ROOT-FEEDING CURCULIONID BEETLES IN UNMANAGED AND MANAGED LOBLOLLY PINE STANDS OF THE GEORIGA PIEDMONT AND DETECTION OF THEIR ASSOCIATED PHORETIC OPHIOSTOMATOID FUNGAL COMMUNITIES

by

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(Under the Direction of Caterina Villari)

ABSTRACT

Grosmannia blue-stain fungi and their root-feeding beetle vectors (*Hylastes*, *Hylobius* and *Pachylobius* species) have been associated with areas of loblolly pine mortality in the Piedmont region of Georgia, yet their potential role in host mortality is unestablished. Study objectives were to determine phenology of loblolly pine associated root-feeding beetles in Georgia and assess potential variation of the blue-stain fungal community associated with these beetles. Beetles were live-trapped in two loblolly pine stands with differing management histories from May 2017-October 2018. Across vectors, abundance varied over time and site was found to have significant effect on the number of root-feeding beetles collected. Ophiostomatoid fungal composition was found to vary between vector species in 2017; fungal species were primarily isolated in spring regardless of vector species or site. Results of this study will provide useful insights for future study of these fungal vectors and inform forest health management in the southeastern U.S.

INDEX WORDS: Ophiostomatoid fungi, Root-feeding beetles, Hylastes, Hylobius, Pachylobius, Phenology, Grosmannia, Loblolly pine

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DEDICATION

Dedicated to the loving memory of John Baptiste Motto; I think of you daily and would not be who I am without your influence and example.

For my parents Jerry and Julie Buland and my brothers Ethan and Nolan; the boundless love, support and encouragement you have always and unfailingly shown are a perpetual source of strength and inspiration. Thank you for always encouraging me to make the world more beautiful.

And for Eric Millard; thank you for consistently encouraging me to move past my limits, for believing in me, even when I don’t believe in myself, and for pushing me up hills, mountains and to meet my goals.

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THESIS INTRODUCTION AND LITERATURE REVIEW

1.1 Mortality of Loblolly Pine in the Southeastern United States

Loblolly pine (*Pinus taeda* L.) is the most commercially significant tree species in the southeastern United States, and along with shortleaf pine, *P. echinata* Mill. is the top plantation cultivated species in the region at 30 million acres. Plantation cultivated trees in this region account for 43% of the total loblolly-shortleaf inventory in the country (Smith et al., 2007; Wear & Greis, 2002), and in 2007 loblolly pine accounted for 32,873 million cubic feet in net volume of growing stock in the southeastern U.S (Smith et al., 2007). Additionally, the species is a primary source of wood pulp for the paper industry (Wear & Greis, 2002). Lately, loblolly pine is experiencing elevated levels of mortality around the Fall-line and Upper Coastal Plain regions in Alabama and Georgia (Eckhardt et al., 2004; Eckhardt & Menard, 2009; Eckhardt et al., 2007), which raises a high economic and environmental concern given the significance of the species. Thus, determination of the factors contributing to this phenomenon has become a crucial task as related to informing future management strategies and economic practices of southeastern U.S. forests and plantations.

Root-feeding beetles belonging to the family Curculionidae (*Hylastes, Hylobius* and *Pachylobius* spp.), and their Ophiostomatoid fungal obligates, also known as blue-stain fungi (in this case *Leptographium* and *Grosmannia* spp., hereafter referred to as the

Grosmannia complex) have been associated with areas of decline Eckhardt et al., 2004; Eckhardt et al., 2007; Matusick et al., 2013b; Sullivan et al., 2003; Zanzot et al., 2010); however, the actual role of these beetles and their fungal obligates in loblolly pine mortality has yet to be established (Coyle et al., 2015). Indeed, *Leptographium* and *Grosmannia* species are mostly considered, with some notable exceptions, only weak pathogens and, together with their beetle vectors, known to be secondary colonizers of their tree host and not viewed as primary drivers of mortality (Coyle et al., 2015; Jacobs & Wingfield, 2001). Thus, the role ascribed to the root-feeding beetles and to the *Grosmannia* fungal complex as a primary driver of mortality in *P. taeda* (Hess et al., 2002) is unexpected and controversial.

Though *Grosmannia* complex fungi may potentially be of significance in the forests of the southeastern United States, too little is still known of their actual species composition and ecology. Prior research into this complex has largely focused on pathogenicity of the fungi (Eckhardt et al., 2004), presence or absence of *Grosmannia* complex species in the galleries of root-feeding beetles (Eckhardt et al., 2007; Matusick et al., 2013a) and the question of whether the root-feeding beetle associated *Grosmannia* complex is driving mortality of loblolly pine on a stand level (Hess et al., 2002; Zanzot et al., 2010). Similarly, very little is known concerning the root-feeding beetle vectors associated with these Ophiostomatoid fungi in the southeastern United States. The Pales weevil (*Hylobius pales*) and the Pitcheating weevil (*Pachylobius picivorus*) are recognized as forest and in particular pine regeneration pests throughout the southeast due to their proclivity to feed upon regenerating pine seedlings (Davis & Lund, 1966; Warner, 1966), however very little is known regarding their phenology, habitat or ecology outside of the context of regeneration stands. Similarly, the *Hylastes* species of the southeast; *Hylastes salebrosus, Hylastes porculus* and *Hylastes tenuis*, though all recognized as native species to the region and as confirmed vectors of Ophiostomatoid fungi in the region, are relatively unknown in terms of their life history or ecology. Finally, little consideration has been given to the nature of the relationship between root-feeding beetles and the *Grosmannia* complex (L. G. Eckhardt et al., 2007), despite extensive studies of the relationships between bark beetles and Ophiostomatoid fungi in other systems, which have showcased a wide range of possible relationships (Kirisits, 2004; Klepzig & Six, 2004; Lieutier et al., 2009). Fungal-vector relationships such as that between the European elm bark beetle *Scolytus multistriatus* and the native (to North America) elm bark beetle *Hylurgopinus rufipes* with the Dutch elm disease pathogens *Ophiostoma ulmi* (Buisman) Nannf.and *O. novo-ulmi* have, for example, been the subject of extensive research and study (Brasier et al., 1998; Webber, 2000).

Interestingly, the Dutch elm disease complex is one of the few such relationships centered around a hardwood tree host, meanwhile there are multiple instances of insect-fungal relationships which have significant negative effects upon conifer hosts. For example, the mountain pine beetle, (*Dendroctonus ponderosae*) Hopkins has a mutualistic relationship with the Ophiostomatoid species *G. clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., and *Ophiostoma montium* (Rumbold) Arx, Antonie van Leeuwenhoek, which have been shown to increase nitrogen levels in the phloem of host trees Ponderosa pine (*P. ponderosa*) Lawson & C. Lawson and lodgepole pine (*P. contorta*) Douglas ex Loudon by ~40% (benefitting the beetle vectors) while having marked negative effects upon the host tree (Bleiker & Six, 2009;

Gibson et al., 2009). In other examples, such as the relationship between the southern pine beetle (*D. frontalis*) with *O. minus* or that of the spruce bark beetle (*Ips typographus* L.) with multiple species of Ophiostomatales in Europe in association with their host Norway Spruce (*Picea abies* L.) Karst. (Six & Klepzig, 2004; Wermelinger, 2004) the host usually experiences mortality as a result of the beetle-fungal infection.

1.2 Loblolly Pine Mortality and its Presumed Association with *Grosmannia* fungi in the Southeastern United States

Unexpected mortality of mature *P. taeda* was first described in overly mature (50+ years of age) loblolly pine stands of the Talladega National Forest during the 1950s; coined 'loblolly pine die-off' (Brown & McDowell, 1968) such mortality became a recurring phenomenon across the southeastern U.S. Lateral root deterioration and feeder root death were determined to be some of the first symptoms of mortality, followed by stress cone production a year prior to death (Brown & McDowell, 1968). Foliar symptoms associated with such mortality of *P. taeda* include reduced radial growth, shortened chlorotic needles and needle drop/sparse crowns of affected trees (Eckhardt et al., 2007; Hess et al., 1999; Hess et al., 2002). Initial studies as to the cause of mortality on this site was attributed to a number of potential causes, including *Heterobasidion irregulare* Garbelotto & Otrosina, *Phytophthora cinnamomi Rands, Meded. Inst. Plantenziekt.* and *Pythium* spp., which were present in root samples collected from the affected trees (Roth & Peacher, 1971). However, a subsequent study in 2000 evaluating potential causes of decline on loblolly pine sites in Alabama did not support these finding, with *P. cinnamomi* found inconsistently and *H. irregulare* completely absent from collected root samples (Hess et al., 2002). While according to the same study, *Leptographium* and *Grosmannia* spp. were isolated from the majority of both root and soil samples collected at these sites, leading to the hypothesis that *Grosmannia* complex species might have played some role in the observed mortality of mature loblolly pine (Hess et al., 2002). The latter possibility was pursued in a study published in 2007, which documented the association between root-feeding beetles and *Grosmannia* complex fungi in loblolly pine stands in a state of decline, leading to the supposition that this vector-pathogen relationship was a primary driver of loblolly pine mortality (Eckhardt et al., 2007). Such observations led to the supposition that this mortality of loblolly pine is a regionally occurring phenomenon that has since been commonly referred to as Southern Pine Decline (SPD) (Eckhardt et al., 2007).

A subsequent study has, however, found little support for this theory, rendering the concept of a widespread SPD syndrome a subject of some debate (Coyle et al., 2015; Jacobs & Wingfield, 2001). Firstly, according to Ciesla and Donaubauer's 1994 publication, the term decline as it relates to forest health is best described as: "*an episodic event characterized by premature, progressive loss of tree and stand vigor and health over a given period without obvious evidence of a single clearly identifiable causal factor such as physical disturbance or attack by an aggressive disease or insect"* (Ciesla & Donaubauer, 1994). The proclivity of mature *P. taeda* to preferentially succumb to loblolly pine mortality therefore does not follow from this definition, as immature (or rather not overly mature) loblolly pine does not seem to be affected by such mortality. Furthermore, commonly accepted theories of what constitutes a decline do not fully match what is known of loblolly pine mortality. One such concept is that of

"Predisposing", "Inciting" and "Contributing" factors, (Manion, 1987) which suggests that such problems are the results of the interaction of three or more sets of factors. Predisposing factors are long-term factors like site and climate which affect the trees' ability to withstand agents of disease. Inciting factors are short duration influences, such as defoliating insects or drought. Contributing factors are those additional factors which further weaken and kill the trees, such as bark beetles, and root decay fungi which are often ultimately credited with the death of the tree (Manion, 1987, 1991). Another theory, proposed by Houston (1981, 1992), defines decline as disease caused by repeated stress factors, compounded by "organisms of secondary action" (pathogens, pests, etc.) which can only successfully attack weakened hosts (Houston, 1992; Houston, 1981).

Observed mortality of loblolly pine, under the definition of SPD, though acknowledging the relevance of potential Predisposing or Inciting factors, does not consider them to nearly the same extent as Contributing factors, attributing SPD primarily to fungal and insect influence, and thus does not evaluate the entire scope of why elevated mortality of loblolly pine could potentially be in occurrence. Additionally, decline syndromes generally persist over extended periods of time and tend to progressively advance. Yet, in the case of the stands in which SPD was initially observed, tree condition had improved the year following the initial observation of mortality (Brown & McDowell, 1968) and five years after the initial observations by Brown and McDowell, decline associated mortality in the stands had almost completely ceased (Roth & Peacher, 1971).

In summary, there are currently, two predominant hypotheses as to the cause of loblolly pine mortality as it relates to root-feeding beetles and blue stain fungi. 1) either there is a regional Southern Pine Decline (SPD) syndrome occurring in the southeastern U.S. including the Piedmont region caused by root-feeding beetles and their associated fungal complex (in this case *Leptographium* and *Grosmannia* spp.) (Eckhardt et al., 2007; Zanzot et al., 2010), or 2) root feeding beetles and their associated *Grosmannia* complex fungi are contributing factors in loblolly pine mortality, taking advantage of already weak and stressed trees with compromised resistance resulting from multiple environmental and anthropogenic factors (Coyle et al., 2015).

1.3 Biology and Physiology of *Grosmannia* and *Leptographium* Fungi

Fungal species belonging to the *Grosmannia* and *Leptographium* genera (the latter being an asexual form of the *Grosmannia* genus), are members of the Ophiostomataceae family in the phylum Ascomycota (or sac fungi). According to the current literature, the Ophiostomatoid species associated with loblolly pine in the southeastern U.S. are *L. procerum* (Kendrick)*, L. terebrantis* (S.J. Barras and T.J. Perry)*, G. alacris* (Goidanich) and *G. huntii* (R.C. Robinson) (Eckhardt et al., 2007; Hess et al., 2002; Matusick et al., 2013b).

The sexual form, *Grosmannia*, is characterized by flask-shaped sexual structures (perithecia) which release sticky sexual spores (ascospores); in addition to the asexual spore bearing conidiophores. Meanwhile *Leptographium*, the asexual form, lacks perithecia and instead bears asexual spores (conidia) only. Those are produced in sticky, globose matrices on the end of macronematous (i.e., morphologically different from the vegetative hyphae) and mononematous (i.e., consisting of a single hypha) stalks (conidiophores) of varying length and composition (Jacobs and Wingfield, 2001). Since the sexual form of many species in the *Leptographium* complex have yet to be observed in field or laboratory settings, the majority of the complex is classified within the *Leptographium* genus. Even those complex member species that have a known sexual form (and are hence technically classified as *Grosmannia*), have in the past commonly been referred to as *Leptographium* spp. (Jacobs & Wingfield, 2001), though this classification system may soon be changing as advancing gene sequencing techniques further determine how we classify fungal species (Stielow et al., 2015; Taylor, 2011).

Sticky spores, long "neck" perithecia and protruding conidiophores, which are typical of the majority of Ophiostomatoid fungi, are considered evolutionary traits adapted to facilitate the relationship with insect vectors (Malloch & Blackwell, 1993). Fruiting bodies of these fungi, either sexual or asexual, grow in beetle galleries and spores adhere to adult vectors at the time of their emergence from the host trees. Beetles can carry spores of Ophiostomatoid fungi either in specific structures called mycangia, such as in the case of ambrosia fungi and their associate ambrosia beetles (Batra, 1963), or by adhering to the exoskeleton, in shallow pits or integumental folds (Six, 2003), which is the case for *Leptographium* and *Grosmannia* species (Jacobs and Wingfield 2001). Emerging infected beetles will seek out a new host tree to colonize and inoculate the host with the fungi by boring into the plant tissue.

Fungi will colonize the phloem tissue as beetle adult and larval activity create galleries and will produce new fruiting bodies in pupal chambers and galleries, where they are subsequently most likely to be encountered by adult vectors (Six & Wingfield, 2011). Host tissues colonized by the fungi show a typical blue or gray discoloration, from which the name "blue-stain" fungi is derived (Seifert, 1993). Heavily melanized mycelia growing within the host tree's phloem leads to blue to gray to occasionally black pigmentation and is a common diagnostic trait of Ophiostomatoid fungal presence in infected trees (Jacobs & Wingfield, 2001). Though such phloem discoloration is indicative of Ophiostomatoid fungal infection, it is not in and of itself, a driver of tree mortality and in some cases is considered cosmetically attractive in the commercial lumber industry for the unique quality it lends to some carpentry work (Robinson et al., 2013).

1.4 Root-feeding beetles of the *Hylastes*, *Hylobius* and *Pachylobius* genera

Several genera of native rhizophagous beetles (i.e., feeding and reproducing in the roots of trees) have historically been characterized as non-aggressive colonizers of pine species across North America. Species known to be found in association with loblolly pine in the southeast are the native species *Hylastes salebrosus* Eichoff*, H. porculus* Erichson*, H. tenuis* Eichoff*, Hylobius pales* (Herbst) and *Pachylobius picivorus* (Germar) (Coleoptera: Curculionidae, Scolytidae) (Fig. 1.1), as well as the introduced species *H. opacus* Erichson (Eckhardt et al., 2004; Eckhardt & Menard, 2009; Helbig et al., 2016; Zanzot et al., 2010). All these species have been associated with Ophiostomatoid fungi, but lacking mycangia, spores of associated microbes are vectored exogenously on pits of their integument (Francke-Grosmann, 1967).

Of the above described beetle species, none have been characterized as aggressive, primary colonizers of healthy pine trees. *Hylobius pales, P. picivorus* and *H. porculus*, and their associated *Grosmannia* fungi, for instance, have been historically documented in association with red pine (*P. resinona* Aiton) mortality in Wisconsin and throughout the Great Lakes region of the northeastern US (Klepzig et al., 1991). However, it has been suggested that these genera, with their known attraction to and preference for stressed and sickened host trees, should not be categorized as primary drivers of pine mortality (Johnson & Murray, 2008).

Hylastes species are root-feeding bark beetles, feeding on the root-collar of pine trees (Scott & King, 1974). Species belonging to the *Hylastes* genus are known as secondary colonizers of weak, stressed and dying pine trees (Coyle et al., 2015; Klepzig et al., 1991; Six, 2003), reproducing in the collar and in the primary and secondary roots of host trees (Klepzig & Six, 2004; Matusick et al., 2013a; Zanzot et al., 2010). Though there are some cases of this genus being linked to fungal pathogens capable of inducing large-scale mortality in North America [e.g., *L. wageneri*, a pathogen of *Pseudotsuga menziesii* in western North America has been linked to *H. nigrinus* (Mannerheim) and *L. terebrantis* and *L. procerum* have been implicated with their vector *H. porculus* as potential drivers of red pine mortality in Wisconsin] the genus is not considered to be an aggressive, primary driver of host mortality (Harrington et al., 1985; Klepzig et al., 1991; Klepzig et al., 1995). The genus *Hylastes* is fairly ubiquitous throughout North American, occurring in association with coniferous tree hosts from Mexico into Canada (Blackman, 1941). Observations of the flight phenology of this genus have been documented in other systems, and conclusions as to appropriate management practices drawn from such results (Reay & Walsh, 2001). Within the southeastern U.S., the phenology of these species has been observed in longleaf pine (*P. palustris* Miller) habitats near Ft. Benning Georgia, however data for this study was recorded for only one year, thus no determination of whether flight phenology is variable based upon the year was reached (Zanzot et al., 2010). Additionally, *H. porculus* and *H. salebrosus* were binned in this study and not assessed as separate species. Some members of this genus (*H. salebrosus* and *H. tenuis*) have shown attraction to sites recently control burned, that collection of the number of beetles in these species was positively related to burn severity, and that the number of insects collected was greatest immediately post-burning, with the number of insects collected steadily decreasing from this point forward in time (Sullivan et al., 2003).

The two pine regeneration weevils, *P. picivorus*, the Pitch eating weevil and *H. pales*, the Pales weevil, feed upon phloem tissues of pine trees above ground in the adult stage, but mating and larval feeding occur in the primary roots of stressed pines. *Hylobius pales* and *P. picivorus* are considered a significant economic pest of regeneration stands in the southeastern and northeastern U.S., feeding upon regenerating seedlings and saplings in natural stands, plantations and nursery settings (Nord et al., 1982). Recently thinned stands with cut stumps and stressed trees have been shown to be attractive to these genera for brood development; after adults have overwintered, they emerge in the spring, mate and oviposit in hosts on such sites (Bliss Jr & Kearby, 1970; Fox & Hill, 1973; Rieske & Raffa, 1990). In previous studies, both species of regeneration weevils have been found to occur in greater numbers in pine stands that have experienced moderate to high-severity burns, lending further credence to their role as colonizers of stressed, damaged pine trees (Fox & Hill, 1973; Hanula et al., 2002). In a 1989 study of *H. pales* and *P. picivorus* flight activity in plantations of 5 – 6-year-old Scot's pine (*Pinus sylvestris* L.) in Wisconsin, it was determined that flight activity for both species occurred from mid-May to mid-August, with flights peaking in mid-July in the northeastern U.S. (Rieske & Raffa, 1990). Studies of the effect of temperature on egg, larval and pupal stages of *H. pales* have been carried out in Arkansas, yet this study did not monitor the flight phenology of adults over the course of multiple years and seasons (Salom et al., 1987). And in a pair of 1970 studies, *H. pales* and *P. picivorus* adult emergence and oviposition in relation to temperature variation was assessed in the Georgia Piedmont (Franklin & Taylor, 1970; Taylor & Franklin, 1970), though these studies did not examine differences in beetle attraction to sites with different management histories. Since an analysis of the flight phenology and behavior of these beetles has not been assessed in the past fifty years in the Piedmont, an up to date assessment of flight activity in these species is prudent.

Weevils are generally diurnal; the European species *Hylobius abietis* (L.) has demonstrated a proclivity for elevated afternoon activity, correlated to high relative humidity (85-95%) and warm temperatures (17-21C^{o}), while during extremely warm periods weevil activity was restricted to twilight and nighttime conditions (Christiansen & Bakke, 1971; Sibul et al., 1999). Adult females of *H. abeitis* are known to oviposit their eggs in either niches excavated in the bark of host trees below ground level or (more consistently) laid directly in the soil near the roots where the soil is drier (Scott & King, 1974). First instar, newly hatched larvae then migrate from the soil to feeding sites within the bark of the host tree's primary roots, where they feed upon cambial tissues, mature and diapause overwinter (Nordlander et al., 1997; Pye & Claesson, 1981; Scott & King, 1974). Though not fully understood it is likely that management practices, climactic variation, presence or absence of predator beetles and monoculture vs mixed species stands may affect mortality of host trees, as well as partially dictating the species composition and abundance of beetles and their vectored fungi present in a system.

1.5 The Vector-Pathogen Relationship

Symbiotic relationships, of which bark and woodboring beetles and their associated fungi characterize a range of concepts from mutualistic, to commensalistic to antagonistic, can be defined as '*the acquisition and maintenance of one or more organisms by another that results in novel structures and (or) metabolism*' (K. Klepzig et al., 2001; Zook, 1998). Fungi associated with bark and woodboring beetles (Coleoptera: Curculionidae, Cerambycidae, Scolytidae) can be carried within specialized structures of the vector beetle, termed mycangia, which are believed to have evolved as a direct result of symbiosis between beetle and fungal species or may be vectored exogenously directly upon the exoskeleton or in shallow pits of the insects' integument. Those fungi carried within beetle mycangia tend to exhibit closely mutualistic relationships with their beetle partners, while fungi vectored exogenously seem to exist in more plastic relationships with their insect vectors, in which the fungal species associated with a specific beetle are often variable and are more often tree pathogens or wood-staining fungi than those that are known as mycangial fungal species (Klepzig et al., 2001; Klepzig et al., 2001). In some cases, such as that of the southern pine beetle (SPB), *Dendroctonus frontalis* Zimmerman (Coleoptera: Scolytidae), both mycangial as well as phoretic fungi are associated with the vector. SPB is considered to be one of the most damaging forest insects in North American and is exceptional among bark and woodboring beetles of the southeastern US in its ability to mass attack and kill healthy living pine trees (Paine et al., 1997). Three fungal species are known to be associated with SPB, the blue-stain fungus *O. minus* (Hedgc.) (vectored by phoretic mites of *D. frontalis*) (Hofstetter et al., 2014; Klepzig et al., 2001), as well as the mycangial fungi *Ceratocystiopsis ranaculosus* Perry and Bridges and *Entomocorticium sp*. A (somewhat unique in its status as a mycangial basidiomycete), and all seem to play separate yet necessary roles in the maturation of SPB larvae (*Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A) or adults' ability to overwhelm tree defenses (*O. minus*) (Klepzig et al., 2001).

The role of bark and wood-boring beetle associated fungi seem to be variable depending upon the context and species involved. Whether the fungi are vectored in the mycangia or phoretically upon the beetles' cuticle, fungal partners in these relationships benefit from being carried to new host trees by their beetle vectors. As previously discussed, these fungi grow in the galleries of their beetle partners and their spores are borne in sticky matrices which are most conducive to sticking to beetle cuticles. Such life history indicates the reliance of the Ophiostomatoid fungi upon vector beetles to colonize new host material. The beetles, meanwhile, may benefit in a variety of ways from their fungal partners. Fungi may be necessary for: maturation feeding of beetle larvae (Paine et al., 1997), overwhelming of host tree resin and chemical defense responses (Lieutier et al., 2009), increases in nitrogen levels in the phloem to increase vigor of newly matured adults (Kirisits, 2004; Kirisits, 2007), or as recently suggested, to aid in the production of vector semiochemicals (Cale et al., 2019).

There are two primary hypotheses concerning the relationship between *Grosmannia* fungi and the beetles that vector them. One is that the relationship is commensalistic, where while the fungi benefit from being vectored to new potential hosts (hence the above-mentioned evolutionary traits), the beetles receive no benefit from the fungi they transport. The second hypothesis, which is (as described above) considered true for most bark beetles and non-mycangial associated Ophiostomatoid fungi, is that the relationship is mutualistic despite the lack of mycangia, and both parties gain some advantage from the association (Jacobs & Michael Wingfield, 2001; Kirisits, 2004; Lieutier et al., 2009; Six, 2012). In particular, associated non-mycangial fungi may still play some role in beetle brood development (which is a known role of mycangial fungi), or benefit the beetles by stimulating (and subsequently leading to the accelerated exhaustion of) the host tree's defenses (Kirisits 2004, Lieutier, Yart et al. 2009, Six 2012). However, given that the fungal species focused upon in this study are associated with non-aggressive, secondary colonizer beetles, (Coyle et al. 2015; Klepzig and Six, 2004), their ability to stimulate their host defenses has yet to be explored.

1.6 Identification of *Leptographium* and *Grosmannia* Species

As far as is known, in all studies evaluating *Leptographium* and *Grosmannia* fungi associated with loblolly pine in southeastern U.S., fungal isolates were obtained either by isolating spores from beetle galleries (Matusick et al., 2013a) (where fungal species with a low incidence of occurrence could easily be missed), or by rolling adult beetles on a selective media plate (Matusick et al., 2013a) (in which case many fungal spores could be left behind on the beetle and their presence undetected). However, classical methods of isolation and growth of fungi on media, as described above are problematic. Such methods are time intensive, financially deleterious due to the large amounts of materials used (media, petri dishes, etc.) and potentially prone to biased results regarding the composition of the complex.

Moreover, nothing is known about the frequency of association of the different fungal species with these vectors in Georgia, or if the complex composition is affected by environmental and/or anthropogenic factors. A more comprehensive knowledge of the *Grosmannia* complex by means of molecular analysis would thus be essential. In fact, when working exclusively with classical methods of isolation and growth of fungi on media, faster-growing species may out-compete and mask the presence of other slower growing species, preventing the latter from being detected. And hence, it could happen for instance that non-native *Grosmannia* species, to which loblolly pine has no coevolved resistance and thus could potentially play a role in elevated mortality of loblolly pine in the southeastern U.S., would go undetected or less frequently detected (Duong et al., 2012). Bearing these factors in mind, a more in depth and targeted analysis of the Ophiostomatoid fungi associated with root-feeding beetle genera in the southeastern U.S. would be deemed prudent if a more comprehensive knowledge of the fungal species associated with these vectors, and their potential role, is desired.

In addition to the isolation methods, a further source of potential error is also the identification of different species within the complex. Identification of *Leptographium* and *Grosmannia* species based solely on morphological features is relatively difficult, as morphological taxonomic features in this genus can be quite variable within a single species, or largely overlapping between different species (Jacobs & Wingfield, 2001).

While molecular tools have been previously used to successfully identify *Leptographium* and *Grosmannia* spp. in some US regions (Bracewell & Six, 2014;

Jacobs & Wingfield, 2001), species identification of *Grosmannia* fungi isolated in Georgia is still predominately based on classical isolation techniques and morphological features, lending significant justification for the view that some *Grosmannia* species in the region may be currently misidentified or unknown (Duong et al., 2015; Duong et al., 2012; Duong et al., 2012). Classic PCR techniques utilizing primer sets designed around segments of fungal DNA have proven to reliably identify *Grosmannia* and *Leptographium* fungal DNA to the species level. The internal transcribed spacer (ITS) of fungi is highly conserved and ITS primers generally provided reliable and consistent amplification within fungi, lending ITS primers some notoriety within the realm of fungal genetics as a barcoding region (White et al., 1990). However, the conserved nature of the internal transcribed spacer does not allow for species specific differentiation within the Ophiostomatales, thus ITS is not favorable for species-level identification within the Ophiostomatoid fungi. Rather, primers on the β-tubulin, calmodulin, large subunit (LSU) and transcription elongation factor 1-α (TEF1α) portions of the fungal genome have proven to accurately elucidate species level identification of Ophiostomatoid fungi (Duong et al., 2015; Duong et al., 2012; Glass & Donaldson, 1995; Stielow et al., 2015).

1.7 Thesis Objectives

The purpose of this research is to facilitate a better understanding of the root-feeding beetles in the Piedmont of Georgia and their associated fungal complex, and to determine if and how environmental and anthropogenic factors might affect beetle ecology. Specifically, understanding of the life history and ecology of the root-feeding beetles and screening of the actual fungal species associated with them is essential.

Furthermore, potential significant nuances of the vector-pathogen relationship, such as the possible role of temperature and seasonal variation (Roe et al., 2011; Six & Bentz, 2007) or stand management history (Sullivan et al., 2003) on the activity and prevalence of root-feeding beetle species are as yet unexplored in this region, presenting an incomplete picture of the relationship between root-feeding beetles and the *Grosmannia* complex.

A 2003 study in South Carolina found that some species of *Hylastes* beetles are attracted to recently burned forest stands, potentially indicating a higher incidence of *Grosmannia* complex fungi in stands that are burned as a portion of their management objectives (Sullivan et al., 2003). Thus, determination of the incidence and composition of the *Grosmannia* complex in Georgia by sampling in stands with various management histories, isolating directly from the beetle vectors and preliminarily identifying fungi via the employment of molecular techniques could advance understanding of the etiology of loblolly pine mortality and ultimately influence the management strategies used to mitigate its effects.

Therefore, the goal of this research is to determine the flight phenology of the root and root-collar feeding bark beetles and weevils *H. pales*, *P. picivorus*, *H. salebrosus*, *H. porculus* and *H. tenuis* in the Georgia Piedmont and to assess whether individual vector species show preference for loblolly pine stands with differing management histories. Secondly, to conduct preliminary identification of phoretic Ophiostomatoid fungi associated with the aforementioned root and root collar feeding bark beetles and weevils in the Georgia Piedmont. Results of this research will inform future studies concerning these fungal complexes and their vectors in the Piedmont and will inform cultural management strategies when pertinent.

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Figure Legend

Figure 1.1. Rhizophagous beetles *Hylastes porculus, Hylastes salebrosus, Hylastes tenuis, Hylobius pales* and *Pachylobius picivorus* studied in this research. All species are native to the southeastern U.S. and found in association with *Grosmannia* and *Leptographium* fungi in declining loblolly pine stands. Image credit: *Hylastes porculus*: Pest and Diseases Image Library, Bugwood.org; *Hylastes salebrosus*: Jeffrey W. Lotz, Florida Department of Agriculture and Consumer Services, Bugwood.org; *Hylastes tenuis*: J.R. Baker & S.B. Bambara, North Carolina State University, Bugwood.org; *Hylobius pales*: Jennifer C. Giron Duque, University of Puerto Rico, Bugwood.org and *Pachylobius picivorus*: Natasha Wright, Cook's Pest Control, Bugwood.org.

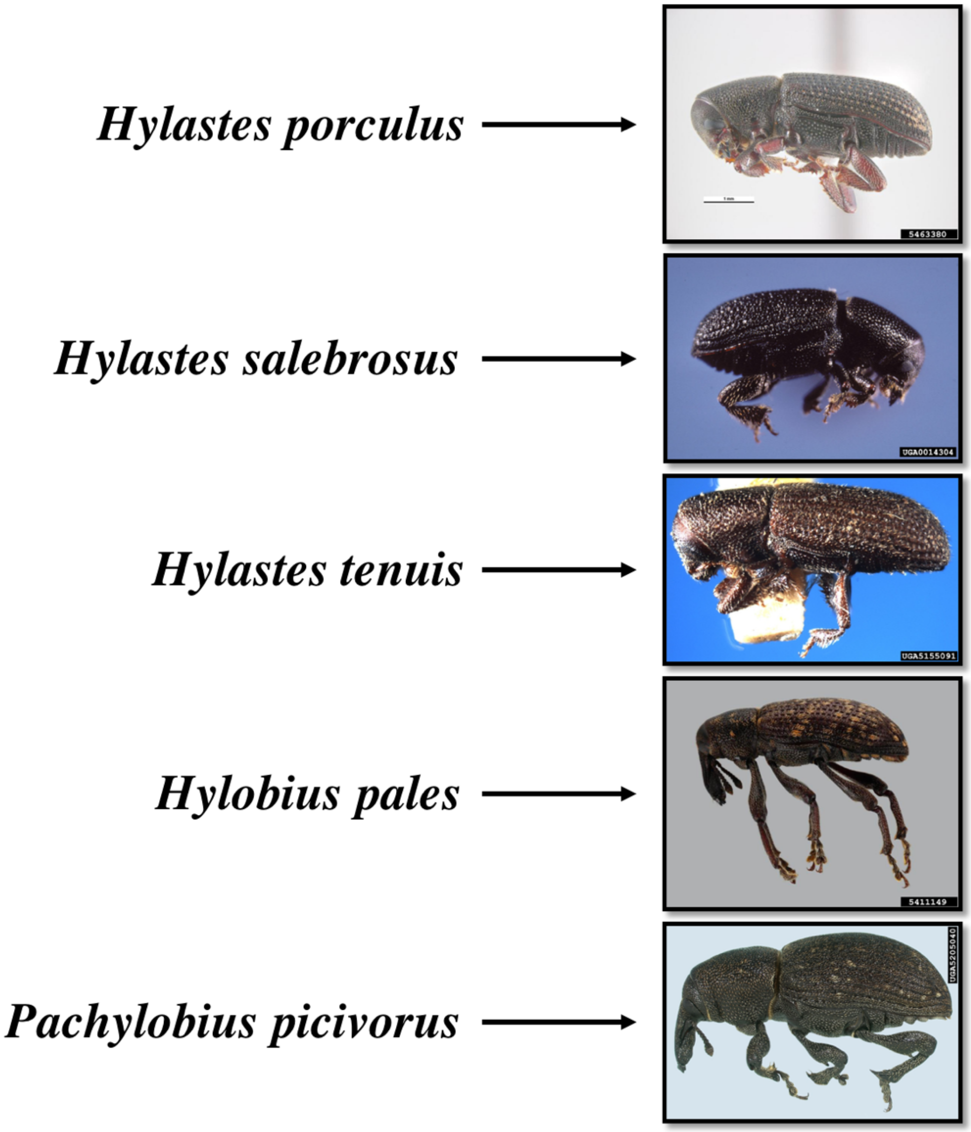
Hylastes porculus
5463380
Hylastes salebrosus
UGA0014304
Hylastes tenuis
UGA5155091
Hylobius pales
5411149
Pachylobius picivorus
UGA5205040

Figure 1.1

CHAPTER 2

DETECTION OF ROOT-FEEDING CURCULIONID BEETLES IN MANAGED AND UNMANAGED LOBLOLLY PINE STANDS OF THE GEORGIA PIEDMONT[1]

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[1] Buland, M., Barnes, B., Klepzig, K., Gandhi, J.K., and Villari, C. To be submitted to *Forest Ecology and Management*.

Abstract

Over the last several decades, periodic elevated mortality of loblolly pine has been observed in loblolly and longleaf pine forests of Georgia and Alabama. Root and root-collar feeding bark beetle species *Hylastes porculus, Hylastes salebrosus, Hylastes tenuis, Hylobius pales* and *Pachylobius picivorus* have been linked to these areas of decline. Additionally, these beetles are often found in association with *Leptographium* and *Grosmannia* blue-stain fungal species and together these beetles and their associate fungi have been suggested to be implicated with the observed elevated mortality. Yet, implication of these organisms in increased pine mortality is problematic as neither the vector beetles nor their fungal associates have been characterized as aggressive or primary drivers of pine mortality in the southeast. In order to understand the etiology of this phenomenon, a better basic understanding of potentially involved organisms is necessary, thus, the objectives of this study are to elucidate the phenology of loblolly pine associated root-feeding beetles in Georgia and assess the potential variation of the blue-stain fungal community associated with these beetles. Beetles were live-trapped in two loblolly pine stands with differing histories (managed vs. unmanaged) from May 2017 to October 2018. Across vectors, abundance varied over time and site was found to have a significant effect on the number of root-feeding beetles collected. Fungal composition was found to vary between vector species in 2017 and fungal species were primarily isolated in the spring regardless of vector species or site. Results of this study will provide useful insights for any future study of these fungal vectors and inform forest health management in the southeastern U.S.

INDEX WORDS: Bark beetles, loblolly pine, forest health, forest management, fungi

2.1 Introduction

As natural and managed forests are increasingly affected by environmentally and anthropogenically induced change the concept of forest health continues to be of significant relevance for both production forestry as well as ecological processes. Though there is no one definition of what constitutes forest health, several overarching themes are present (Abrams et al., 2005; Edmonds et al., 2011). Generally, a healthy forest could be considered one where current and future management objectives of forests are not undermined by pests, pathogens or abiotic conditions, that is resilient to change, that can recover from stress on an appropriate timescale for the system and where a fully functioning ecosystem can maintain complexity while providing for human needs (Edmonds et al., 2011; Rapport et al., 2009; Samson & Knopf, 2013).

From an economic and management-based viewpoint, a healthy forest would be one where management objectives are not threatened by biotic or abiotic conditions (Area., 1994). Yet, as Wagner (1994) pointed out, the above definition can be troublesome, because although healthy forest depends on meeting management objectives, forest health is in and of itself often considered a management objective (Wagner, 1994) and as Kolb et al. (1994) suggest in response to this definition, such an approach to defining forest health "suffers from circular logic, where a desired state of forest health depends on the occurrence of a healthy forest" (Kolb et al., 1994). Under the above 1994 definition, bark beetles (Coleoptera: Curculionidae) would most definitely be considered threats to a healthy forest, as they have demonstrated the capacity in some systems to have devastating effects on forest management objectives (Bakke, 1983; Gibson et al., 2009; Klepzig et al., 1991).

Though some species of bark and ambrosia beetles such as southern pine beetle (*Dendroctonus frontalis*) Zimmerman (Lorio Jr, 1986; Thatcher, 1960), the European elm bark beetle (*Scolytus multistriatus*) Marsham (Brasier & Gibbs, 1973; Gibbs, 1978) and the European Spruce bark beetle (*Ips typographus* L.) (Bakke, 1983; Wermelinger, 2004) have a well-documented record of threatening management objectives, and often times forest health, for many other members of Curculionidae their status as drivers of host mortality is less clear cut. One such example are the rhizophagous (root-feeding and breeding) beetles and weevils of the genera *Hylastes, Hylobius* and *Pachylobius* in the southeastern United States. *Hylastes salebrosus* Eichoff, *H. porculus* Erichson, *H. tenuis* Eichoff, *Hylobius pales* (Herbst) and *P. picivorus* (Germar) are root and root-collar feeding bark beetles and weevils of the family Curculionidae, native to North America, which, together with their associated Ophiostomatoid fungi, have been associated with loblolly pine (*Pinus taeda* L.) mortality in the southeastern U.S. (Eckhardt et al., 2007; Matusick et al., 2013b; Zanzot et al., 2010). According to the current literature, genera of root and root-collar feeding bark beetles and weevils native to the southeastern U.S. have been associated with *Grosmannia* complex fungal species: *Leptographium procerum* (Kendrick) M.J. Wingfield*, L. terebrantis* S.J. Barras and T.J. Perry*, G. huntii* (Robinson-Jeffrey and Grinchenko) Zipfel, de Beer and Wingfield and *G. alacris* T.A. Duong, Z.W. de Beer & M.J. Wingfield (Duong et al., 2012; Eckhardt et al., 2004; Eckhardt et al., 2004). The above listed fungal species are all considered native to North America, with the exception of *G. alacris* which, though being widely distributed globally, has undetermined origins (Duong et al., 2015).

Localized mortality of loblolly pine, characterized by lateral root deterioration, feeder root death, reduced foliar radial growth, shortened chlorotic needles and spare crowns, has been a periodic yet repeated occurrence throughout the southeastern U.S. over the past seventy years (Brown & McDowell, 1968; Hess et al., 1999). First reported in overly mature (50+ years of age) loblolly pine stands of the Talladega National Forest in the 1950s, such mortality has since occurred in other areas of the southeast repeatedly, termed as Southern Pine Decline (SPD) (Brown & McDowell, 1968; Eckhardt et al., 2004; Eckhardt & Menard, 2009; Eckhardt et al., 2007; Hess et al., 2002). Yet this title may be problematic, as observed mortality of loblolly pine does not meet the criteria of a true decline syndrome (Ciesla & Donaubauer, 1994) and root-feeding beetles were found to be secondary and not primary colonizers of pine trees (Coyle et al., 2015).

All of the above described beetle species have been associated with Ophiostomatoid fungi which are vectored exogenously on pits of the beetle's integument, since the beetles lack true mycangia (Francke-Grosmann, 1967). Ophiostomatoid fungi are filamentous Ascomycota thought to have a long and rich co-evolved history with the beetles of family Curculionidae (Farrell et al., 2001; Spatafora & Blackwell, 1994). None of these fungal species have been characterized as aggressive, primary colonizers of healthy pine trees, though they or other members of their genera have in some cases been associated with conifer disease and mortality in other regions of the U.S. (Harrington et al., 1985; Klepzig et al., 1991; Klepzig et al., 1995). Rather, it has been suggested that these genera, with their known preference for sick and stressed host trees, should not be categorized as primary drivers of pine mortality (Johnson & Murray, 2008). Nevertheless, it has been proposed that these fungal associates, more than the beetles themselves, might be implicated as drivers of mortality in loblolly pine (Matusick et al., 2013b).

Hylastes species are root-feeding bark beetles known as secondary colonizers of weak and stressed trees, feeding on and breeding in the root-collar of conifers, especially pines (Coyle et al., 2015; Klepzig & Six, 2004; Scott & King, 1974). The genus *Hylastes* is relatively ubiquitous throughout North America; from Mexico north through Canada (Blackman, 1941). In the southeastern U.S. the phenology of these species has been assessed in longleaf pine (*Pinus palustris* Miller) habitats of south Georgia, yet the study was limited to a single year and any differences in phenology of *H. porculus* and *H. salebrosus* were inconclusive based upon the methods of the study (Zanzot et al., 2010). Some members of the *Hylastes* genus, (*H. salebrosus* and *H. tenuis*) have shown preference to sites recently subjected to controlled burns, further indicating preferentiality for sites with stressed trees in this genus (Sullivan et al., 2003).

The Pales weevil (*H. pales*) and the pitch eating weevil (*P. picivorus*) feed on phloem tissue of pine species above ground as adults, with mating and larval feeding occurring in the primary roots of stressed trees. These two species, which are often considered together due to their similar host preferences and life history strategies, are considered a significant economic pest of pine regeneration stands throughout the eastern U.S. (Nord et al., 1982). Often, these species are found feeding upon regenerating seedlings in plantations, natural stands and nursery settings (Nord et al., 1982; Nordlander et al., 1997). Brood development has been shown to preferentially occur in recently thinned stands with cut stumps and stressed trees; adults overwinter in cut stumps within these sites, before emerging to mate and oviposit in the spring (Bliss Jr &

Kearby, 1970; Fox & Hill, 1973; Rieske & Raffa, 1990). Additionally, both species have been found to occur in greater numbers in pine stands that have experienced moderate to high-severity burns, lending further validity to their role as colonizers of stressed, damaged pine trees (Fox & Hill, 1973; Hanula et al., 2002). Though *H. pales* and *P. picivorus* adult emergence and oviposition in relation to temperature has been previously assessed in the Piedmont, the effect of management history on flight phenology was not investigated; additionally, as this analysis took place over fifty years prior to the current study, an up to date assessment of flight activity in these species is prudent (Franklin & Taylor, 1970; Taylor & Franklin, 1970).

To gain a better understanding of microbial communities associated with root and root-collar feeding beetles in the Piedmont region, a more comprehensive assessment of the flight phenology of these species and their preference for sites of different management history is needed. In fact, fungal communities vectored exogenously upon their hosts' cuticle stand a far greater likelihood of being influenced by external factors, such as seasonal and geographic influences (Roe et al., 2011; D.L. Six & Bentz, 2007). Knowledge of when and in what type of stand vectors are at peak activity at the species level, and the potential variability of their associated fungi, will be beneficial for conducting future studies of these genera and their fungal symbionts. Thus, objectives of this studies are: firstly, to determine the flight phenology of the root and root-collar feeding bark beetles and weevils *H. pales*, *P. picivorus*, *H. salebrosus*, *H. porculus* and *H. tenuis* in the Georgia Piedmont and to assess whether individual vector species show preference for loblolly pine stands with specific management histories (managed vs. unmanaged). Secondly, to elucidate the phoretic Ophiostomatoid fungal community associated with the aforementioned root and root collar feeding bark beetles and weevils in the Georgia Piedmont.

2.2 Methods

2.2.1 Study Sites

Two study sites were established in the Spring of 2017 at the University of Georgia’s Whitehall (33°52’33.0”N 83°21’21.4”W) and Hardman Forests (34°04’39.2”N 83°26’26.4”W), in Clarke County, Georgia. The study sites were located in the Piedmont region of Georgia. The Piedmont region is located above the Fall Line, which separates the Piedmont in the northern portion of Georgia from the Atlantic coastal plain region of Georgia and Alabama located below the Fall Line. The climax forests of the Piedmont were dominated by hardwood species prior to colonization by European settlers and subsequently heavily transitioned to agricultural production – primarily the cotton industry (Nelson, 1957). The Fall Line, which runs longitudinally though Georgia, serves as a southern range boundary for some tree species frequently found in the southern Appalachian Mountains, and as a northern range boundary for multiple tree species common to the Coastal Plain, and delineates one of the physiographic provinces of the southern Appalachian Highland Realm (Shankman & Hart, 2007). The underlying geology of the Piedmont is that of the ‘Carolina complex’; composed primarily of granites, quartzites, granodiorites, schists and amphibolites, along with granite outcrops, which forms the basis for the lithologic composition of the region (Woodruff & Parizek, 1956). The soils of the Piedmont region are primarily Ultisols and clay based, overlaying a granite/gneiss bedrock and possessing a distinct red coloration as result of the iron found in bedrock materials of the region (Hodler & Schretter, 1986). Isotopic data indicates a Pleistocene age residence time for the formation of most Piedmont soils (Markewich et al., 1990). Climate of the region is temperate to sub-tropical (Hodler & Schretter, 1986). Dominant vegetation of the region includes hardwoods such as white oak (*Quercus alba* L.), southern red oak (*Quercus falcata* Michx.), sweetgum (*Liquidambar stryaciflua* L.), hickory species (*Carya* spp.), northern red oak (*Q. rubra* L.) and pine species like shortleaf pine (*P. echinate* Mill.) loblolly pine (*P. taeda* L.) and in some cases Virginia pine (*P. virginiana* Mill.) (Hoover & Parker, 1991).

Each site had six plots, 40 m in diameter each, with > 250 m between plots. Plots were selected to meet the following criteria: 1) loblolly pine stands that have been either not burned in the past five years (Hardman Forest) or burned in the past 1-2 years (Whitehall Forest); and 2) loblolly pine stands that are 15-20 years old (at both Whitehall and Hardman forests). These relatively unmanaged and managed forests represent the two land-area treatments. In total, twelve plots were established for this study.

Canopy cover at the unmanaged site (Hardman forest) was ~87% and stand Basal Area (BA) was determined to be ~155 ft^2/acre using a 10 Basal Area Factor (BAF) prism, measured within a 22.6-meter diameter sample plot. For all stems > 6.35 cm DBH, stems at Hardman were ~96% pine and 4% hardwood. Mean DBH of trees at Hardman was 22 cm ± 0.75 cm and ranged as 7.1 – 26.9 cm DBH. At the managed sites (Whitehall forest), canopy cover was ~82% and stand BA was ~130 ft^2/acre using a 10 BAF prism, measured within a 22.6-meter diameter sample plot. For all stems > 6.35 cm DBH, stems at Whitehall were 96% pine and 4% hardwood; 26 hardwood stems > 6.35 cm were found in the understory of the plot. Mean DBH of trees at Whitehall was 33.6 cm ± 1.18 cm, the DBH range was 26.9 – 41.4 cm.

Site level temperature data was regularly recorded every six hours for the duration of each trapping period utilizing Hobo temperature data loggers (Onset Computer Corporation, Bourne, Massachusetts, USA). Data loggers were placed at two randomly selected, but not adjacent to one another plots within each site, corresponding to two Hobo units/site, and four units total for this study. Area rainfall and temperature (°C) over the course of all study periods was analyzed using data from the National Oceanographic and Atmospheric Association (NOAA).

2.2.2 Beetle Collections

To attract root-feeding beetles, four loblolly pine trees at the managed (mean DBH of girdled trees for all collection periods 33.3 ± 0.46 (SE) cm) and unmanaged sites (mean DBH of girdled trees for all collection periods 25.5 ± 0.41 cm) per plot were selected and double girdled within each 40 m plot (Table 2.1). The upper girdle on each tree was placed at ~152 cm above the root-collar of each pine tree, and the lower girdle placed ~46 cm below the upper girdle. The exposed cambial layer of the lower girdle was treated with 52% glyphosate (Monsanto Company, St. Louis, Missouri, USA) to accelerate the death of the tree (Burke et al., 2012). Canopy conditions of girdled trees at each plot were determined every two weeks and a 1-5 rating system was used to quantify levels of tree dieback. The rating system included the following metrics: 1 = 100-75% live canopy; 2 = 75-50% live canopy, 3 = 50-25% live canopy, 4 = 0-25% live canopy; and 5 = 0% live canopy, or a dead tree.

Four pitfall traps were placed adjacent (<1 m away) to the girdled trees within each cluster of pine trees. Pitfall traps were constructed out of 10 cm diameter PVC pipe cut to 20 cm lengths. Eight holes were drilled around the circumference of the pipe, approximately halfway down the length of the PVC. Both ends of the pitfall trap were covered with PVC caps and the interior of the traps coated with Insect-a-Slip Insect Barrier – Fluon® (BioQuip, Rancho, Dominguez, California, USA) to prevent the escape of collected beetles. Design of pitfall traps was as described in Hunt & Raffa (1989) and Klepzig et al (1991). Pitfall traps were baited with two 15 ml slow release poly vials (WestGreen Global Technologies, Langley, British Columbia, CA) filled with 200 proof Ethyl alcohol (Decon Labs, Inc., King of Prussia, PA, USA) diluted with deionized water to a 95% solution, and pure gum spirits of turpentine (Sunnyside Corporation, Wheeling, IL, USA) as attractants. Ethanol and turpentine have been shown to serve as effective attractants to species of *Hylobius, Pachylobius* and *Hylastes* in previous studies; it is pertinent to note that both compounds have been shown to be much more effective in attracting bark beetle and weevils in synergy with one another, than either chemical on its own (Phillips, 1990; Raffa & Hunt, 2017). Pitfall traps were positioned with the eight entrance holes just above ground level, following the method described in Eckhardt et al. (2004) and Hess et al. (2002).

In addition to the four pitfall traps, two 12-unit Lindgren funnel traps were also placed at each plot (Lindgren, 1983). As with pitfall traps, funnel traps were baited with 15 ml slow release poly vials containing pure gum spirits of turpentine and 200 proof ethyl alcohol diluted with deionized water to a 95% solution. One of the two funnel traps per plot was also baited with a packet of *exo*-brevicomin at a release rate of 4mg/day (Synergy Semiochemical, Burnaby, BC, Canada), an aggregation pheromone of beetles belonging to the genus *Dendroctonus*, but which has been shown to be attractive for *Hylastes* beetles as well (Phillips, 1990; Pureswaran et al., 2014). *Exo*-brevicomin packets were replaced every three weeks; turpentine and ethanol vials were filled as needed.

Plots were established in early May of 2017, and trapping took place over four time periods across 2017 and 2018. Each trapping event took place in the same areas, with a fresh set of four loblolly pine trees girdled > 20 m away from any existing plot at the beginning of subsequent trapping periods, and with pitfall and funnel traps moved adjacent to the newly girdled trees. Trapping periods took place at the following time intervals: Experiment I: May 5th, 2017 – August 3, 2017; Experiment II: August 23rd, 2017 – November 21st, 2017; Experiment III: February 23rd, 2018 – June 26th, 2018 and Experiment IV: July 13th, 2018 – October 29th, 2018. The length of the trapping period was determined when the trees were dead for a few weeks and catches were reduced to almost zero. This trapping schedule served to document potential changes in the vector-pathogen relationship over the course of multiple seasons during a two-year period.

During each trapping period, traps were emptied every three days, to minimize cross-contamination of the beetle-associated fungi within the traps. Collected beetles were labeled by trap, plot and date of collection. Samples were subsequently identified to the species-level for target species of the *Hylastes*, *Hylobius* and *Pachylobius* genera based on morphological features (Blatchley & Leng, 1916); samples were stored individually in 0.5 mL microcentrifuge tubes at -20 °C until further processing.

2.2.3 Preliminary screening of *Grosmannia* complex species associated with *Hylastes, Hylobius* and *Pachylobius* root-feeding beetles

For every month of field collection in 2017, the microbial community associated with target root-feeding beetles was screened for potential Ophiostomatoid isolates. To assess the Ophiostomatoid community associated with these vectors one collected specimen of each target species/plot/site (for a total of 30 beetles/site) was vortexed at 40 Hz for 60 seconds in 200 µl of 1% Tween 80 solution (Sigma-Aldrich, Burlington, MA, USA) to dislodge fungal spores carried by the beetles (Battisti, 1999; Villari, 2012) and the resulting solution spread on selective CSMA media [2% malt extract agar (10g Malt Extract (VWR Life Science, Radnor, PA, USA),) and 17 g Agar (VWR Life Science) per liter of solution) (MEA) amended with 100 mg/ml in DMSO cycloheximide (Sigma-Aldrich) and streptomycin sulfate salt (Sigma-Aldrich)](Zhou et al., 2007). Amending the plates with streptomycin serves to reduce growth of unwanted bacteria, while adding cycloheximide selects for Ophiostomatoid fungi, which are tolerant to this antifungal antibiotic (Harrington, 1981). Plates were stored at room temperature and *Grosmannia*-like fungal colonies were transferred to a new 1.5% MEA plate and identified to the species level using a combination of morphological observations (Jacobs et al., 2001) and molecular approaches (Duong et al., 2012).

To extract DNA, pure colonies were grown on 1.5% MEA plates overlaid with cellophane, and mycelia was scraped after 5-10 days, dependent upon the growth rate of the culture. Genomic DNA was extracted with a salting-out protocol (Patwary et al., 1994). In brief, samples were crushed in a solution of extraction buffer [1 M EDTA (Promega, Madison, WI, USA), 0.05 M, Tris pH 8 (Promega)], and incubated overnight at 55 °C with 10% SDS (Promega) and 20g/mL proteinase K (Promega). Two µl of Rnase A (10 mg/ml) (Invitrogen, Carlsbad, CA, USA) were then added and samples incubated for five minutes at 37 °C. Forty µl of a saturated solution of NaCl (>6M) was added to each sample, which was then vortexed for 20 minutes and centrifuged at 4°C, 14000 rpm for 30 minutes. Supernatant was transferred to a new tube and DNA was first precipitated in 200 µl of chilled isopropanol (VWR Life Science), and then washed with 500 µl of 70% ethyl alcohol (VWR Life Science). The pelleted DNA was finally re-dissolved in 20 µl of sterile water and stored at -20 °C until further use.

PCR was conducted in a reaction mixture containing, in a total volume of 25 µl: 1X colorless GoTaq Flexi buffer (Promega), 2mM dNTPs (Promega), 2 mM MgCl2 (Promega), 1 U of Go Taq Polymerase (Promega), 2.5 µM of each forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa, USA) and 1 µl of template DNA. Preliminary tests showed that template DNA amounts over 1 µl were inhibitory to successful PCR, potentially due to the abundant dark pigments typical of the fungi, and that are not removed by the salting-out DNA extraction protocol. Primers Bt2a (forward) (5-GGTAACCAAATCGGTGCTGCTTTC-3) and Bt2b (reverse) (5-ACCCTCAGTGTAGTGACCCTTGGC-3) (Integrated DNA Technologies) were used to amplify a portion of the β-tubulin gene region (Glass & Donaldson, 1995). PCR was run with the following thermocycler conditions: (94^{o}C – 5 minutes, 94^{o}c – 1 minute, 58^{o}c – 1 minute, 72^{o}c – 1 minute) 35x, 72^{o}c – 10 minutes.

Amplification was confirmed by running at 70 volts for 30-50 minutes 5 µl of PCR product and 1 µl of 1 kb DNA ladder (Promega) into a 1.5% agarose (VWR Life Science) gel with 1 µl SYBR Safe DNA gel stain (Invitrogen). Products were then submitted for post-PCR cleaning and sequencing to the Georgia Genomics and Bioinformatics Core (GGBC), University of Georgia, Athens, Georgia. Tentative identity was assigned by matching the sequence results to the ones of known species through the application of Basic Local Alignment Search Tools (BLAST) of the National Center for Biotechnology Information (NCBI) database.

In the case of *L. profanum*, identity of the isolates was further confirmed via sequencing of the Calmodulin partial gene using the primers

CL3F (forward) (5′-CCGARTWCAAGGAGGCSTTC-3′) and CL3R (reverse) (5′-TTCTGCATCATRAGYTGSAC-3′) (de Beer et al., 2016), which was amplified using similar conditions as described previously for the β-tubulin gene region.

2.2.4 Statistical Analysis

2.2.4.1 Determining Number of Beetles per Species Collected by Site

To determine the number of beetles per species found at the unmanaged and managed sites, we utilized Poisson regressions. All statistical analyses were performed in R studio statistical software version 3.3.3 for MAC OS X (R Core Team 2017). Due to the large number of zeros present in the data set, resulting from observation periods being set at three-day intervals and thus yielding an overabundance of recorded zeros, our data was non-normal (non-parametric). This was confirmed with Q-Q (quantile-quantile) plot analysis in Rstudio (function: qqPlot, package: car). In this case linear regressions would have been inappropriate; therefore, Poisson regressions were employed to correct for the non-normality of the data set. Sample size was considered to be the number of beetles collected/collection date/site. Poisson regressions were run in Rstudio using the modified loglink function to determine whether site history (managed and unmanaged) could potentially have any effect on the number of beetles of a particular species collected there. Dummy variables were created for the dependent variable of collection site.

However, preliminary analyses showed that variance indicated under-dispersion for all species, with the exception of *Hylobius pales*, where the data was over-dispersed. Hence, to correct for under or over dispersion of our data we used a modified generalized linear model (GLM) Poisson model, the quasi-Poisson model, also run in Rstudio (R Core Team 2017). The quasi-Poisson model is more robust than the Poisson model in itself and attempts to correct for over or under dispersion in the data (Zeileis et al., 2008). To adjust for the heteroskedasticity of the data, we used the Heteroskedasticity-Consistent Covariance Matrix Estimation test (function: vcovHC; package: Sandwich) in R to calculate robust standard errors for the quasi-Poisson models; $\alpha = 0.05$. The response variable of our models was beetle collected at each site.

2.2.4.2 Polynomial Regression Graphing of Beetle Flights and Sum of Individuals per Vector Species Collected per Experiment

To visualize the flight activity of each beetle species, we used a local polynomial regression (LOESS function) (R Core Team 2017) to visualize how collection rates of beetles equated to changes and peaks in flight over time. Since our data was nonparametric over time, the LOESS function allows for us to perform least squared regressions in localized subsets, thereby smoothing our numeric vector (in this case number of beetles/species collected on a particular day) over time for ease of interpretation (Cleveland et al., 1992). A smooth parameter of $\alpha = 0.25$ (a range of 0-1 is possible for this parameter) was employed to smooth the fitted curve for each species. For each year of collection data, all flight curves were analyzed separately, and then fitted to a single plot for a given site and time (i.e. Hardman, 2017); it should be noted that in 2018 there was a period of time between Experiments III and IV during which no beetles were collected, and this must be considered when reading the graphs for 2018 collections. For each species, in each year of analysis, the percentage of total insects collected/species under peak flights was assessed (function: trapz; package: pracma) (R Core Team 2017) (Borchers, 2016). The sum of individual beetles for each vector species collected over the course of each experiment was also assessed, without separating beetles based upon study site. Means and standard errors were calculated for the sum of each experiment for each species and the total number of individuals for each vector species was calculated.

2.2.4.3. Beetle species attraction to trap type and *exo*-brevicomin

To better understand the effectiveness (or lack thereof) of methods utilized for sample collection in our experimental design, Student's t-tests were run to examine the difference in collection counts of beetles of all five species between pitfall traps and funnel traps. Experiments I and II (2017) were assessed together as were Experiments III and IV (2018); managed and unmanaged sites were not assessed separately in this analysis. Data was standardized to account for the difference in number of pitfall (4) and funnel traps (2) located at each site. Prior studies have indicated that some *Hylastes* species may be attracted to the *Dendroctonus brevicomis* pheromone *exo*-brevicomin, yet no such data is available in the southeast for any *Hylastes* species with the exception of *H. salebrosus*, which has previously exhibited an attraction to this pheromone. In addition, response of the three monitored *Hylastes* species*; H. salebrosus, H. porculus* and *H. tenuis* to *exo*-brevicomin lures was assessed.

2.3. Results

2.3.1 Mortality of Glyphosate Treated Trees and DBH of Treated Trees by Site

It was observed that the DBH of girdled and glyphosate treated trees was consistently higher across all plots at the managed site (Whitehall) in contrast to the unmanaged site (Hardman) where the DBH of girdled and treated trees was smaller across all plots and time periods (Table 2.1). Observations of crown mortality of treated loblolly pines during Experiments II, III and IV, along with means and calculated standard errors of observed mortality, indicated that in experiments III and IV observed crown mortality occurred more rapidly at the managed site than at the unmanaged site (Fig. 2.1). During Experiment II such correlation was not observed, and differences in rate of crown mortality between the managed and unmanaged site were not as easily observable (Fig. 2.1).

2.3.2 Determining Number of Beetles per Species Collected by Site

Results of quasi-Poisson regressions provide an assessment of the effect of Hardman vs Whitehall (unmanaged vs managed) on beetle counts over time. For all results, the smaller coefficient denotes the site in which fewer beetles of each species were collected, and the higher coefficient indicates the site in which more beetles of each species were collected. For *H. salebrosus*, it was determined that a beetle of this species was less likely to be found at the unmanaged site of Hardman (coef. = -1.488), and more likely to be found at the managed site of Whitehall (coef. = -0.111). However, the results for this species at the managed site (Whitehall) were not statistically significant (*p* = 0.51*)*, therefore we must be cautious when interpreting this result. For all other monitored beetle species, the abundance of beetles collected was significantly higher at the managed site (Whitehall) than at the unmanaged site (Hardman) (Fig. 2.2). In relation to *H. tenuis,* (coef. = -3.44) a beetle of this species was less likely to be found at the unmanaged site and more likely to be found at the managed site (Whitehall) (coef. = 0.58). Though results for *H. tenuis* were statistically significant (*p* = 0.042*),* low total counts of *H. tenuis* (75 beetles total over the course of all four experiments) calls for some conservative interpretation of these results.

H. porculus had a decreased likelihood (coef. = -2.09) of being detected at Hardman and an increased likelihood of being detected at Whitehall (coef. = 0.47). Results for *H. porculus* were statistically significant (*p* = 0.024*).* For the weevil species *P. picivorus* (coef. = -2.58) was less abundant at Hardman and more abundant (coef. = 1.02) at Whitehall. Results for this comparison were considered to be statistically significant (*p* <0.001*).* Lastly, *H. pales* was less likely to be detected at the unmanaged site (coef. = -0.63) and more likely to be detected at the managed site (coef. = 0.59). Differences in attraction of *H. pales* to sites with different management histories was statistically significant (*p* <0.001). Total number of beetles per vector species found at each site was calculated and compared (Fig. 2.2). With regards to *H. salebrosus* 171 beetles were collected at Whitehall vs 191 collected at Hardman, for *H. porculus* 168 beetles were collected at Whitehall, vs 105 beetles collected at Hardman, *H. tenuis* had 48 beetles collected at Whitehall vs 27 beetles collected at Hardman, with regards to *H. pales* 812 beetles were collected at Whitehall vs 449 beetles collected at Hardman and for *P. picivorus* 177 beetles were collected at Whitehall vs 64 collected at Hardman.

2.3.3 Polynomial Regression Graphing of Beetle Flights

In 2017 (Fig. 2.3), *H. pales* showed two peaks in number of beetles collected; once from late June – mid-July, though it should be noted that collection rates were very high at the beginning of the collection season as well, (35% of total collections occurred from May 5th – June 16th at Hardman and 51% from June 1st – mid-July) and again in the fall during the month of September at both Whitehall (57% of total collections took place between August 3rd – October 16th) and Hardman (40% of total collections took place between August 3rd and October 16th). In 2018 (Fig. 2.4), *H. pales* collection counts remained high over extended periods of time (though again showing a bivoltine flight phenology) peaking mid-April – mid-May (28% of total collections took place from March 22nd – April 21st at Hardman and 51% from March 22nd – May 6th at Whitehall) and again (as the year prior) to a lesser extent from late June to mid-July at both sites. *H. pales* was the only monitored species to exhibit a bivoltine phenology.

P. picivorus peaked from late-June to late-July in 2017 (Fig. 2.3) at both sites (65% of total collections took place between July 1st – July 31st at Hardman, 40% of total collection took place in this same time at Whitehall). And in 2018 (Fig. 2.4) from mid to late May at both Hardman (30% of total collections took place from April 21st – May 21st) and Whitehall (31% of total collections took place from April 21st – May 21st); though *P. picivorus* did exhibit a second, smaller peak, at Whitehall in the fall of 2018 (with 30% of total collections at Whitehall in 2018 occurring between June 20th and August 3rd). In 2017 (Fig. 2.3), *H. salebrosus* peaked in late June at Hardman (69% of total collections took place from June 1st – July 1st) and mid-July (38% of total collections took place in collection events from June 16th – July 31st) at Whitehall – though it should be noted that at Whitehall two smaller peaks were observed for *H. salebrosus* in both spring and fall of 2017. In 2018 (Fig. 2.4), *H. salebrosus* peaked from late April – early May at both Hardman (49% of total collections occurred between

March 22nd – May 6th) and Whitehall (36% of total collections took place from March 22nd – May 6th).

H. porculus exhibited nearly identical flight phenology to *H. salebrosus* at both sites in 2017 (Fig. 2.3), peaking in late June at Hardman (67% of total collections took place from May 5th – July 1st) and mid-July at Whitehall (65% of total collections took place from June 16th – July 31st). In 2018 (Fig. 2.4) *H. porculus* peaked in late July – early August at both sites (20% of total collections took place from May 21st – July 19th) at Hardman, and 54% in collection periods from July 19th – August 18th at Whitehall) though, much like *H. salebrosus*, *H. porculus* counts were already high at the beginning of 2018 monitoring at Hardman (though not at Whitehall). Finally, *H. tenuis* collection counts were exceedingly low in both years (2017 and 2018), though there were two slight peaks in 2017 (Fig. 2.3) collection counts at Hardman from early – mid-July (51% of total collections took place May 5th – June 16th and 42% of total collections occurred from June 25th – July 31st with a complete drop off in collections in between), and at Whitehall did not peak. In 2018 (Fig. 2.4), *H. tenuis* counts were close to zero, and no flight phenology peaks were observable. Total numbers of individuals for each vector species collected per experiment and over the course of all experiments determined that across species, the greatest number of individuals were collected in the spring, regardless of year (2017 or 2018) (Table 2.2). *H. pales* was the most commonly collected vector and *H. tenuis* was least commonly collected (Table 2.2).

2.3.4 Beetle species attraction to trap type and *exo*-brevicomin

Differences in effectiveness of trap type to collecting each species of beetle monitored was assessed with t-tests (Figs. 2.5 and 2.6). In 2017, bark beetle species *H.*

salebrosus ($p = 0.004$), *H. porculus* ($p = 0.009$) and *H. tenuis* ($p = 0.003$) were trapped in higher numbers in funnel traps than pitfall traps. Weevil species *H. pales* ($p = 0.002$) and *P. picivorus* ($p = 0.011$) meanwhile, were significantly more attracted to pitfall traps than funnel traps. In 2018, bark beetle species *H. salebrosus* ($p < 0.001$), *H. porculus* ($p < 0.001$) and *H. tenuis* ($p = 0.005$) were again collected in higher numbers in funnel traps than pitfall traps. Weevil species *H. pales* ($p < 0.001$) and *P. picivorus* ($p < 0.001$) meanwhile, were also, as the year before, significantly more attracted to pitfall traps than funnel traps.

Differences in attractiveness of *exo*-brevicomin to *Hylastes* species was assessed with Two Sample t-tests (Fig. 2.7). None were significant at a p-value <0.05 [*H. tenuis* ($p = 0.93$), *H. porculus* ($p = 0.2$,) and *H. salebrosus* ($p = 0.19$)]. However, F-test for equality of variance yielded a significant p-value ($p = 0.01$) for *H. salebrosus*, while *H. porculus* and *H. tenuis* were still determined to not be statistically significant. Effect of *exo*-brevicomin on *H. pales* and *P. picivorus* was not assessed, as attraction of these species to funnel traps was not significant.

2.3.5 Preliminary Screening of *Grosmannia* fungal species found in association with root-feeding beetles

As the number of individual insects collected for each target species was highly variable over time, and beetles collected during periods of high rainfall were undesirable for microbial community sampling (due to high levels of contamination on plates and little to no isolation of Ophiostomatoid cultures from these vectors) the number of pure cultures obtained that were subsequently determined to be Ophiostomatoid fungi is far less than the number of beetles sampled. Ophiostomatoid fungal species isolated from root-feeding beetles included *Grosmannia huntii, G. alacris* and *Leptographium profanum*. *Leptographium profanum* was found in association with *H. pales, P. picivorus* and *H. tenuis*. Eight isolates of *L. profanum* were found from vectors collected at the unmanaged site (Hardman) and four isolates were taken from beetles collected at the managed site (Whitehall) (Table 2.3). Of these, four isolates were found from insects collected during Experiment I and eight isolates were collected from target vectors collected during Experiment II (Table 2.3). *G. huntii* was isolated from *H. pales* (two isolates)*, P. picivorus* (one isolate) and *H. salebrosus* (two isolates), three isolates from the managed site and two from the unmanaged site and all isolates from Experiment I (spring 2017). A single isolate of *G. alacris* was identified in association with *H. tenuis* in the unmanaged site during Experiment I (Table 2.3). Though other Ophiostomatoid fungal species were isolated from *H. porculus*, no *Grosmannia* isolates were identified associated with this species.

Other Ophiostomatoid fungi isolated from vectors include *Ophiostoma ips* (Rumbold) Nannfeldt, which was isolated from all vectors except *H. tenuis* and was isolated eleven times during Experiment I and only once from a vector collected during Experiment II. Eight of the of the *O. ips* isolates were taken from vectors collected at the unmanaged site and four from vectors collected at the managed site. Based only on the closest match of the β-tubulin partial gene, four isolates from *H. salebrosus, H. tenuis* and *H. pales,* all from the unmanaged site during Experiment I, were tentatively identified as *Ophiostoma pseudotsugae*, (Romb.) von Arx. Two isolates detected at the managed site during Experiment I were tentatively identified as *Ophiostoma pallidulum,* Linnak., Z.W. de Beer & M.J. Wingf. and one as *Ophiostoma nigrocarpum*, (Davidson) De Hoog (Table 2.3).

2.4. Discussion

In this study, we explored the phenology of the root and root-collar feeding bark beetles and weevils *H. pales*, *P. picivorus*, *H. salebrosus*, *H. porculus* and *H. tenuis* in the Georgia Piedmont and to assesses whether individual species show preference for loblolly pine stands with specific management histories. Greater understanding of the flight phenology of the root and root-collar feeding beetles in the Piedmont of Georgia is beneficial for a variety of reasons. Knowledge of time periods during which initial emergence activity occurs within a species may be advantageous for future studies attempting to document fungal species associated with a beetle vector at the beginning of a season. As the Ophiostomatoid fungal species associated with these root-feeding beetles are vectored phoretically (Zanzot et al., 2010), contamination and variation within the exogenous fungal community has an increased potential of occurring as beetles travel between the tree in which they matured and a new host tree in which to reproduce and interact with other beetles and their fungal communities. Knowledge of the approximate time frame during which monitored beetle species initiate their first flight activity for the year, and collection of vectors during this time period, will enable identification of those fungi which may overwinter with beetle vectors in their galleries, and thus may be more closely associated with vectors. Our results indicate that in the case of *H. pales, H. salebrosus* and *H. porculus*, initial emergence flights for a year begin as early as late-March, while for *P. picivorus* and the *H. tenuis* flight activity for the year begins much later in the season; additionally, it was found that *H. pales* exhibited a bivoltine flight phenology. It should be noted that collection counts of *H. salebrosus* were already high at the beginning of 2018 monitoring at both sites, suggesting that initial flights of *H. salebrosus* may have begun before the spring 2018 sampling period was initiated. Because the time period of monitoring of the microbial communities vectored exogenously on these beetle species may vary depending upon the species of interest, rather than sampling over the course of nearly an entire year, knowledge of when flight activity peaks for different vectors will enable short term collection of increased counts of a vector species.

Beyond increased accuracy in sampling of phoretic fungal communities associated with vector species, elucidation of flight phenology of these vector species in the Piedmont may be beneficial from a management perspective as well. *Hylobius pales* and *P. picivorus* are known as significant economic pests of pine regeneration stands (Franklin & Taylor, 1970; Taylor & Franklin, 1970), girdling pine seedlings during feeding and causing stunting and deformation of regeneration stock of *P. taeda, P. strobus* and *P. palustris* among other species. Cultural and chemical management of these pests within regeneration stands may be more effectively executed with greater knowledge of when activity of these species is highest. Additionally, as climactic patterns exhibit increasing variability in recent years, understanding how current phenology varies from historical trends is beneficial for management of these pest species. It should be noted that it is generally understood that phenology within a species is often variable between years based upon environmental conditions (Reed et al., 2009). Thus, flight phenology of root-feeding beetle vectors as documented in this study should not be considered as absolute, and the inherent variability that may occur within the phenology of any species based upon environmental changes that occur from year to year must be recognized (Ellwood et al., 2012).

With the exception of *H. salebrosus*, site was found to have a significant effect on the number of individuals collected for all monitored beetle species. *Hylastes porculus*, *H. tenuis*, *H. pales* and *P. picivorus* were all determined to be significantly more attracted to the managed site (Whitehall) than to the unmanaged site (Hardman). Given what is known concerning these genera of beetles, these results are not surprising. *Hylastes, Hylobius* and *Pachylobius* species are known to show preferential attraction to downed trees, cut stumps and stressed host trees (Blackman, 1941; Franklin & Taylor, 1970; Taylor & Franklin, 1970). Additionally, *H. tenuis, H. salebrosus, H. pales* and *P. picivorus* have previously shown attraction to sites of relatively recent controlled burns, yet another indication of preference for stressed trees and sites (Fox & Hill, 1973; Sullivan et al., 2003). Whitehall, our managed site in this study, is a research forest of the University of Georgia's Warnell School of Forestry and Natural Resources and as such is subject to regular thinning and burning regimens for both research and education purposes. Hence, its stands of loblolly pine are regularly thinned, leaving behind debris and cut stumps, and burning occurs regularly throughout pine stands of this site, leading to increased fine root death of trees in burned stands (Sullivan et al., 2003), which provide favorable conditions to root and root-collar feeding beetles. On the other hand, the unmanaged site (Hardman, also a property of the Warnell School of Forestry and Natural Resources, but largely unmanaged) had both a higher Basal Area (BA) (and consequently a higher density stand) and % canopy cover than the managed site (Whitehall) – characteristics of an overstocked stand. Historically, Southern Pine

Decline (SPD) of *P. taeda* purported to be caused by root-feeding beetles and their Ophiostomatoid fungal associates was often detected in over mature stands of loblolly pine (often stands 50+ years in age) (Eckhardt & Menard, 2009). Generally, *P. taeda* is harvested on a 25-35-year rotation depending upon objectives for the stand (Amateis & Burkhart, 2012; Schultz, 1999), far before the 50-year age mark. From a silvicultural standpoint such over mature stands can often be characterized by high BA and high % canopy cover, markers of an 'unmanaged' stand – such as those at Hardman forest in this study. Thus, the lessened attraction of the unmanaged site (Hardman) to root and root-collar beetles monitored in this study, compared to attraction to the managed site of Whitehall forest, is interesting when taking into consideration the historical criterion of sites experiencing accelerated mortality attributed to SPD.

It must be noted that though the number of beetles collected for nearly all species were higher at the managed site (Whitehall), controlled burning or thinning regimens of the site may not be the only factors affecting increased beetles' collection counts. As with any study conducted in a field setting, control of all factors contributing to study outcomes is not feasible, and other factors beyond the control or scope of this study might have had have an effect on the results.

Future research concerning these genera may be conducted more effectively thought clarification of attractiveness of different trap types and pheromone lures to target beetle species. Sampling of *Hylastes* species, for example, will not benefit from the addition of pitfall traps, as these beetles show little attraction to this trap type. Rather, the use of funnel traps should be preferred, as *Hylastes spp*. are preferentially attracted to this trap type. As shown in previous studies, *H. salebrosus* shows significant preferential attraction to funnel traps baited with the aggregate pheromone *exo*-brevicomin. Yet, other *Hylastes* species native to the southeastern U.S., *H. porculus* and *H. tenuis*, do not show any preferential attraction to this pheromone. This difference in attraction in species of the same genus, native to the same areas and with similar life histories to *exo*-brevicomin is an interesting result.

Finally, the isolation and identification of *G. alacris, G. huntii* and especially *L. profanum* from root-feeding beetles in the Piedmont is beneficial to guide further research into and understanding of the role these fungi and their vectors may play in loblolly pine health in Georgia. *Grosmannia alacris* and *G. huntii* have previously been isolated from both root-feeding beetles and from the roots of loblolly pine trees in the southeastern U.S., and their isolation and identification from beetles collected in this study was expected (Eckhardt et al., 2007; Zanzot et al., 2010). However, *L. profanum* was an unexpected species found in association with these beetles. *Leptographium profanum* has, until now, only been isolated from the roots of blackgum (*Nyssa sylvatica* Marshall), hickory (*Carya sp*. Nutt.) and flowering dogwood (*Cornus florida* L.) trees in Alabama and has not been associated with an insect vector (Jacobs et al., 2006). *Leptographium profanum* has been determined to be closely related to *L. procerum* (Duong et al., 2013), a species known to be native to the southeastern U.S. and to be associated with root-feeding bark beetles in loblolly pine (Eckhardt et al., 2007; Matusick et al., 2013b; Zanzot et al., 2010), yet they are considered to be separate species (Yin et al., 2015). Therefore, the isolation of *L. profanum* from root-feeding beetles in loblolly pine stands is an unexpected and interesting result. Moreover, in our preliminary screening we never isolated *L. procerum*, which according to the literature should have been a common species to find (Eckhardt et al., 2007; Zanzot et al., 2010). Perhaps, with their high degree of similarity*,* some of the previous reports of *L. procerum* in this region could have actually been *L. profanum*, in an unusual instance of a *Grosmannia* species being associated with both a hardwood and coniferous host. Other Ophiostomatoid species not previously reported in Georgia were isolated during this study, however, their identity remains only at a tentative level, as the sequencing of the β-tubulin partial gene alone in this case was not enough for a positive species identification (Mitchell & Zuccaro, 2006).

Overall, it was found that the activity of root-feeding beetle species varied over the course of a year based upon species, but was fairly consistent within a species between sites and experiment years (2017 vs 2018), that the majority of vector species were found in significantly higher numbers at the managed site than at the managed site and that trap type, and in the case of *H. salebrosus* pheromone lures, are significantly different in their collection efficacy depending upon the target beetle species. The Ophiostomatoid fungal community associated with root and root-collar beetles was found to be variable between species and far more isolates were collected during Experiment I (spring 2017) than during Experiment II (fall 2017). Ophiostomatoid fungal isolates obtained from root-feeding beetles differed from what was expected in their composition, with *L. terebrantis* not isolated at all, *G. alacris* only isolated once and the unexpected fungus *L. profanum* found repeatedly across vector species and sites. Results of this study will contribute to future research of root-feeding beetles and their microbial communities in the southeastern U.S. and may be beneficial to inform future silvicultural practices and management of loblolly pine in regard to these beetle species and their associate fungal communities.

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Table 2.1. Mean diameter at breast height (DBH) (cm) ± SE of all trees girdled at both Whitehall and Hardman Forest for all plots in all treatment periods

Site	**Plot**	**Mean DBH (cm)**	**± SE**
Whitehall	1	36.54	0.987
Whitehall	2	33.49	0.828
Whitehall	3	34.27	1.035
Whitehall	4	33.04	0.995
Whitehall	5	31.75	0.952
Whitehall	6	31.15	1.429
Hardman	1	29.48	0.707
Hardman	2	27.13	0.866
Hardman	3	25.87	1.206
Hardman	4	24.32	0.618
Hardman	5	22.97	0.647
Hardman	6	22.84	0.731

Table 2.2. Sums and means (± SE) of beetles collected for each target beetle species for Experiments I, II, III and IV and the total number of beetles per species collected across all experiments

Vector Species	Experiment I Sum	Experiment I Mean (± SE)	Experiment II Sum	Experiment II Mean (± SE)	Experiment III Sum	Experiment III Mean (± SE)	Experiment IV Sum	Experiment IV Mean (± SE)	Total Experiment(s) Sum
H. salebrosus	129	4.16 ± 0.03	45	1.45 ± 0.05	165	3.93± 0.04	23	0.62 ± 0.02	362
H. porculus	158	5.09 ± 0.03	36	1.16 ± 0.02	34	0.81 ± 0.05	45	1.21 ± 0.02	273
H. tenuis	33	1.06 ± 0.02	10	0.32 ± 0.01	17	0.40 ± 0.01	15	0.41 ± 0.01	75
H. pales	155	5.0 ± 0.04	179	5.77 ± 0.08	684	16.28 ± 0.06	243	6.56 ± 0.12	1261
P. picivorus	101	3.26 ± 0.02	15	0.48 ± 0.02	75	1.78 ± 0.03	50	1.35 ± 0.03	241

Table 2.3. Ophiostomatoid fungi isolated from root-feeding beetle species *H. porculus, H. salebrosus, H. tenuis, H. pales* and *P. picivorus* at managed and unmanaged sites during Experiment I (spring 2017) and Experiment II (fall 2018). Number of isolates per fungal species for each vector at each site and time period were assessed. Tentative identity was assigned based on the sequencing of a portion of the β-tubulin gene, except for *Leptographium profanum*, whose identity was confirmed via additional sequencing of a portion of the Calmodulin gene

Site	**Season**	**Vector**	**Tentative Identity**	**Number of Isolates**
Unmanaged Site	Experiment I	*H. porculus*	*Ophiostoma ips*, (Rumbold) Nannfeldt	2
		H. salebrosus	*Ophiostoma pseudotsugae*, (Romb.) von Arx **	1
			Ophiostoma ips, (Rumbold) Nannfeldt	2
		H. tenuis	*Grosmannia alacris*, T. A. Duong, Z. W. de Beer & M. J. Wingf.	1
			Ophiostoma pseudotsugae, (Romb.) von Arx **	1
			Leptographium profanum, K. Jacobs et al. *	2
			Ophiostoma rectangulosporum, Ohtaka, Masuya & Yamaoka **	1
		H. pales	*Leptographium profanum*, K. Jacobs et al. *	2
			Grosmannia huntii, (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingfield	2
			Ophiostoma ips, (Rumbold) Nannfeldt	2
			Ophiostoma pseudotsugae, (Romb.) von Arx **	1
		P. picivorus	*Ophiostoma ips*, (Rumbold) Nannfeldt	2
	Experiment II	*H. pales*	*Leptographium profanum*, K. Jacobs et al. *	4

Managed site	Experiment I	*H. porculus*	*Ophiostoma pallidulum*, Linnak., Z.W. de Beer & M.J. Wingf.**	2
			Ophiostoma ips, (Rumbold) Nannfeldt	1
		H. salebrosus	*Grosmannia huntii,* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingfield	2
			Ophiostoma ips, (Rumbold) Nannfeldt	1
		H. pales	*Ophiostoma nigrocarpum*, (Davidson) De Hoog **	1
		P. picivorus	*Ophiostoma ips*, (Rumbold) Nannfeldt	1
			Grosmannia huntii, (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingfield	1
	Experiment II	*H. pales*	*Ophiostoma ips*, (Rumbold) Nannfeldt	1
		P. picivorus	*Leptographium profanum*, K. Jacobs et al. *	4

*Identity confirmed via sequencing of the Calmodulin partial gene. **Tentative identity to be confirmed.

Figure Legend

Figure 2.1. Crown mortality of double girdled, glyphosate treated loblolly pine at each study site, Hardman and Whitehall and the mean calculated for each site. Trapping periods took place at the following time intervals: Experiment I - May 5th, 2017 – August 3rd, 2017; Experiment II - August 23rd, 2017 – November 21st, 2017; Experiment III - February 23rd, 2018 – June 26th, 2018 and Experiment IV - July 13th, 2018 – October 29th, 2018. Data were not collected during Experiment I (spring 2017). Mortality was determined as 1 = 100-75% live canopy; 2 = 75-50% live canopy, 3 = 50-25% live canopy, 4 = 0-25% live canopy; and 5 = 0% live canopy, or a dead tree.

Figure 2.2. T-test results, mean value and standard error ± (SE) of *Hylastes porculus, Hylastes salebrosus, Hylastes tenuis, Hylobius pales* and *Pachylobius picivorus* collected at managed vs unmanaged sites across all collection periods.

Figure 2.3. *Hylastes porculus, H. salebrosus, H. tenuis, Hylobius pales* and *Pachylobius picivorus* flight phenology in spring and fall 2017 collection periods. Flight curves are visualized using a local polynomial regression function.

Figure 2.4. *Hylastes porculus, H. salebrosus, H. tenuis, Hylobius pales* and *Pachylobius picivorus* flight phenology in spring and fall 2018 collection periods. Flight curves are visualized using a local polynomial regression function.

Figure 2.5. Mean value and standard error ± (SE) of *Hylastes porculus, Hylastes salebrosus, Hylastes tenuis, Hylobius pales* and *Pachylobius picivorus* attracted to funnel traps vs pitfall traps during Experiment I and Experiment II (2017) and across study sites.

Figure 2.6. Mean value and standard error ± (SE) of *Hylastes porculus, Hylastes salebrosus, Hylastes tenuis, Hylobius pales* and *Pachylobius picivorus* attracted to funnel traps vs pitfall traps during Experiment III and Experiment IV (2018) and across study sites.

Figure 2.7. Mean value and standard error ± (SE) of *Hylastes porculus, Hylastes salebrosus* and *Hylastes tenuis* attraction to funnel traps with or without the pheromone *exo*-brevicomin across all collection periods and study sites.

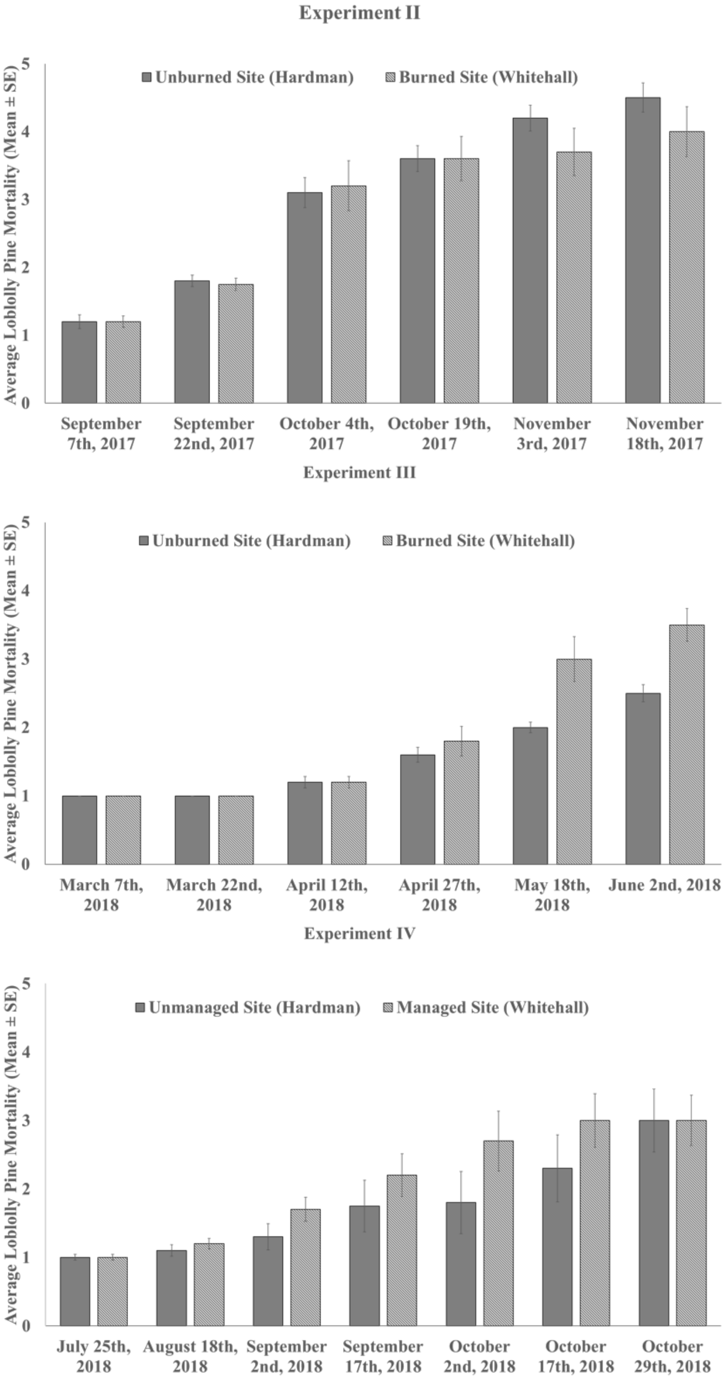
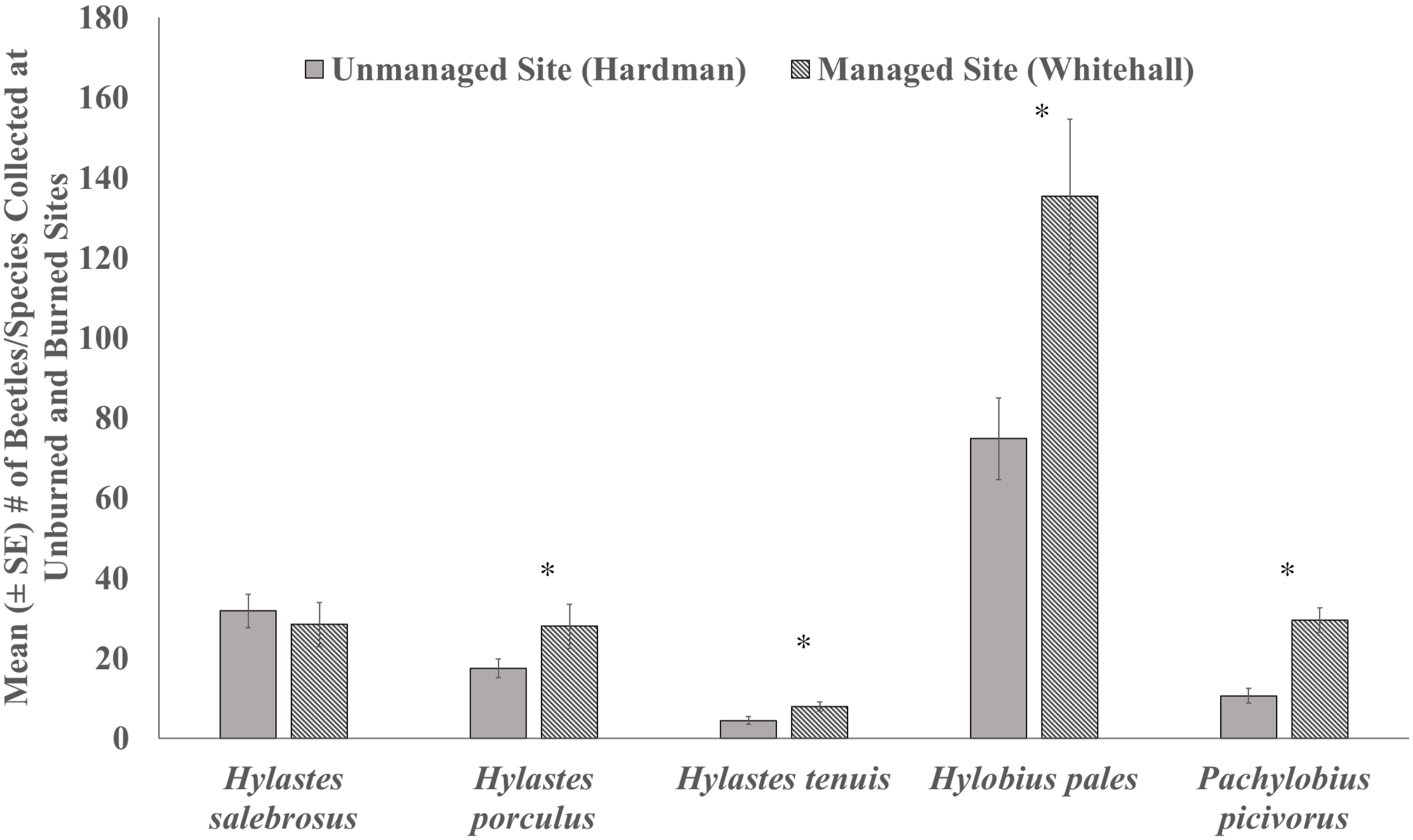

Experiment II
Average Loblolly Pine Mortality (Mean ± SE)
Unburned Site (Hardman)
Burned Site (Whitehall)
0
1
2
3
4
5
September 7th, 2017
September 22nd, 2017
October 4th, 2017
October 19th, 2017
November 3rd, 2017
November 18th, 2017
Experiment III
Average Loblolly Pine Mortality (Mean ± SE)
Unburned Site (Hardman)
Burned Site (Whitehall)
0
1
2
3
4
5
March 7th, 2018
March 22nd, 2018
April 12th, 2018
April 27th, 2018
May 18th, 2018
June 2nd, 2018
Experiment IV
Average Loblolly Pine Mortality (Mean ± SE)
Unmanaged Site (Hardman)
Managed Site (Whitehall)
0
1
2
3
4
5
July 25th, 2018
August 18th, 2018
September 2nd, 2018
September 17th, 2018
October 2nd, 2018
October 17th, 2018
October 29th, 2018

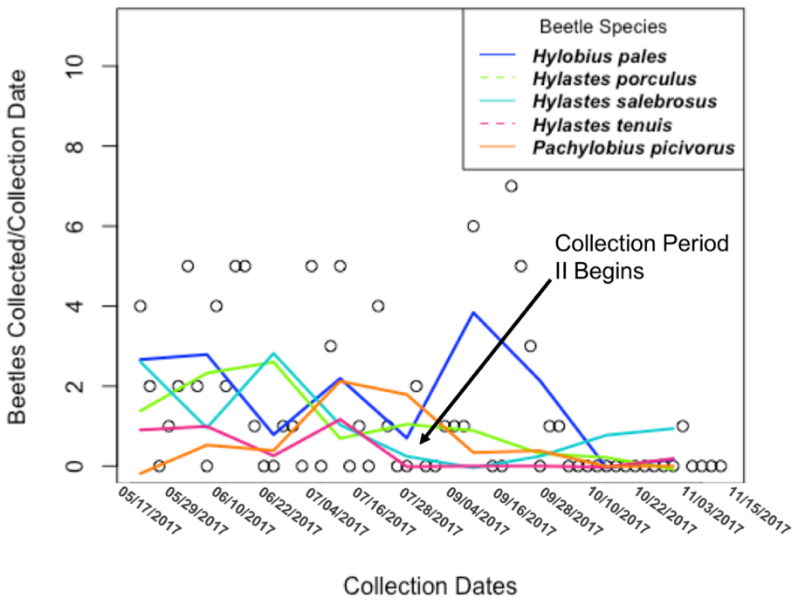
Figure 2.1



Mean (± SE) # of Beetles/Species Collected at Unburned and Burned Sites
180
160
140
120
100
80
60
40
20
0
Unmanaged Site (Hardman)
Managed Site (Whitehall)
*
*
*
*
Hylastes salebrosus
Hylastes porculus
Hylastes tenuis
Hylobius pales
Pachylobius picivorus

Figure 2.2

Unmanaged Site (Hardman) Experiment(s) I & II



Managed Site (Whitehall) Experiment(s) I & II

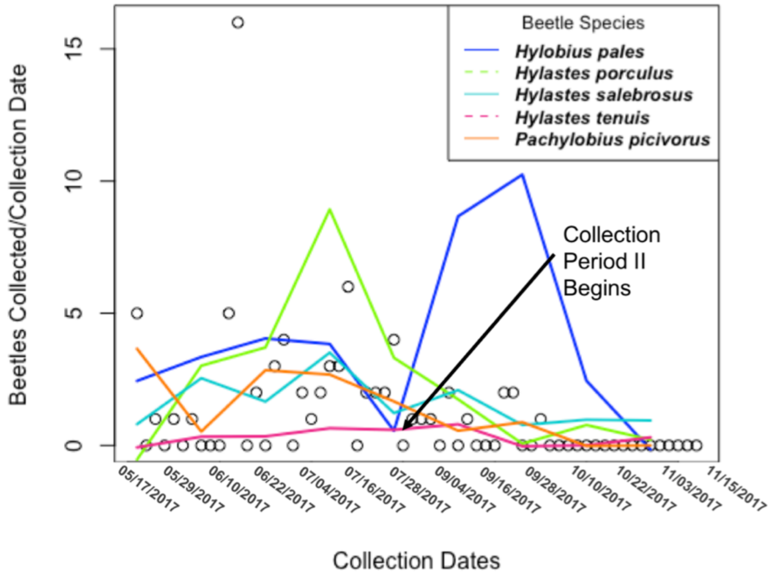
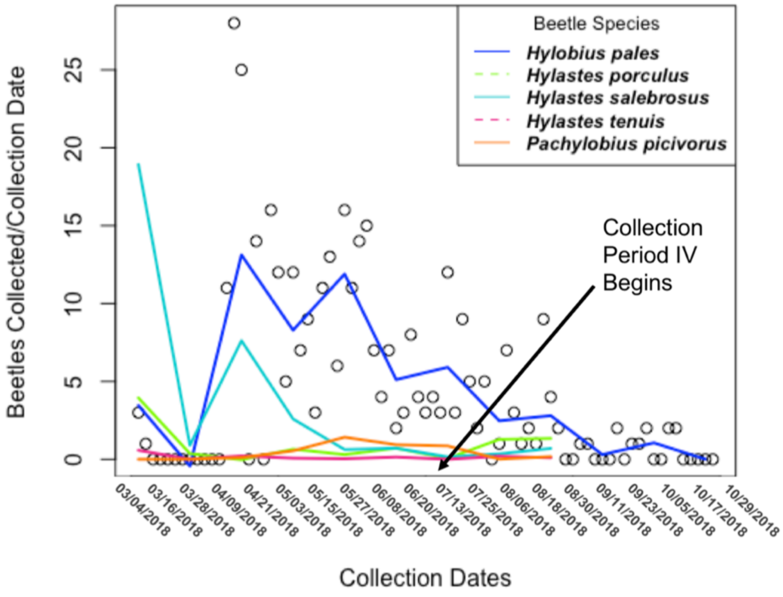


Figure 2.3

Unmanaged Site (Hardman) Experiment(s) III & IV



Managed Site (Whitehall) Experiment(s) III & IV

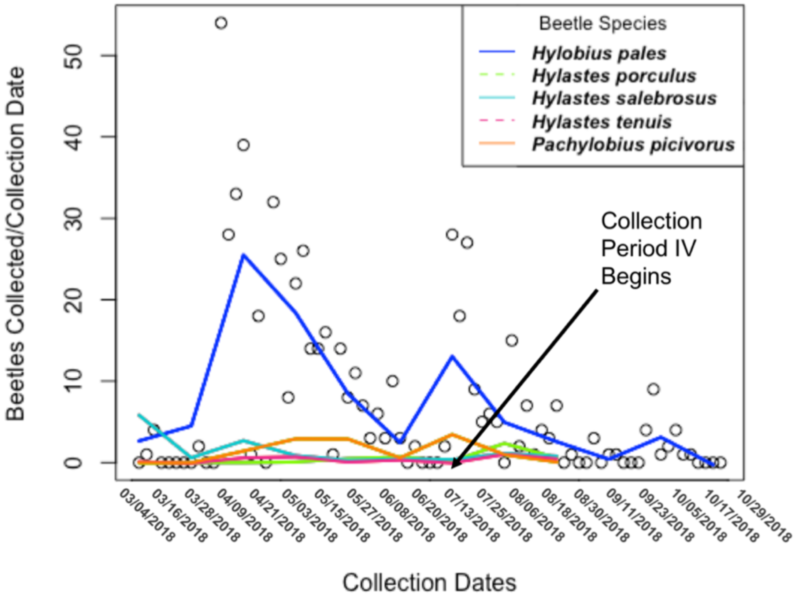


Figure 2.4

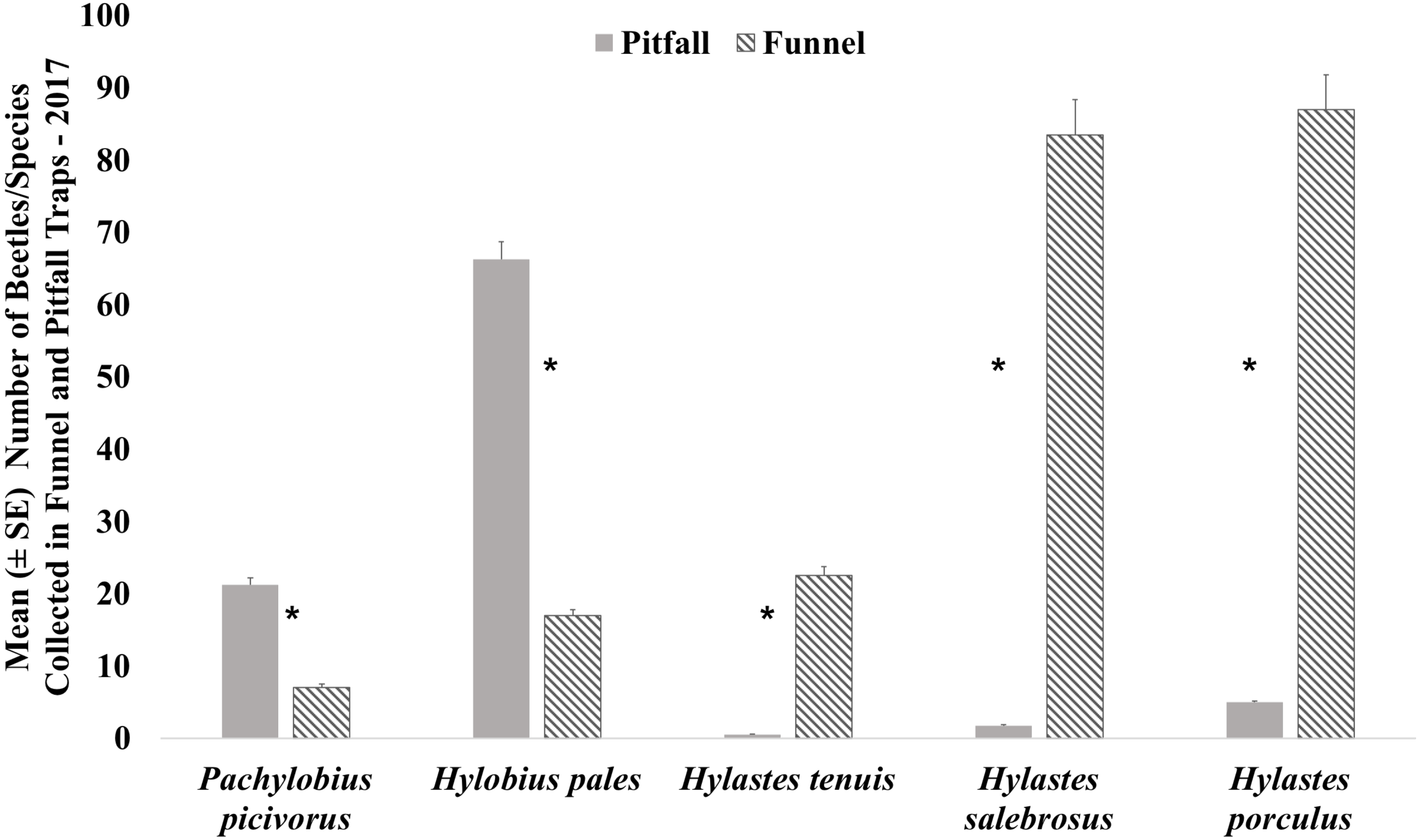
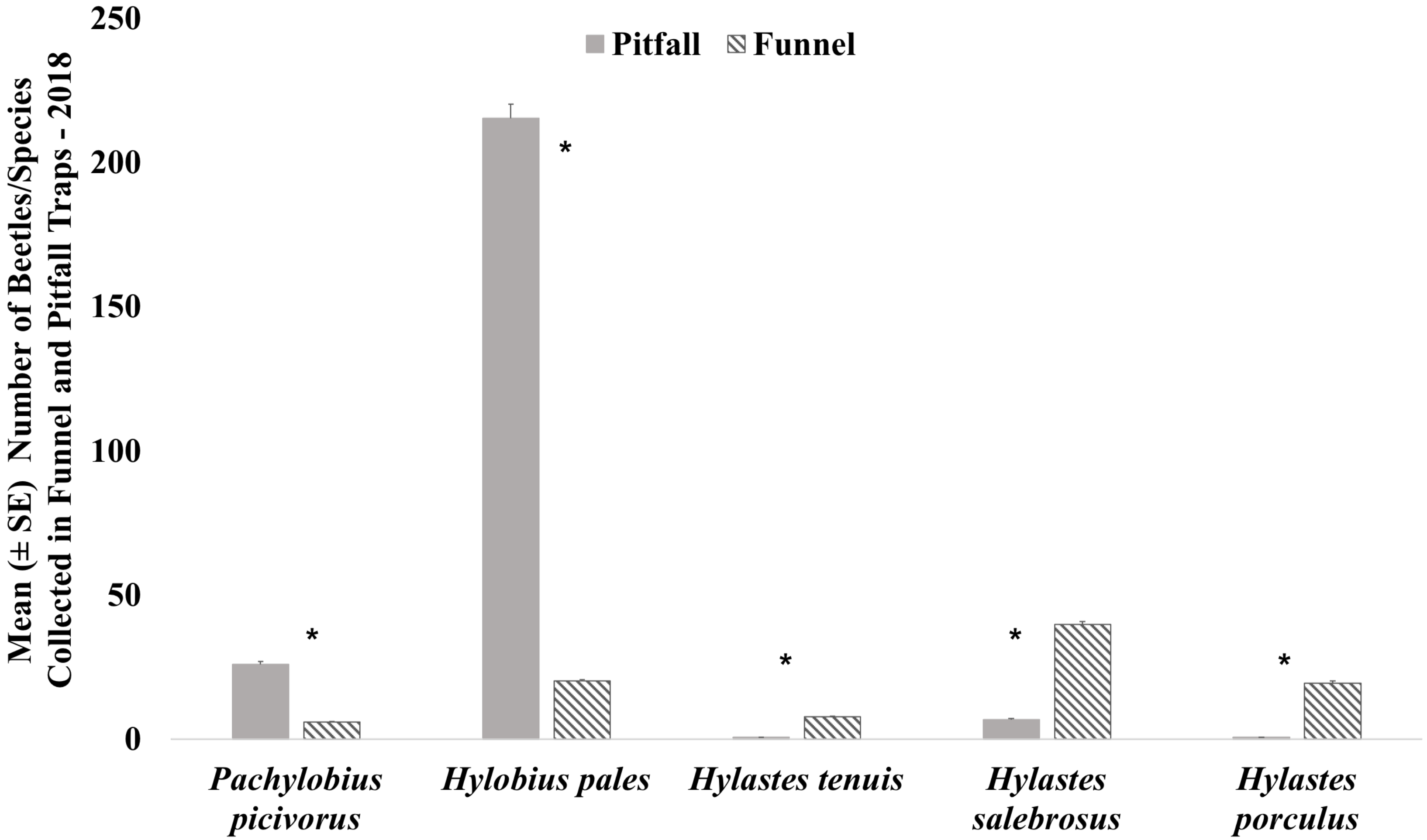

■ Pitfall
▨ Funnel
Mean (± SE) Number of Beetles/Species Collected in Funnel and Pitfall Traps - 2017
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
*
Pachylobius picivorus
Hylobius pales
Hylastes tenuis
Hylastes salebrosus
Hylastes porculus

Figure 2.5



Pitfall
Funnel
Mean (± SE) Number of Beetles/Species Collected in Funnel and Pitfall Traps - 2018
250
200
150
100
50
0
*
*
*
*
*
Pachylobius picivorus
Hylobius pales
Hylastes tenuis
Hylastes salebrosus
Hylastes porculus

Figure 2.6

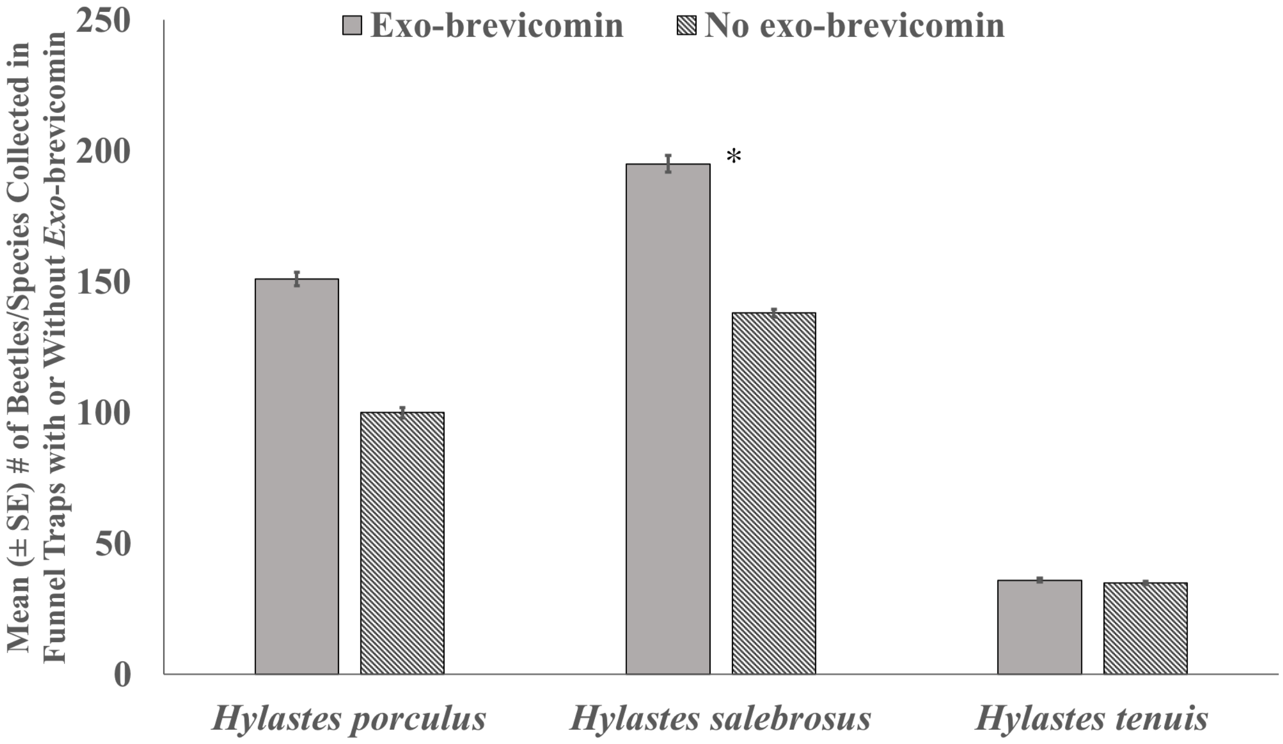

Exo-brevicomin
No exo-brevicomin
Mean (± SE) # of Beetles/Species Collected in Funnel Traps with or Without *Exo*-brevicomin
250
200
150
100
50
0
*
Hylastes porculus
Hylastes salebrosus
Hylastes tenuis

Figure 2.7

CHAPTER 3

THESIS CONCLUSIONS

3.1 Conclusions

With the effects of environmental and anthropogenically driven climate change increasingly evident in both managed and natural forest settings, a more thorough understanding of the role bark beetles and their associate fungi play in affecting forest health has become of increasing pertinence. The objective of this thesis was to develop a more in-depth picture of how silvicultural practices and management strategies in loblolly pine (*Pinus taeda* L.) stands may affect presence of root and root-collar feeding beetles within these sites, to determine the flight phenology of these species and to conduct preliminary screening for *Leptographium* and *Grosmannia* Ophiostomatoid fungal species that may be associated with these root-feeding beetles in the Georgia Piedmont. To this end, root and root-collar feeding bark beetle species *Hylastes salebrosus* Eichoff, *Hylastes porculus* Erichson, *Hylastes tenuis* Eichoff, *Hylobius pales* (Herbst) and *Pachylobius picivorus* (Germar) (Coleoptera: Curculionidae) were collected over a two-year period. Beetles were collected in loblolly pine stands that were classified as either managed or unmanaged based upon site management history and collection counts of the beetles were analyzed to determine how site and time influenced variability in the number of beetles collected in each species. It was found that in the managed site, which was regularly control burned and had been thinned, significantly greater counts of all beetle species were documented, with the exception of *H. salebrosus*. Collection peaks within beetle species was found to occur within a consistent time period across collection years, but peaks were found to occur at different times for different beetle species, including within the same genus. Overall flight phenology of beetles was found to be mostly consistent between years for the same time periods. Weevil species (*H. pales* and *P. picivorus*) were found to be significantly more attracted to pitfall traps than to funnel traps across all sites and collection periods. Blue-stain fungal species *G. alacris, G. huntii* and *L. profanum* were isolated from beetles collected in this study and identified via morphological and molecular techniques in order to gain a preliminary understanding of *Grosmannia* species associated with root-feeding beetles in the Piedmont and to inform future research into these fungi. Though *G. alacris* and *G. huntii* are not an unexpected result, the isolation and identification of *L. profanum* was unexpected, as *L. profanum* had previously only be identified in association with the roots of hardwood trees in Alabama and did not have a recognized insect vector.

3.2 Future Research

To further an understanding of the potential roles of root-feeding beetles and their fungal associates in mortality of loblolly pine, further study beyond the scope of this research is warranted. Suggested next steps of this research would be as follows:

1) Identification of other factors that could explain root and root-collar feeding beetle preference for sites with a management history of controlled burning and thinning. Clarification of which factors affect the number of beetles found at sites with differing management histories would be beneficial from both a research and management perspective.

2) Assessment of the fungal community associated with root-feeding bark beetles at the beginning of the year, to clarify which fungal species are most closely associated with a beetle vector would be informative. As the beetle species which are the focus of this research lack mycangial structures, vectoring their fungal symbionts exogenously in pits on their cuticle, the potential for contamination of the associate fungal community is significantly increased over time. Collection of root and root-collar feeding bark beetles at the beginning of the year, when they initiate their emergence flight, would be useful in identifying the fungal species which overwinter in beetle galleries and which are subsequently most likely much more closely associated with the vectors of interest.

3) Response of loblolly pine to *Leptographium* and *Grosmannia* species isolated and identified in this study may facilitate further understanding of the potential pathogenicity of these fungi to loblolly pine. A measure of the response of loblolly pine to inoculation with fungi identified in this research will drive understanding of if and how these fungi negatively affect the health of loblolly pine in the southeast. Tree response, both chemical and physical, to the presence of fungal inoculum may provide indicators of whether or not these fungal species exhibit pathogenic traits in loblolly pine.