen feeding daily for yeast extract and aphid diets across trials 1-3.

Diet	# Feeding
Yeast Extract-Mesh	5.43[A]
Yeast Extract	3.78[B]
Aphid diet-Mesh	4.04[B]
Yeast Extract-Food coloring	3.28[B]
Aphid diet-Food coloring	3.14[B]
Aphid diet	3.23[B]
Aphid diet-Food coloring-Mesh	2.88[B]
Yeast Extract-Food coloring-Mesh	4.10[B]

* Means in the same column followed by the same letter are not significant according to α=0.05, according to Tukey Kramer HSD procedure.

Table C.3 Number of aphids seen feeding daily for diet x trial*.

Diet	Trial 1	Trial 2	Trial 3
Yeast Extract	2.9 (0.697) ^{ABCD}	3.3 (1.633) ^{ABCD}	4.8 (0.717) ^{ABC}
Yeast Extract- Food coloring	5.6 (0.726) ^{A}	2.4 (1.458) ^{ABCD}	0.9 (0.793) ^{CD}
Yeast Extract-Mesh	5.6 (0.720) ^{A}	5.3 (0.847) ^{AB}	5.4 (0.695) ^{A}
Yeast Extract- Food coloring-Mesh	0.3 (1.339) ^{D}	5.3 (0.821) ^{A}	2.5 (1.905) ^{ABCD}
Aphid diet	4.1 (0.782) ^{ABCD}	3.4 (0.847) ^{ABCD}	0.8 (1.214) ^{D}
Aphid diet- Food coloring	2.5 (0.720) ^{ABCD}	4.4 (0.785) ^{AB}	0.8 (1.214) ^{D}
Aphid diet-Mesh	5.2 (1.196) ^{ABCD}	4.4 (0.847) ^{AB}	1.8 (1.214) ^{BCD}
Aphid diet- Food coloring-Mesh	2.1 (0.821) ^{ABCD}	4.9 (1.223) ^{ABCD}	2.0 (1.011) ^{BCD}

* Means in the same column followed by the same letter are not significant according to α=0.05, according to Tukey Kramer HSD procedure. Mean (Std Error).

THESIS CONCLUSIONS

The balsam woolly adelgid (BWA; *Adelges piceae*) is an exotic pest that has killed thousands of acres of Fraser fir, *Abies fraseri*, an important resource that only occurs naturally in the Southern Appalachians and is also a prominent Christmas tree species. The research presented in this thesis was aimed to determine the necessary requirements to successfully develop artificial rearing techniques for BWA and has presented a novel approach in rearing an insect species. The best substrate and optimum environmental conditions for BWA development and reproduction in the laboratory was determined (Chapter II) and future rearing of BWA should be done with seedlings in a large enough area to permit adequate airflow, set at 20°C and 75 percent RH. Logs that contain branches with a lot of needles should also be included to see if that increases the infestation rate and provides an optimal rearing substrate for BWA.

Several findings in this research helped in understanding the induced responses from the BWA and Fraser fir relationship. BWA feeding on Fraser fir modifies the bark and initially causes a rapidly induced response that increases protein content of the bark and adelgid survival (Chapter III). BWA may cause an induced response in the tree is to force the tree to generate more glucose, which allows BWA to survive because they (or the symbionts in their gut) use the high glucose levels as an energy source in glycolsis. It is also possible that BWA possibly ingest higher sugar levels than initially thought. Artificial diets tested to this point may have been insufficient because it was thought BWA didn't consume as much sugar as aphids. Further research is needed to explore the glucose relationship in BWA that could provide a basis for diet development containing the correct percentage of

nutrients to sustain BWA populations. IC comparisons should also be done with Turkish fir, *Abies bornmuelleriana*, and Veitch fir, *Abies veitchii,* both tolerant and resistant to BWA attack, respectively, to see how their sugar levels correspond to Fraser fir. Infested bark was found to be twice as hard as un-infested bark, suggesting an induced response and possibly the trees' defense mechanism against adelgid attack. Hardness comparisons should also be done with *A. bornmuelleriana*, and *A. veitchii* to see if hardness acts as a defense mechanism.

Results from our juvabione trial (Chapter IV) showed that juvabione or other compounds present in the Fraser fir petroleum ether extract delayed reproduction. We believe this can be seen as a form of resistance and a possible population control method. When reproduction is delayed, adults are exposed to the elements and predators (in their natural range) longer, both of which can kill them. This delay also gives the tree or plant time to recoup and mend itself, possibly preventing its death. The juvabione or a juvenogen is an effective control against several insects. These chemicals could provide a convenient and environmentally safe control method for aphids and the balsam woolly adelgid.

While none of the diets were successful in rearing BWA (Appendix A), great strides have been made in showing favorable substrates that may aid in developing a successful artificial diet and rearing system. Overall, crawlers inserted into the rubber more than the films. The rubber may also be very porous allowing for easy insertion for BWA and thus provide a more favorable substrate than the films and should be used as a cover for the diets in future trials. LC/MS/MS analysis of the digested adelgid gut sample revealed a large number of high quality MS/MS spectra of high abundance (Appendix B) but due to the very limited number of protein entries specific to BWA in any of the available protein sequence

databases, most of the resulting MS/MS spectra could not be matched and assigned to any protein. If the genome for BWA were fully sequenced and the corresponding protein database available, many of these unassigned spectra could be matched and would give insight into the nutritional requirements of BWA. Four proteins were identified that are involved in the glycolysis/glucose metabolism pathway and all of these enzymes generate ATP from D-glucose in the glycolysis pathway. The research I presented has provided the groundwork for further research to better understand the feeding mechanisms of BWA. A multidisciplinary approach has provided several different findings that are all connected, and have provided a slightly larger look at potential rearing strategies for BWA and a direction for future research.