Ophiostomatoid Fungal Infection and Insect Diversity in a Mature Loblolly Pine Stand

by

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Abstract

Root-feeding beetles and weevils are known vectors of ophiostomatoid fungi, such as *Leptographium* and *Grosmannia*, that have been associated with a phenomenon called Southern Pine Decline in the Southeastern United States. One of these fungi, species name *Leptographium terebrantis*, has a well-known effect on pine seedlings, but the effect on mature, field-grown trees and associated insect populations is still to be determined.

This study examined changes in insect diversity one year pre- and post-inoculation of mature loblolly pine trees with varying levels of a *L. terebrantis* isolate, giving special attention to monitoring insects of concern. Three different insect traps of two types – pitfall and airborne – were used during the twenty-five month study. Insects were collected every two weeks, identified to family where possible, and further sorted to morphospecies.

Of 9,748 insects collected, we identified 16 orders, 149 families, and a total of 676 morphospecies. Of these, less than ten individuals were each *Hylastes*, Hylobiini, and *Ips* species of concern. We collected over 60 individual ambrosia beetles in nine species. However, all but one had six or less individual beetles. Only one species, the invasive *Xylosandrus germanus*, had a total of 44 individuals.

Ground-based pitfall traps were more efficient at capturing Hylobiini weevils, but airborne panel traps caught overwhelmingly more ambrosia beetles. Insect diversity did not vary with treatment, but instead showed seasonal variation between the two years. A drought that occurred at the end of the first year may be a possible explanation for this. Antifreeze-based pitfall traps were marginally significant between treatments for the year they were

out. Additional monitoring may provide a greater understanding of inoculum load on insect diversity.

Methods of ophiostomatoid fungal identification have initially relied on manual and molecular methods. These often require growing fungal cultures that can take weeks to develop. Other options, including using near-infrared light waves to size features on bark beetles, including fungal spores, have just become a potential avenue to investigate. Our study addressed the possibility of identifying fungal species with these light waves on the surface of a beetle.

We used spores from three fungal species: *Grosmannina alacris*, *Leptographium procerum*, and a *Graphium* species. *Hylastes salebrosus* beetles were collected from neighboring wildlife areas, sterilized before use, and rolled in corresponding fungal cultures to mimic vectoring of spores. Prepared beetles were then transported to CytoViva, Inc., for imaging.

To help identify regions of the beetle's surface where spores could be found, we devised a code to be used with ImageJ and called it "FFT map" after basing it on the fast fourier transform algorithm. After consulting wavelengths of associate species, we could set thresholds to identify areas that match reflecting spores.

Of 52 fungal spores identified and measured for size, we found an average mean spore size of 3.15 µm, 3 µm, and 2.31 µm for *G. alacris*, *L. procerum*, and the *Graphium* species, respectively. We showed that two of our three species can be identified with hyperspectral interferometry. Though our map eliminated much of the beetle's surface that did not contain spores, narrowing our search scope and quickening identification, additional automation could provide a simple, optical tool for identifying spore loads on insects. This process was made more challenging by the presence of setae and other beetle surfaces that cause interference.

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Introduction and Literature Review

1.1 Forestry in the Southeastern U.S. with a focus on Loblolly Pine

The forest industry makes up a large constituent of the Southeast's current economy (Schultz 1997). A top choice for lumber plantations is that of Loblolly pine, *Pinus taeda* Linnaeus. Loblolly pine is one of over a hundred species of pine in North America and historically ranges from eastern Texas stretching east to central Florida and as far north as New Jersey (Schultz 1997). Naturally, it is found growing with mixed hardwoods or other pines and it is a minor species when found out of moist sites.

Prior to the planting of loblolly pine, many areas in the South now reserved for forestry were primarily lands for growing crops. Early knowledge of proper soil and management techniques were lacking, which contributed greatly to erosion and the subsequent loss of nutrients (Trimble 1974). Today, loblolly pine is the most heavily cultivated pine in the southeastern United States. Its success lies in its ability to grow in a large variety of sites and its fast growth, which has led to its wide planting and use in the timber industry (Baker and Langdon 1990). Because of this hardy nature, its planting dramatically increased in the 1900s after the cotton industry declined, so where it once covered only 2 million hectares, after the Europeans arrived in America it grew to reside in 13.4 million hectares (Schultz 1997). Thus, loblolly pine went from a minor species to becoming the dominant pine species in less than a century (Schultz 1997).

1.2 Insects Associated with Commercial Forests in the Southeast

As a major component of all life on Earth, insects continue to play a major role in ecosystem function. Insects provide food to humans and other animals, prey on pest insects and vegetation, pollinate plants and facilitate seed dispersal, aerate soil, and contribute to the nutrient cycle by aiding in decomposition (Edmonds and Eglitis 1989; Belovsky and Slade 2000). However, the benefits of insects in commercial forest are often overlooked when pests are involved.

Fallen trees and other debris can provide a potential fire hazard if not properly considered and can provide refuge for unwanted insects (Brown et al. 2003). Through their habits, these insects affect rates of nutrient recycling, introducing fungal decomposers and adding nutrients back to the environment (Edmonds and Eglitis 1989). Wood-boring beetles, bark beetles, and termites increase the rate of decomposition in felled or fallen trees (Edmonds and Eglitis 1989; Ulyshen 2016). This rate is affected through several means, including enzymatic digestion, substrate alteration, nitrogen fertilization and biotic interactions (Ulyshen 2016). In addition to this, herbivory by insects on plants also has the capacity to contribute to nitrogen cycling, potentially affecting plant abundance (Belovsky and Slade 2000).

Forest pests in the United States continue to be, in a large part, non-native, with over 400 species originating from outside the country, costing homeowners and local governments up to billions of dollars in damages (Aukema et al. 2011). While not all non-native pests cause economic damage, infamous invasive insects, such as the emerald ash borer (*Agrilus planipennis* Fairmaire), hemlock woolly adelgid (*Adelges tsugae* Annande), gypsy moth (*Lymantria dispar* Linnaeus), and Asian longhorn borer (*Anoplophora glabripennis* Motschulsky) cause noticeable effects, altering ecosystems, sometimes irrevocably (Kenis et al. 2009). Ecosystem impacts from invasive insects include eating native species, parasitizing native insects, introducing and vectoring pathogens, pollination disruption, competing for resources, and hybridizing with native

species (Kenis et al. 2009). These actions have cascading effects on the ecosystem and food webs.

In addition to this, there are still plenty of native insects that cause economic impacts in the southeastern United States. The region can be especially conducive to insects due to the warmer temperatures and longer growing season. Species such as the infamous southern pine beetle, *Dendroctonus frontalis* Zimmerman, are known to have as many as five generations in their northern range of the United States, but this number nearly doubles in areas such as Florida and South America. This beetle is widely considered as one of the most important bark beetle pests in the country, and economic costs average around $43 million a year for timber producers (Pye et al. 2011).

The large insect family, Curculionidae, which includes southern pine beetle, also is host to thousands of other species that prey on commercial trees. In addition to this, the families Cerambycidae and Buprestidae include wood boring beetles that cause damage to tree tissues. Between 1985 and 2005 alone, 25 exotic species of beetles representing these three families were introduced to the United States (Haack 2006). Fungal associates of such insects complicate matters, adding a new variable into an otherwise known equation.

1.3 Insect – Fungal Associations and Other Vectors

Millions of years of evolution have created ecological relationships between insects and microorganisms. In commercial forests, some of these relationships are especially well known. Insects play host to different microbes and fungi, acting as inadvertent and direct vectors, introducing pathogens and aiding in decomposition and nutrient recycling.

Indeed, in the case of ambrosia beetles (Coleoptera: Scolytinae) they excavate galleries in weakened or fallen trees, sowing fungal spores, and feeding exclusively on the byproduct of their

fungal crops. The ambrosia beetle, *Xyleborus ferrugineus* Fabricius, has been shown to carry a mix of fungi, yeast, and bacteria, including *Graphium* species that enhance the tissue's nutrient content (Baker and Norris 1968). Still, other insects feed on both wood and fungal tissues, and in the case of the southern pine beetle, some invertebrate vectored fungi are inhibitory to the beetle's preferred fungal food source (Klepzig and Hofstetter 2011), going against the mutualistic relationships provided by mycangial fungi. The beetles' preferred fungi make wood more palatable, increasing nutrient content in terms of nitrogen and phosphorus and increasing survival for offspring (Beaver et al. 1989; Ayres et al. 2000; Eckhardt et al. 2004a).

Despite the overall benefits of insects to the environment, insects are also known to contribute to disease. By attacking plants and trees, they cause stress, resulting in allocation to defenses and affecting normal physiological processes (Christiansen et al. 1987). The fungi and pathogens they carry can cause great harm to the host plant, even at a benefit to the insect. Trees weakened by insect attack can further become susceptible to fungal pathogens that move in post-attack (Christiansen et al. 1987). Fungi are known agents of tree disease and perpetuate the cycle of tree decline (Manion 1981). The introduced red bay ambrosia beetle, *Xyleborus glabratus* Eichhoff, carries *Raffaelea* species, and was first discovered in the United States through Georgia in 2002 (Haack 2006). One of its fungal symbionts, *Raffaelea lauricola* T.C. Harr Fraedirich & Aghayeva, is the casual pathogen of laurel wilt, affecting plants in the Laurel family, including red bay, avocado, sassafras, and cinnamon. In the case of beech bark disease, caused by *Neonectria* species of fungi and vectored by the invasive beech scale, *Cryptococcus fagisuga* Lindinger, it results in annual cankers from scale feeding that eventually girdle and kill trees. Harrington (1993) acknowledged two possible hypotheses for the relationship between fungal species and bark beetles in specific: either the fungi serve as a food source or provide an advantage to the beetles' offspring, or second, the fungi are weeds that happen to coincide with

the beetles habits. Pests like these therefore gain an advantage as they attack trees, the fungi they bring unintentionally weakening trees (Christiansen et al. 1987; Eckhardt et al. 2007).

1.3.1 Ophiostomatoid Fungi

In the case of ophiostomatoid fungi, cultures in the roots need a way to infect new trees if they are originally from root tissue in the soil. Beneath the ground, air and rain dispersal are inhibited, and so spores are introduced into susceptible tissues by the wounding of insect feeding (Harrington and Cobb 1983). These conidia – asexual fungal spores – are sticky, allowing for adherence to the bodies of insects, but some insects use specialized mycangia for transportation of spores (Harrington 1993). The adhesiveness owes to a mucilage covering that protects from desiccation and sunlight, while also allowing spores to travel as discrete masses (Dowding 1969). There's also support that this mucilage protects the spores as they pass through the internal digestive tracts of beetles, allowing for yet another mode of dispersal into tree tissues as beetles create galleries (Francke-Grosmann 1963).

Insects associated with ophiostomatoid fungi – *Hylastes salebrosus* Eichoff, *Hylastes tenuis* Eichoff, *Pachylobius picivorus* Germar, *Hylobius pales* Herbst and *Hylastes porculus* Erichson – have all been significantly correlated with declining trees, and have been shown to carry fungi such as *Leptographium terebrantis* Barras and Perry, *Leptographium procerum* (Kendr) Wingf., and *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf. (Klepzig et al. 1991, Jacobs and Wingfield 2001; Eckhardt et al. 2007; Matusick et al. 2013). These *Leptographium* species carried by the beetles have been shown to increase their reproductive capacity when present (Eckhardt et al. 2004a; Menard et al. 2010).

In addition to these species, the black turpentine beetle, *Dendroctonus terebrans* Olivier, that attacks the lower bole of pine trees, has also been found to carry ophiostomatoid species that include *L. terebrantis* (Rane and Tattar 1987). In other areas of North America, the red

turpentine beetle, *Dendroctonus valens* LeConte, has also been found to carry *Leptographium* species in addition to the pine root collar weevil, *Hylobius radices* Buchanan, and *Hylobius rhizophagus* Millers, Benjamin, and Warner (Wingfield 1983; Owen et al. 1987). Beetles gain a foothold in trees as the fungi they inadvertently bring further weaken trees (Christiansen et al. 1987; Eckhardt et al. 2007).

Beetles are, however, not the only possible carriers of ophiostomatoid fungi. Phoretic mites (Trombidiformes: Tarsonemidae) also are known carriers of blue stain fungal spores, such as *Ceratocystis minor* Hedgcock (Bridges and Moser 1983) and *Leptographium abietinum* Peck (Cardoza et al. 2008) depending on the host beetle species (Hofstetter and Moser 2014). These mites live in the galleries created by bark beetles and other insects and can feed on the fungi and collect spores in pouches on their heads called sporothecae (Moser 1985; Hofstetter and Moser 2014). These mites manage to cling to bodies of different species of beetles and inadvertently are vectored to other trees and parts of the same tree. In fact, 90% of mites leave their hosts or die between arrival and their host mating and laying eggs, and can survive over 30 days without a host once they do leave (Klepzig and Hofstetter 2011). This unexpected transportation can cause *Leptographium* species and other closely related fungi to be found in unexpected places of the tree, such as the upper and mid bole, negatively affecting some populations of bark beetles – which in turn become inadvertent vectors themselves (Hofstetter and Moser 2014; Bridges and Moser 1983).

Besides the roots, *Leptographium* and other ophiostomatoid fungi are also found in the lower bole of the tree, according to the feeding habits of their associated beetles (Klepzig et al. 1991). In addition to the longitudinal and radial growth of the fungi, the spread of these fungal species can be supported by the existence of phoretic mites that also carry the fungi and are not limited to one part of the tree or one specific host insect (Hofstetter and Moser 2014). Because of this

fact, previous work with these ophiostomatoid fungi utilized both stem and root inoculation tests and determined pathogenicity was similar though damage potential may not be (Matusick et al. 2016).

Likewise, there is evidence to suggest that trees affected and weakened by ophiostomatoid fungi may in turn be more susceptible to southern pine beetle as these trees are weakened and giving off terpenes. The southern pine beetle perpetuates the disease cycle as a vector by picking up fungal spores and transporting them to healthy trees that they can attack in epidemics (Ostrosina et al. 1997). Furthermore, roots infected with *Leptographium* species have been observed to have increased populations of subterranean termites, which may also be another vector of these fungi (Riggins et al. 2014). To further compound the problem, animals such as feral hogs damage pines in plantations through rooting and chewing of the tree roots. Hogs in areas with declining trees can carry *Leptographium* species on their snouts to further infect trees through wounding (Eckhardt et al. 2016).

While plants and animals are limited in their range due to temperature and hosts, climate change is expected to change this. Rising temperatures and introduced hosts and pathogens may expand ranges, extend breeding seasons, and give rise to additional vectors and mutualistic associations that aid in tree decline by insects (Pautasso et al. 2015, Wong and Daniels 2017).

1.3.2 *Leptographium terebrantis*

Of the species associated with pine decline in the southeastern United States, *Leptographium terebrantis* is considered a moderate to severe pathogen (Harrington and Cobb 1983; Matusick and Eckhardt 2010). Like other ophiostomatoid fungi (Repe and Junc 2010), it has been shown to cause lateral root damage and resin-filled lesions that extend into the xylem, occluding water uptake (Raffa and Smalley 1988; Matusick and Eckhardt 2010; Matusick et al. 2012). While lesions can appear in older trees, Harrington and Cobb (1983) showed this fungus has the ability

to kill pine seedlings.

This fungus also has a history of being associated with pines that are dead or declining, including not only loblolly pine, but also shortleaf, longleaf, slash, and red pine (Klepzig et al. 1991; Harrington and Cobb 1983; Jacobs and Wingfield 2001). In turn, it has been found on the bodies of the root-feeding beetles (Coleoptera: Scolytinae) *H. tenuis, H. salebrosus, H. pales,* and *P. picivorus,* where these insects are more abundant where southern pine decline is present (Eckhardt et al. 2007). In addition to this, it has also been inoculated from the roots of trees attacked by southern pine beetle, *Dendroctonus frontalis,* suggesting that southern pine beetle may act as a contributing factor to decline (Bridges and Moser 1983; Eckhardt et al. 2004b; Ostrosina et al. 1997).

1.4 Management of Insect Pests in Commercial Forests

1.4.1 Abiotic

1.4.1.1 Fire and Prescribed Burns

Fire can have both negative and positive impacts on insect populations. Herbivore accumulated mortality from insects, along with leaf litter, can increase the risk of wildfires (Fettig et al. 2007; Schowalter 2008). Fires in already stressed areas can allow for bark beetle infestations to occur, as emerging beetles have a ready source of weakened and stressed food nearby (Ferrell 1996; Kolb et al. 2007). Other insects, too, such as wood borers (eg. Cerambycidae, Anobiidae, Elateridae), species that feed on fungi (eg. Latridiidae, Cryptophagidae, Leiodidae), and species that are associated with soil and leaf debris (eg. Carabidae, Byrrhidae) increase in recently burned areas, reacting to damaged trees, fungi utilizing fresh debris, and the fodder insects resulting from these (Muona and Rutanen 1994). Some species, such as the red turpentine beetle, *Dendroctonus valens* LeConte, will readily search out and reproduce in scorched trees, and therefore care must be used when considering the

area to be burned (Ferrell 1996). In a study undertaken by Maloney et al. (2008) in a mixed-conifer stand, bark beetle attacked trees were higher in number in burned compared to unburned plots, and similar results were obtained in Alabama longleaf plots following burns in a study by Campbell et al. (2008). Evans and Kuster (1980) studied the infrared receptors in cavities adjacent to mesocoxal cavities in the black fire beetle (*Melanophila acuminata* DeGeer) and suggested the beetle uses these to search for sources of heat, including those of forest fires to seek out the affected weakened or dying conifers to oviposit eggs.

Fire can injure roots, especially fine feeder roots near the soil surface, leading to wounds that provide entry points for insects (Schwilk et al. 2006). Root-feeding species such as *H. salebrosus* and *H. tenuis*, along with some ambrosia beetles and weevils – including species carrying *Leptographium* spores – are attracted to recently burned plots (Sullivan et al. 2003). Smaller trees can be more readily injured or killed by severe fires, providing substrate for bark beetles, though earlier season burns under higher moisture conditions may reduce injury and therefore reduce the risk of bark beetle attack (Schwilk et al. 2006). In the case of red pine, *Pinus resinosa* Sol. ex Aiton, which has coevolved with fire, burns can initially lower resin defenses and trees take 7-10 days to return to pre-fire levels, but eventually can increase two fold over the next 55 days (Lombardero et al. 2006). Though fires may result in an increase in these insect populations, however, these usually do not persist long and relatively disappear after the first year (Werner 2002).

Prescribed fires are a method used to reduce bark beetle infestations (Fettig et al. 2007). While fires can also stress trees and potentially attract beetles, higher intensity fires can weaken trees yet render them less than optimal for beetles (Ferrell 1996). Fire exclusion can lead to a change in ecosystem structure and the method of prescribed fire has often been used as a tool to restore ecosystem function as well as to control pest populations (Schwilk et al. 2006), especially

in regards to longleaf forests that are adapted to fire.

1.4.1.2 Thinning

Density management of trees can change plant species composition, allowing for the entry of early successional species (Thomas et al. 1999; Wilson and Puettmann 2007). Insect populations can also be affected, decreasing populations through the removal of cover and the subsequent increase in temperature and loss of humidity (Kremen et al. 1993). Selective removal of older trees – in the goal to harvest them before they are attacked – has been successful in some areas in regards to reducing tree mortality and resulting pest populations (Ferrell 1996). Thinning measures can increase remaining trees' radial growth and constitutive resin defense, water, and carbon uptake, therefore making trees less susceptible to pests (Kolb et al. 2007).

Likewise, previous work has shown that thinning prevents further insect outbreaks and encourages tree growth (Fettig et al. 2007). Variations of thinning are generally accepted as reducing tree competition and increasing tree vigor, which decreases susceptibility to pests (Sartwell 1971). Mechanical control of trees in this way has proven effective for control of southern pine beetle populations that are newly started. Techniques such as cut-and-remove and cut-and-leave also involve felling a buffer strip of trees in addition to infected trees in order to hinder further population growth during the high risk months (Billings 2011). Ultimately, however, thought should be given to surrounding areas as pest populations may still affect managed areas if the populations are high enough (Schmid and Mata 2005).

On the opposite side, thinning can also benefit other insect species in the short term, such as some Hymenoptera, Lepidoptera, Diptera, and Coleoptera species, some of which may attack trees (Taki et al. 2010). As tree densities increase, trees compete with each other, weakening their defenses and allowing for opportunistic insects to move in. While alleviating these densities, remaining stumps and injuries left behind can further increase insect populations

(Schowalter 2008). Felled trees can provide substrate for wood boring beetles and termites, as well as for flies and wasps, and as decomposition sets in, the fungal communities can change, shifting the insect communities accordingly (Vanderwel et al. 2006). Thinning operations involve heavy machinery that can lead to soil compaction and damage to surrounding trees, all sources of additional stress for trees (Ferrell 1996). This can affect roots, causing wounds that attract insects (Ferrell 1996; Eckhardt and Menard 2008). If insects don't directly invade at first, pathogens such as *Heterobasidion* and *Armillaria* species can invade injured roots and cut stumps, weakening trees for future insect attack (Ferrell 1996; Harrington 1993). Likewise, thinned areas can have higher temperatures and increased wind, where beetle diversity may actually increase (Hindmarch and Reid 2001). Cut material left in a thinned stand can provide ready breeding sites while creating new niches for biodiversity (Peltonen et al. 1998). Thinning can also affect nutrient input as well as water availability, adding to the nutrients available as well as allowing more rainfall to enter the leaf litter, influencing understory growth and thus the insects associated with those (Kremen et al. 1993; Thomas et al. 1999).

Clearcutting is another technique used to remove plant cover, decreasing tree densities, paving the way for early successional species, as a way to increase food and habitat resources for wildlife, or as a way to prepare the land before planting or conversion. While this can result in soil erosion and a loss of biodiversity, species richness and diversity of some insects may actually be increased, as seen with ground beetles (Coleoptera: Carabidae) in a boreal mixed forest ecosystem (Duchesne et al. 1999).

1.4.1.3 Drought

As bark beetles and other phytophagous insects respond to stressed plants, drought is one inciting factor that can result in increased plant mortality and may aid in insect dispersal due to the increased availability of hosts (Mattson and Haack 1987; Ferrell 1996). The factors affecting

attacks during drought may stem from several factors, including lowered plant defenses and an increase in nitrogen in plant tissues (White 1984; Mattson and Haack 1987). Plant tissues can acquire more minerals such as calcium, magnesium and nitrogen due to increasing soil temperatures, decrease in water and ion movement (Mattson and Haack 1987). Because of these nutrients, plants can be especially attractive to insects. Even these changes can influence the growth of microorganisms, providing more sugars for their growth, especially in the sapwood and inner bark (Mattson and Haack 1987). These sugars are especially conducive for growth of blue stain fungi, such as *C. minor* which is carried by the southern pine beetle, *D. frontalis* (Barras and Hodges 1969). In addition, insect pathogens may be hindered during drought, where fungal spores are unable to spread far in the low humidity, bacterial and viral components are damaged by the higher ultraviolet radiation, and parasites are unable to develop correctly in higher temperatures (Mattson and Haack 1987). Where these natural enemies fail, insect populations are released.

Severe droughts may decrease pest populations, especially after a certain point, limiting outbreaks as the moisture present in the field drops below what is required for insect survival (Schowalter et al. 1981). Some insects are especially sensitive to changes in humidity and temperature and have methods to detect each (Altner and Loftus 1985). While some nutrients may increase in plant tissues during drought and other factors can be favorable for insects, chemicals such as terpenes may have their concentrations change (Heikkenen and Hrutfiord 1965). What may be a repellant during moisture-sufficient times may become an attractant during drought. Likewise, just as tissues can become more attractive to some insects, the same tissues can also be less toxic during adverse conditions, such as with the chemicals myrcene and limonene (Cates and Alexander 1982).

1.4.1.4 Flooding

One of the most common concepts of flooding benefitting insects is with mosquitoes (Diptera: Culicidae) and their aquatic larvae. Studies with rice fields have shown flooding can increase nutrients and mosquitoes, but also other herbivorous and predatory insects (Lawler and Dritz 2005). Compared to areas that are regularly exposed to flooding, areas that are flooded unexpectedly may have insect populations with a surprising resilience. In riprarian sites around the Rio Grande in New Mexico, species richness was unaffected by non-historical flooding, and ground beetles (Coleoptera: Carabidae) and ants (Hymenoptera: Formicidae) can actually increase (Ellis et al. 2001). Likewise, a similar study in the southeastern United States with loblolly pine showed unaffected beetle richness and abundance with seasonal flooding in areas (Ulyshen 2014). However, with flooding as a stressor, ambrosia beetles can preferentially attack affected trees compared to non-affected ones (Ranger et al. 2013), with flooding intolerant trees being the most susceptible (Ranger et al. 2015).

Caddisflies (Trichoptera) and mayflies (Ephemeroptera) can, however, be negatively affected in the short term by flooding, despite their aquatic larvae, decreasing population densities of some species up to fifty percent (Hendricks et al. 1995). Compared to unflooded forests, flooded forests can have substantially less subterranean termites (Isoptera: Rhinotermitidae: *Reticulitermes* species), which are important decomposers of wood (Ulyshen 2014).

For plants that are not adapted to periods of extended water logging, flooding can cause hypoxia and mortality. Some plants can counter flooding by growing quickly to escape the rising water, but this can leave them susceptible to herbivores such as galling insects that feed on new growth (Ribeiro et al. 1998). This is in line with the idea that flooding is a stressor and if plants are putting more resources into avoiding the stressor, they have less to devote to defense. While

loblolly pine can grow in a wide variety of soils, very poorly drained or waterlogged areas can produce poor growth results (Baker and Langdon 1990). In fact, loblolly pine seedlings have been shown to be negatively affected by flooding when compared to control plants, including exhibiting reduced stomatal conductance, carbon assimilation, and dry weight (Pezeshki 1992). These results support the idea that flooding is a stressor and may leave plants susceptible to insect herbivores.

1.4.1.5 Frost

Insects' tolerance to frost can be related to their region and range (Addo-Bediako et al. 2000). Often times, insects can overwinter in a variety of forms as either egg, larva, pupa, or adult and can be accomplished by a variety of strategies. Most insects rely on their environment to regulate their body temperatures, and as their hemolymph is water at its base, they can be.subject to freezing (Zachariassen 1985). A sudden or prolonged drop in temperature, especially at the wrong time in an insect's life cycle, can cause mortality in affected individuals.

Insects adapted to regions with extreme temperatures use strategies such as supercooling their own fluids and having some kind of frost resistance in place. Physiologically, this involves regulating concentrations of solutes, and other agents in their hemolymph (Zachariassen 1985; Block 1990). Insects may enter diapause and employ these strategies as winter months approach. Some adapt their life cycles around the winter, overwintering in one stage or another. During this time, it may appear that insect abundance and diversity has decreased. Supporting this is previous work with spruce, *Picea abies* Karst, where barklice (Psocoptera) and aphids (Aphidoidea), in addition to butterflies and moth (Lepidoptera) larvae, dropped sharply from autumn to winter (Jansson and Bromssen 1981). Likewise, the reason some insects can be found in greater abundance in seasons following a particularly mild winter compared to a severe one

can provide evidence for predicting future pest outbreaks (Bale 2002). On the opposite end, there are increasing reports of insects being found farther out of their historical range and breeding longer into the season than expected, showing that colder temperatures not only affect populations in the short term (Crozier 2003; Battisti et al. 2005). Despite insects having temperature limits, warming global temperatures provide for possible range expansions.

1.4.1.6 Fertilization

Fertilizing stands with nutrients is based in the belief that giving extra resources than what's naturally found in the soil can boost the growth of trees, providing a better product at harvest time, or provide an increase in reserves to use in defense against pests and pathogens. Yet, tree growth can be constrained by defenses against herbivores and pathogens, under the theory that plants have only so many resources available at one time and so to commit to one must mean a tradeoff on the other (Herms and Mattson 1992). Stands treated with fertilization were found to have higher resin content in their needles, suggesting a possible ability to have an increased defense to defoliators (Stark 1965), yet phenolic concentrations may actually be higher in unfertilized trees, despite having lower resin concentrations (Bjorkman et al. 1998; Sampedro et al. 2011). Likewise, previous research with ammonium nitrate fertilization gave neutral results with increased nitrogen and resin acid concentrations, leading to sawfly predators being unaffected, suggesting perhaps because the nitrogen increase benefitted them while the resin acid concentration did not (Bjorkman et al. 1990). These results may be supported by looking to other plant species. Studies with maize showed that increasing nitrogen fertilization only resulted in an increase in the number of leafhopper pests (Power 1987) and a meta-analysis done by Koricheva et al. (1998) with woody plants showed that phosphorus fertilization has no effect on carbon- based secondary compound concentrations, such as phenolics and terpenoids.

As a natural defense against bark beetles such as the southern pine beetle, a higher resin

flow can be preferred in areas where there is a high risk. When it comes to loblolly pine, it has been suggested that constitutive oleoresin flow is higher in adverse conditions such as drought, when trees have less resources available, but induced flow is higher during the seasons of greater growth, where resources are plentiful and defense isn't a priority until damage is created (Lombardero et al. 2000). In contrast, other work has suggested fertilization increases constitutive resin flow with younger, growing trees exhibiting a sustained induced defense after wounding, suggesting an acquired resistance may be present (Knebel et al. 2008). Ultimately, these complexities can be environmental and can depend on the nutrient or compound considered, and therefore reports should be considered carefully (Bjorkman et al. 1998; Wright et al. 2010).

Fertilization can have important impacts on potential insect herbivores. In the case of maritime pine, *Pinus pinaster* Aiton, fertilization actually increased damage from the pine weevil *Hylobius abietis* Linnaeus, causing a loss of benefits from the initial fertilization (Zas et al. 2006). Work with other plants has shown that some pests prefer tender shoots which may be more palatable and have increases nutrients due to developing tissues (Krauss et al. 2006). Indeed, rice is one such example that has shown to have an increase in herbivory when nitrogen fertilizers are applied (Lu et al. 2007). However, in regards to pine, it should be noted that resin is considered the primary defense against bark beetles such as *D. frontalis* (Strom et al. 2002) where both constitutive and induced defenses are valued.

1.4.1.7 Semiochemicals

When trees become stressed, such as through a lack of optimal conditions, they release chemicals, such as terpenes, that insects respond and aggregate to. Compounds, including α-pinene, β-pinene, limonene, camphene, α-terpineol, and geraniol, have been shown to attract various bark beetles (Rudinsky 1966). Likewise, these same compounds in different

concentrations and combinations may have the opposite effect, repelling insects (Heikkenen and Hrutfiord 1965). People made use of these chemicals before the advent of technology by using "trap trees" intentionally felled and allowed to attract insects before removing them.

Insects, too, also use chemicals to convey messages between and across species, and can be used to attract mates, attract others regardless of sex, or deter other individuals. When aggregation pheromones were first discovered, such as Brevicomin in the frass of the western pine beetle, *Dendroctonus brevicomis* LeConte (Silverstein et al. 1968), this opened up new avenues for bark beetle control. The idea that these messages can be manufactured and used against them is a driver of such research. Dozens of compounds have been isolated and have been successful in lures and other tools to monitor and manage insect populations (Witzgall et al. 2010). Regardless if it's from a tree or insect, lures can allow for the confirmation of an insect's presence in an area, can tell the start of a flight period, or can attract individuals only to have them captured or killed by insecticide. Deterrents discourage insects from attacking hosts in that area or confuse one sex from finding members of the opposite sex. These uses for mass trapping, "lure and kill," and mating disruption have even been applied to the eradication of invasive species, but are usually most effective when populations are small or newly emerging (El-Sayed et al. 2006).

In the example of southern pine beetle, there are several semiochemicals that have been used mostly to survey and monitor for outbreaks as they are not yet ready for prevention in wide scale areas due to a number of factors (Strom and Clarke 2011). Like tree stress chemicals, these insect semiochemicals may have varying responses based on environmental factors, concentrations, and combinations (Heikkenen and Hrutford 1965; Strom and Clarke 2011). Current market lures can vary in these concentrations, purities, and release rates, which combined with ageing in the field can produced mixed results (Arn et al.1997). Regardless,

insects captured this way can provide invaluable data about ranges, flight patterns, and infestation rates while being target specific. Pheromone traps used for gypsy moth, *Lymantria dispar* Linnaeus, are invaluable when population densities are low and the coverage area is large, allowing for early intervention and monitoring of population trends (Elkinton and Carde 1981).

1.4.2 Biotic

1.4.2.1 Tree Defenses Against Insects

Sometimes, human interference is not required to deal with insect outbreaks in commercial forests. Outbreaks may be prevented by natural tree defenses before a problem is even detected. Tree bark represents an initial physical defense, and its effectiveness depends on a combination of the species involved as well as biotic and abiotic factors that influence the insects present. After bark, trees can also use chemicals for defense, and just as there are stress chemicals that attract insects, these chemicals can act as repellants (Hanover 1975). The ratio of compounds may also play a part in the difference between attracting and repelling insects. For example, β-pinene has been shown to repel the Douglas fir beetle, *Dendroctonus pseudotsugae* Hopkins, but α-pinene attracts it, and so susceptibility and response depends on the concentrations in tree tissue (Heikkenen and Hrutfiord 1965). Pressure within the tree and sap may also play a role (Hanover 1975).

If insects do succeed at invading tree bark and tissues, pine trees in particular can utilize resin, especially oleoresins, as a means of pushing invaders out (Hanover 1975; Strom et al. 2002). Indeed, it is only when these defenses fail and insects become pests that further controls are considered. Because tree defenses are the preferred mode of management for outbreak prevention, there has been substantial effort put into tree genetics and breeding, combining knowledge gained from physiologists, entomologists, and plant pathologists (Painter 1958; Hanover 1975). This reasoning rests in the belief that a good foundation can prevent future costs

on management and control of pests.

1.4.2.2 Disease Outbreak

Increased dead and dying material in forests can provide habitat material for increasing populations of insects. Species from *Pissodes*, *Hylobius*, *Ips*, and *Pachylobius* are found associated with woody debris, but ultimately, moisture, size, and temperature of the material all affect insects and their ability to use it (Hanula 1993). In the case of disease outbreak, trees can be increasingly stressed – attracting insects that favor this – or die, providing ready material to these insects. Footholds like these can allow for species to grow their populations up large enough to result in outbreaks, as is the case with the infamous *D. frontalis*. In the cases of diseases vectored by insects where mortality is unequal or only certain age classes are affected, outbreaks can occur as tree cohorts age and the disease takes hold once more. An example of this is with beech bark disease, the fungus *Neonectria coccinea var. faginata* (Pers.:Fr.) Fr. Var. Lohman, A.M. Watson, & Ayers vectored by the scale *Cryptococcus fagisuga* in Maine, where mature trees were reduced to resprouting thickets that are susceptible to the disease and can provide more material for insects (Houston 1975). Another example of an invasive disease outbreak is chestnut blight, caused by the fungus *Cryphonectria parasitica* (Murrill) Barr, and while not vectored by an insect, trees are still reduced to resprouting stumps that provide tender shoots and stressed wood for herbivorous insects. Certainly, the transmission and success of these disease-causing fungi allow for their associated insects to grow and thrive.

Felled trees, one possible result of a disease outbreak, also can provide breeding grounds and food to insects. In pines, the first eight weeks of felling seems to be the most important time for bark and ambrosia beetle colonization, where beetle populations drop off sharply after that (Flechtmann et al. 1999). This is evidence to support the value of dying and recently dead wood to these species, where competition is high as succession continues. In felled loblolly and slash

pines in Louisiana, *Ips* beetles were allowed to colonize trees and it was revealed that predators and parasites of these insects decreased *Ips* survival by 30.8% while their own populations grew three fold (Riley and Goyer 1986). Felled trees left in the area can increase insect diversity compared to only leftover stumps, allowing for dozens more species over the course of four years (Jonsell and Weslien 2003).

1.4.2.3 Animal Damage and Competitors

Trees damaged by animals provide wounds that attract herbivorous insects, as trees release semiochemicals such as terpenes (Rudinsky 1966; Hanover 1975). Some animals use this fact to their advantage, as in the case of sapsuckers, *Sphyrapicus* species, which initially drill holes into certain trees to feed on sap, but also feed on the resulting insects that are attracted to the wounds (Zobrist 2014). Other animals also damage and kill trees, including rodents such as squirrels, through feeding and gnawing behaviors, creating entry points for fungi and insects (Sandro 2008). Girdling in this way can fatally wound trees, resulting in dead material that can attract new insect pests. Invasive species, too, add to the list of animals that can injure trees. Non-native feral hogs disturb and chew roots, causing wounds and may introduce fungi which are both stressors that can attract insects (Eckhardt et al. 2016).

As insects don't all prefer the same food source, they cannot always be found in the same area. Even bark beetles that may attack the same species of tree don't attack the same parts of the tree, instead fitting into their own niche, with some possible overlap (Payne et al. 1991). Beetle competition inside a tree extends to the same species as well as others. Individuals use olfactory senses to perceive where other individuals are, and even once inside the tree, use these senses to avoid intersecting competitors' galleries (Byres 1989). These natural competitions regulate each other's populations.

1.5 Identification of Insects

1.5.1 Morphospecies

Diversity measurements of insects have traditionally revolved around morphological or molecular taxonomy to family level, as further identification of many insects remains questionable and time consuming. This, coupled with morphospecies – identifying an insect as a species based on morphological characteristics – can give estimates of diversities per family (Ulyshen and Hanula 2009). Studies have shown that "morphospecies" are nearly as effective at measuring species diversity in the place of species when morphological differences are present (Kremen et al. 1993; Oliver and Beattie 1996; Barratt et al. 2003). Some limits to measuring diversity should be noted, however, as morphospecies vary largely depending on the invertebrate group and family (Derraik et al. 2002; Krell 2004).

1.5.2 Microscopy of Insects

Microscopy work involving insects has looked at the nano-structures of insect wings and given evidence towards the support of function or looked at structure and how it pertains to coloration (Wagner et al. 1996; Watson and Watson 2004; Seago et al. 2009). Others looked at the coloration in scarab beetles (Coleoptera: Scarabaeoidae) and its correlation with wavelength (Arwin et al. 2014) and structure (Del Río et al. 2014). One study by Vukusic et al. (1999) looked at structure, its effect on coloration, and how it pertained to intra-species interactions.

More specific work involving insects and work with fungal spores has supported identifying fungal-specific areas, such as the pronotum and elytra, on economically important insects such as bark beetles (Livingston and Berryman 1972; Furniss et al. 1990; Lewinsohn et al. 1994). These areas of collection on bark beetles, called mycangia, can be small pits on the bodies of these insects and this feature has evolved multiple times in the Scolytinae lineage (Six 2012). Mycangia can be pit or sac shaped, and are mostly on the exterior of the beetle, though some

Scolytinae beetles may have them in their oral cavities (Batra 1963). These pits can have a wax-resembling lining that can facilitate storage and transport (Livingston and Berryman 1972) and in some species, the fungal cells grow and multiply, nourished by secretions or adjacent cells and protected from desiccation as long as the beetle is alive (Batra 1963). Mycangia such as these may be focused on where spore identification is concerned. It should, however, be noted that mycangia are not always species specific with regards to fungi, and plant pathogens may also ride along (Batra 1963).

1.5.2.1 Hyperspectral Microscopy

Hyperspectral interferometry has had a previous focus in medical research (Ferris et al. 2001; Fox et al. 2006; More et al. 2016), whereas insects are underappreciated. Widjanarko et al. (2012) used this method to look at rough surfaces, but not of insects. Agricultural research has used hyperspectral imaging to look at detecting damage to crops as affected by insects and fungi, particularly of wheat and maize kernels (Singh et al. 2009; Del Fiore et al. 2010) and as an evaluation tool of fruit quality in soybeans (Monteiro et al. 2007; Huang et al. 2013). It has even been used in astronomical surveillance (Hege et al. 2003) and in pharmaceutical drug quality evaluation (Lyon et al. 2002; Rodionova et al. 2005).

Beach et al. (2015) described a method of observing spores on the bodies of bark beetles and hypothesized that hyperspectral interferometry could be used to determine the thickness and size of observed spores. A more refined method of this type could possibly yield spore loads on insects and provide information relating to those. This has relevance for forestry research, where fungal tree infection can result in tree damage that negatively affects the forest industry (Eckhardt et al. 2004a). Currently, spore loads and identification are determined by collecting insects and using molecular techniques such as polymerase chain reaction or DNA fingerprinting (Six et al. 2003; Schweigkofler et al. 2005).

Though Beach et al. (2015) used dead insects to observe spores, it has been shown that small molecules can give unique spectral signatures and that similar signatures can be obtained from both in vivo and in vitro systems (More and Vince 2015; More et al. 2016). This gives support to the hypothesis that imaging results of fungal spores both on and off insects should be similar.

1.6 The Role of Insect Diversity

Comprising most of the world's known organisms, it should come as no surprise that insects are considered excellent candidate indicators of ecosystem function, supported by their species richness, behavioral diversity, and responsiveness to disturbance (McGeoch 2007). Aquatic insects, especially larvae of the orders Trichoptera, Ephemeroptera, and Plecoptera, are sensitive to changes in water parameters, and as such, have been used to satisfy criteria as biological indicators of water quality (Phillips 1980), providing scientists a way to measure the effects of trace elements (Nehring 1976; Burrows and Whitton 1983; Cain et al. 1992) and non-point source pollutants such as fine inorganic sediment (Relyea et al. 2000). Likewise, many terrestrial insects have been shown to serve as indicators of anthropogenic and habitat disturbance, such as ground beetles (Humphrey et al. 1999; Allegro and Sciaky 2003; Rainio and Niemela 2003; Avgin and Luff 2010). Rove beetles (Coleoptera: Staphylinidae)(Bohac 1999) and hover flies (Diptera: Syrphidae)(Sommaggio 1999; Sueyoshi et al. 2003; Maleque et al. 2009) both have been suggested given their diversity, abundant numbers, wide environmental requirements, and known taxonomy. As an alternative to Lepidoptera and birds, tiger beetles (Coleoptera: Cicindelidae) may provide estimates of species richness in a matter of hours instead of months or years (Pearson and Cassola 1992).

With their intrinsic ties to pollination, insects such as honeybees have been shown to be crucial indicators of sustainability and pollution (Kevan 1999). Lepidoptera are affected by forest fragmentation and thinning, given their strong association with understory cover (Barlow et al.

2008; Van Halder et al. 2008; Maleque et al. 2009). Dung beetles (Scarabaeidae: Scarabaeinae) provide valuable decomposition and nutrient recycling services and have been used as bioindicators in research involving forest fragmentation (Estrada and Coates-Estrada 2002; Feer and Hingrat 2005; Maleque et al. 2009), especially due to clear cutting and logging activities (Nichols et al. 2007). Important wood decomposers in forests, Cerambycidae beetles can also serve as pollinators, are affected by understory cover and thinning (Maleque et al. 2009), and therefore have been suggested as excellent indicators of biodiversity (Maeto et al. 2002; Holland 2007).

Given their ubiquity, it's no surprise ants (Hymenotpera: Formicidae) are also a popular choice for indicators, and Maleque et al. (2009) gave evidence supporting their role in anthropogenic disturbance in managed forest plantations at the local scale. Work in Arizona plantations that were burned and thinned revealed ant groups varied with disturbance levels, suggesting that habitat diversity was necessary to maintain diverse communities and to facilitate forest health (Stephens and Wagner 2006). Like similar insects before them, ants have also been shown to be negatively affected by fragmentation and logging activities (Carvalho and Vasconcelos 1999), an effect that persisted for years afterward (Vasconcelos et al. 2001). Converted forest can have decreased species richness, where generalists dominate new commercial lands (Maeto and Sato 2004; Sinclair and New 2004). Given these effects, Underwood and Fisher (2006) concluded from 60 published studies that ants are important in management-based monitoring in regards to detecting invasive species, monitoring endangered, threatened or keystone species, evaluating management actions, and for assessing long term ecosystem changes, making them prime candidates for evaluation when it comes to conservation.

Being closely tied with plant species, insect communities can be affected by decreasing species richness, invasions by non-native species, and spreading fungal diseases (Knops et al.

1999). Indeed, in a study with maize, leafhopper pests' populations and movement decreased with increasing plant diversity, resulting in a corresponding decrease on the spread of disease (Power 1987). In another, a field experiment with increasing plant species richness showed a comparable increase to insect richness (Haddad et al. 2001), and Murdoch et al. (1972) showed a similar result when they compared their overall study area. Even increasing genetics in an area by planting different cultivars can provide a barrier to pathogen spread and infection (Cox et al. 2004) and increase beneficial predaceous insects (Johnson et al. 2006). Ratnadass et al. (2012) explained this effect through a variety of possible mechanisms, including dilution of resources, spatial disruption, temporal disruption, allelopathic effects, resistance from physiology, suppression brought about by soil, physical barriers from plant architecture, and conservation and utilization of natural enemies.

1.6.1 Measures of Diversity

The book, *Biodiversity*, under editorship of E.O. Wilson introduced the concept of "biodiversity" to the world (Magurran 2004), and later on, the United Nations Environment Programme defined biological diversity as "the variability among living organisms from all sources" and including "within species, between species and of ecosystems" (Heywood 1995). Specifying this even further, biodiversity can be identical to species richness and relative species abundance in one point in time (Hubbell 2001). Magurran (2004) defined the species as the basic unit of diversity, equating the number of species to species richness while also stressing the importance of abundance. In addition to this, a similar term – ecological diversity – can be defined as the variety and richness of ecological communities (Pielou 1975) and could traditionally be measured by a diversity index (Magurran 1988). Today, biological diversity and ecological diversity are often used interchangeably with the first taking popularity over the other (Harper and Hawksworth 1995; Magurran 2004).

When considering biological diversity, it is important to consider two components, both of species evenness and richness (Simpson 1949). While species richness can be defined as the number of species, evenness refers to the variability in species abundances (Magurran 2004). A community of the highest evenness would have species with equal numbers of individuals. Blending both concepts is a reflectance of a diversity index, yet the weighting placed on each can influence results greatly (Magurran 2004). Morris et al. (2014) described indices as describing characters of communities in an effort to compare different groups, making them important tools in the fields of conservation and environmental monitoring.

Perhaps the most common diversity index used is the Shannon index, or sometimes known as Shannon-Weiner. This index arise out of the belief that individuals are randomly sampled from a large, infinite community and that all species are included (Pielou 1975). Given that all species are rarely so equal in occurrences and populations, this can lead to bias. Shannon's index is calculated with:

$$H' = - \Sigma p_i \ln p_i$$

Here, p_i is the proportion of individuals sampled from the *i*th species. Usually, this value comes out between 1.5 and 3.5 and rarely 4, where a higher value is a result of a very large number of species. Despite this index taking evenness of species abundances into account, this narrow range of values can make it difficult to compare diversities between samples. Despite its disadvantages, the long tradition of use has ensured this index's popularity. More than one study has used this index to evaluate insect diversity in plantation forests (Humphrey et al. 1999; Allegro and Sciaky 2003) or to compare differently aged loblolly pine plots (Lee et al. 2018).

A second diversity index – referred to as Simpson's – is another commonly used and describes the probability of any two, randomly sampled individuals being a part of the same species (Simpson 1949). Simpson's index is calculated as D where:

$$D = \Sigma p_i^2$$

Here, p_i once again refers to the proportion of individuals in the *i*th species. Increasing diversity results in a lower value of *D*. Therefore, this index is often represented as $1 - D$ or $1/D$, so that as this value increases, the assemblage becomes more even (Magurran 2004). Simpson's is less sensitive to species richness, and instead emphasizes dominance, giving more weight towards heavily abundant species. This index is considered a robust measure of the variance of species abundance distribution and a more intuitive option than Shannon's (Magurran 2004). Allowing for better comparisons of communities, previous studies in commercial forests have evaluated butterfly diversity along habitat boundaries (Lucey and Hill 2012), to compare natural and recently logged areas (Hamer et al. 2003) and to evaluate diversity among timber-quality and non-quality stands (Lencinas et al. 2008).

In addition to diversity indices, measures of similarity and complementarity have been devised to describe a pair of samples or sites (Magurran 2004). With many of the goals centered in conservation and environmental planning, these can be used to select the two sample areas best at preserving the most number of species. Better known as β diversity, complementarity increases with higher diversity (Magurran 2004). An intuitive way to describe this value is to use a similarity/dissimilarity coefficient. A popular similarity one is the Sørensen index, defined as:

$$C_s = 2a / (2a + b + c)$$

Here, *a* is the total number of species in both samples, *b* is the number of species in the first sample, and *c* is the number of species in the second sample. With a resulting value between 0 and 1, signifying no and complete similarity, respectively, this qualitative measure is intuitive. However, while easy to calculate, a disadvantage lies in the lack of weight in regards to the relative abundance of species. Therefore, a dominant species will be accounted for in the same way of rare one. Novotny et al. (2002) used this coefficient to show that out of nine-hundred

herbivorous insects in a New Guinea rainforest, the majority are feeding only on a handful of related plant species, the results of which affect a global estimate of arthropod diversity. Likewise, another study confirmed five-hundred herbivorous species against their host species showed species had a wide distribution (Novotny et al. 2007). New Zealand pine stands showed high similarity among differently aged plots (Hutcheson and Jones 1999) while another study in Turkey showed that pine plantations had less diversity similarity when compared to natural areas (Tecimen et al. 2017).

While species richness seems like a simple concept to define – as the number of species of a given taxon – this term is compounded by the problem that a species is not a universal term. Conflicting views of what constitutes a species abound and species estimates can therefore become inflated or depressed (Magurran 2004). Given that many species are yet to be described, the idea of morphospecies – identification based on distinguishing morphological differences – provides a heuristic solution to unknown species names. Therefore, morphospecies can be considered a faster equivalent to species in regards to richness estimates (Kremen et al. 1993; Oliver and Beattie 1996; Barratt et al. 2003; Magurran 2004).

To estimate species richness in an area, scientists often take a series of samples, either as quadrants, plots, or traps. The rate at which species are added to the overall assemblage provides information about species richness and distribution, and can be plotted in the form of a species accumulation curve (Magurran 2004). Colwell and Coddington (1994) defined these as cumulative species plotted against a function of sampling effort, where effort could be the total number of individuals collected, the total number of samples, or sampling time. Such curves that look at species over area are popular in botanical research (Williams et al. 2005; Williams et al. 2007). A curve's shape can be influenced by the order of samples included, however, which is why randomization is done to smooth the curve by randomly adding samples a specified number

of times before taking the average (Magurran 2004). However, curves do not give direct estimates of species richness without exhaustive sampling; extrapolating past the sample size is one solution (Colwell and Coddington 1994; Magurran 2004). At least in terms of botanical species, given this lack of direct estimation, sampling may be considered sufficient if the rate of species per unit drops below two (Williams et al. 2007). As a disadvantage, it should be noted that in small to moderate sample sizes, especially in cases of disturbance, curves may prove to be inadequate when it comes to ranking communities. Therefore, some recommend the Simpson index as a better tool for this, especially when quick assessments are needed for conservation, and recommend using it along with species richness when comparing communities (Lande et al. 2000).

Chapter 2

Insect Diversity in a Loblolly Pine Stand Infected with *Leptographium terebrantis*

2.1 Abstract

Ophiostomatoid fungi, such as *Leptographium terebrantis*, are vectored by root-feeding bark beetles and cause sap staining, reduced growth, and vascular occlusion in tree tissues they infect. To assess the impact this fungus has on insect diversity, especially root-feeding bark beetles and other insects of concern, we utilized pitfall and panel insect traps to sample these invertebrates in order to capture the widest variety possible. While undoubtedly a pathogen and stressor to trees, it is necessary to understand how it affects overall insect populations to make informed decisions regarding management. Of an initial two year study in a commercial loblolly pine stand, insect diversity varied between years. Pitfall traps captured more Hylobiini, while panel traps were more efficient at capturing ambrosia beetles. Despite this, average morphospecies capture, along with Shannon and Simpson indices, showed consistent diversity and species evenness across trap type. Panel traps were important in capturing different species along with consistent capture rates. Despite *L. terebrantis* inoculum, disparity of annual insect populations during the sampling period seemed due to seasonal variation.

2.2. Introduction

With a major stake in the southeastern United States' economy, loblolly pine is the tree staple due to its hardy nature and fast growth (Schultz 1997). However, despite this, it is not immune to pests and pathogens. While insects are innumerably beneficial, providing critical ecosystem services such as food, pollination, decomposition and nutrient recycling (Edmonds

and Eglitis 1989; Ulyshen 2016), pest insects interfere with human desires for wood products, reducing wood quality through their habits, introducing pathogens, and impacting tree growth.

In forests, insects play host to microbes and fungi, acting as vectors and contributing to disease cycles (Manion 1981). Fungal species can alter tree tissue's nutrient content (Baker and Norris 1968; Beaver et al. 1989; Ayres et al. 2000), increase the insect's reproductive capacity (Eckhardt et al. 2004a), or interfere with insect development (Klepzig and Hofstetter 2011). Already, when insects attack trees, they cause stress, affecting physiological processes – such as growth and defense – and reallocation of resources to those purposes (Christiansen et al. 1987). Weakened trees are further susceptible to more pests through the stress chemicals they release (Rudinsky 1966) and to disease (Manion 1981).

Root feeding bark beetles in particular vector ophiostomatoid fungi that stain wood and decrease tree growth in addition to their feeding habits that create galleries in wood and girdle trees (Repe and Jurc 2010). Beetles such as these include *Hylastes salebrosus* and *Hylobius pales*, and have been found associated with declining pine trees, carrying fungi such as *Leptographium* and *Grosmannia* (Klepzig et al. 1991; Jacobs and Wingfield 2001; Eckhardt et al. 2007; Matusick et al. 2013). Rane and Tattar (1987) showed that the infamous black turpentine beetle, *Dendroctonus terebrans*, was capable of picking up ophiostomatoid fungi by tunneling through infected tissues, further perpetuating the cycle.

As one such ophiostomatoid fungi in the southeastern United States, *Leptographium terebrantis* has been found to result in root damage and resin-filled lesions in the xylem, resulting in occlusion (Raffa and Smalley 1988; Matusick et al. 2012). Several species of pines – including shortleaf, slash, loblolly, longleaf, and red – have shown an association of decline with this fungus (Klepzig et al. 1991; Harrington and Cobb 1983; Jacobs and Wingfield 2001). With

this study we were able to observe how increasing inoculum levels of this fungus affected insect diversity.

Biodiversity has been shown to be an instrumental factor in the invasion of non-native species in an area and enhances the spread of fungal diseases (Knops et al. 1999). Decreased insect species often corresponds to a decrease in plant diversity (Murdoch et al. 1972; Haddad et al. 2001), which in turn can increase pathogen infection (Ratnadass et al. 2012). Monocultures enriched with other plant species or genetic variants has potential to reduce diseases and pests though the increase of barriers and predatory and parasitic insects (Power 1987; Johnson et al. 2006; Ratnadass et al. 2012). Knowledge such as this is critical to developing management tools. This study was initiated to determine the impact *L. terebrantis* has on insect populations in association with varying inoculum levels and to test the hypothesis that root-feeding bark beetle pests increase with increasing inoculum.

2.3 Methods and Materials

2.3.1 Study Site and Plot Measurements

Fifteen plots were established on a loblolly pine, *Pinus taeda*, stand privately owned by a member of the Forest Health Cooperative (Auburn University, AL) in Eufaula, Alabama (Fig. 2.1). Prior to the setup, the land had undergone a third row thin, allowing for paired lined plots and double randomization. One line had five randomly chosen control trees and the second had another five chosen as treatment trees. These two lines together consisted of a single experimental plot and altogether all fifteen made up a split plot completely randomized design with three replicates per treatment (Fig. 2.2).

Figure 2.1. Location of study site in Eufaula, AL in relation to Auburn, AL.

Trees at the time of selection were aged to be between twelve and thirteen years of age. Soil in the study area was identified mainly as Annemaine-Wahee complex (AwA) and minorly as Ocilla loamy fine sand (OcA)(Fig. 2.2).

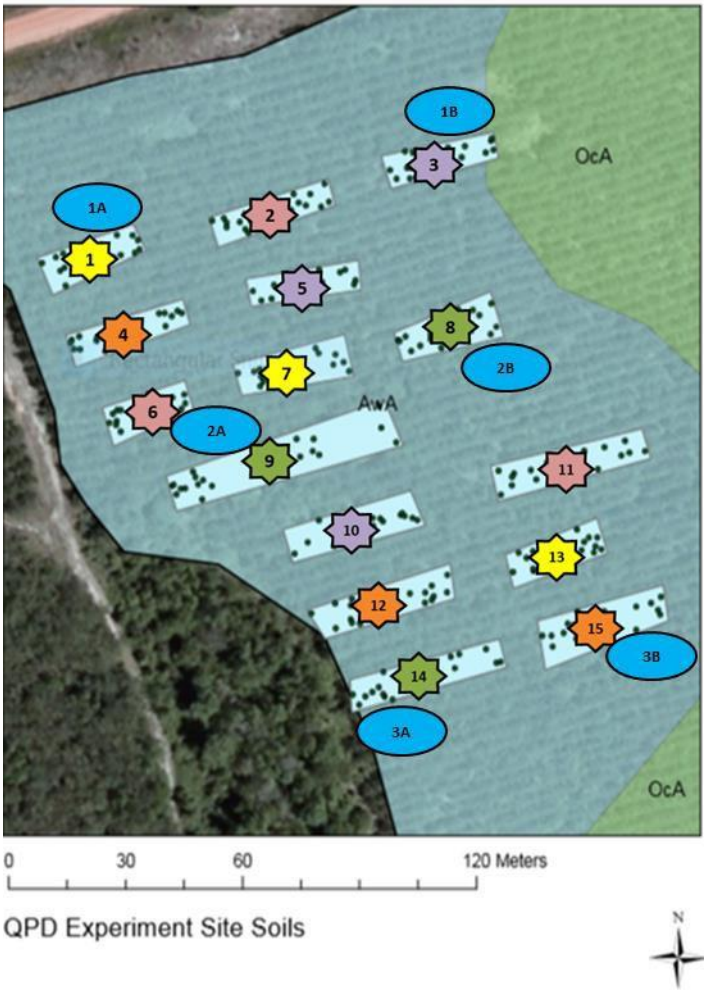

Figure 2.2. Fifteen plots of the study site in Eufaula, AL, with stars corresponding to plots, denoted by number, and ovals demonstrating the location of panel traps and their associated label. Treatment levels were assigned by color where green is control, orange is low, yellow is medium, high is pink, and wound is purple.

2.3.2 Insect Trapping

To monitor changes in insect populations over time, three different traps of two types – pitfall and panel – were used. Panel traps (Forestry Distributing Inc., Boulder, CO) (Fig. 2.3A) were made of black corrugated plastic and Lindgren funnels hung on metal poles and were designed to capture flying insects. Panel traps were installed on poles one meter above the ground and had a plastic cup attached to the bottom that contained a 2:1 mixture of water and antifreeze (Super Tech antifreeze, Bentonville, AR) to preserve captured insects.

Pitfall traps consisted of two types. One consisted of a 20-cm length of a 10-cm diameter polyvinyl chloride plastic pipe with eight holes spaced equally around the circumference

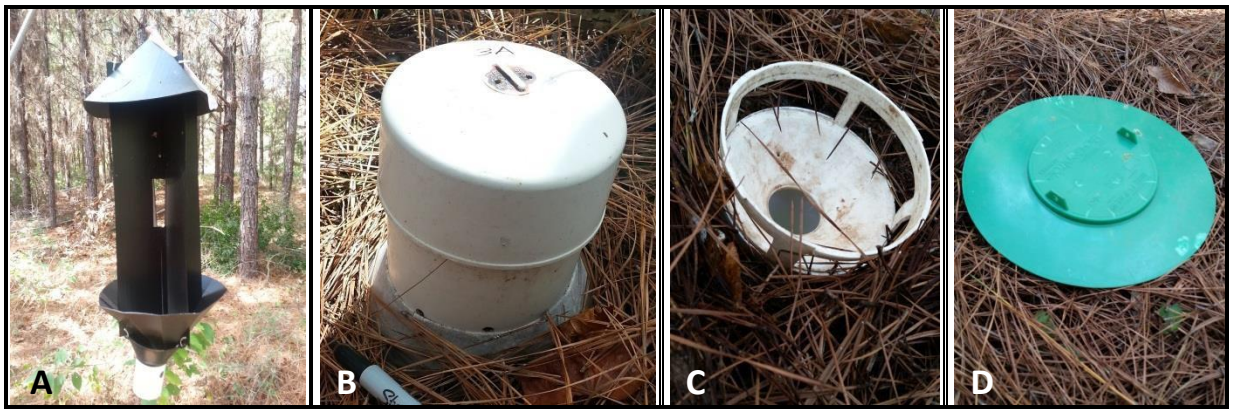

Figure 2.3. (A) Panel trap, (B) pitfall trap to be baited with twigs, and pitfall trap without top (C) and pitfall trap with top (D).

(Klepzig et al. 1991) (Fig. 2.3B). Both ends of the pipe had caps that served as removable lids, with holes drilled into the bottom cap for drainage. Traps were buried into the soil and leaf litter until the entrance holes lined up at ground level. These traps' interiors were coated with a layer of liquid Teflon^{TM} (Northern Products Woonsockets, RI) to prevent insects from climbing out once inside. Each of these pitfall traps were baited with two 3 cm long by 1 cm diameter loblolly pine twigs placed in the bottom of the interior to provide refuge for captured bark beetles.

The second type of pitfall trap (15 cm diameter funnel-type traps, Multi-pher; Bio-Controle, Quebec City, Quebec) (Fig. 2.3C and D) allowed for the entry of larger ground based insects and each contained a 16 oz SOLO plastic cup filled with 50 mL of a 2:1 mixture of water and antifreeze (Super Tech antifreeze, Bentonville, AR) to preserve insects that fell inside. All types of insect traps had cups refilled and twigs replaced every two weeks during the collection period.

Insect collections were taken up every two weeks from February 2016 to March 2018. Each of the fifteen plots held two of the pitfall traps baited with twigs and one pitfall trap with an antifreeze mixture. Panel traps were placed with two on each of the sides and middle of the study area. Both twig-based pitfall traps and panel traps were established in 2016, one year prior to tree

inoculation while antifreeze-based pitfalls were established February 2017 before inoculation. During collection periods, insects caught were placed in polyethylene cups and transported to the Forest Health Dynamics Laboratory at Auburn University (Auburn, AL, USA), placed in the cooler until time for processing, sorted, and identified.

2.3.3 Insect Identification

Specimens captured were identified to family with taxonomic keys (Triplehorn and Johnson 2005) where possible, given a designated morphospecies name according to Ulyshen and Hanula (2009), and curated at the Forest Health Dynamics Laboratory at Auburn University. A voucher collection was also compiled for Louisiana State University. Species of concern were catalogued (Table 2.1) and also identified with taxonomic keys (Wood 1982).

Table 2.1. Species of concern captured and sorted by taxonomy.

Taxonomy Classification	Species Collected
Hylastes	*Hylastes salebrosus*
Hylobiini	*Hylobius pales, Pissodes nemorensis*
Ips	*Ips avulsus*
Ambrosia	*Xyleborus pubescens, Xyleborus ferrugineus, Orthotomicus caelatus, Xyleborinus saxeseni, Xylosandrus crassiusculus, Xylosandrus germanus, Monarthrum fasciatum, Gnathotrichus materiarius, Xyleborus affinis*

2.3.4 Tree Inoculation

Tree inoculations took place in February of 2017. The fungal isolate of *L. terebrantis* was retrieved from the Forest Health Cooperative Laboratory at Auburn University after determining maximum virulence (Devkota et al. 2018b). Root-feeding beetles and weevils often inoculate the roots and lower bole of a tree with fungi, and previous studies showed that inoculation points utilizing the lower stem can have a similar pathogenicity (Matusick et al. 2016). Of the treatment

plots (Fig. 2.2), inoculation procedures were done in a manner similar to Devkota et al. (2018a). Toothpicks used for the experiment were previously sterilized at 121 ºC for sixty minutes, and fungal colonization was allowed for twenty-one days at 25 ºC.

Treatment plots were designated three each as either control, wound, low, medium, or high. Control trees were untouched and wound trees had inoculum-free toothpicks inserted to simulate infection. Treatment corresponded to toothpick density inserted. Low, medium, high, and wound each had inoculations of two, eight, sixteen, and sixteen, respectively, per 6.35 cm of diameter at breast height (DBH)(Fig. 2.2).

2.4 Statistical Analysis

Species richness in the form of total number of morphospecies was conducted for twig- and antifreeze-based pitfalls with a repeated measures analysis of variance (ANOVA). The dependent variable was morphospecies while the independent variables were treatment and year. Compound symmetry (CS) was used for the covariance structure. These statistical analyses were run with the program SAS (SAS 9.4, 2013). Indices for Shannon and Simpson, Sørensen's coefficient, and species accumulation curves, were ran with the EstimateS program (EstimateS, 9.1.0, 2013; Colwell 2013) and Excel (2016). Transformed values for Shannon and Simpson were computed and repeated measures ANOVA ran with SAS (SAS 9.4, 2013).

2.5 Results

A total of 9,748 insects divided into 16 orders, 149 families, and 676 different morphospecies were collected over the duration of this study (Table 2.2). Orders with the most to the least number of morphospecies were as listed: Coleoptera, 299; Diptera, 130; Hymenoptera, 120; Hemiptera, 47; Lepidoptera, 22; Collembola, 19; Trichoptera, 12; Psocodea, 11; Orthoptera, 4; Blattodea, 3; Neuroptera, 3; Microcoryphia, 1; Mecoptera, 1; Mantodea, 1; Phasmida, 1;

Table 2.2. Totals of insect families collected over the entire two-year sampling period (25 months). Of 9,748 total insects collected in 16 orders, there were 676 morphospecies in 149 families.

Coleoptera		Oedemeridae	1	Reduviidae	4	Psocodea	1	Hybotidae	1	Encyrtidae	1
Cerylonidae	1	Silvanidae	4	Miridae	1	Psocoptera	8	Tipulidae	1	Colletidae	1
Ciidae	3	Tenebrionidae	12	Nabidae	1	**Diptera**		UNK Diptera	70	Megaspilidae	1
Sphindidae	2	Zopheridae	2	Cixiidae	1	Bibionidae	1	**Phasmida**		Scelionidae	4
Byrrhidae	1	Trogossitidae	1	Psylloidea	1	Cecidomyiidae	6	Pseudophasmatidae	1	Chrysididae	1
Rhysodidae	1	Throscidae	1	Plataspidae	1	Chironomidae	4	**Thysanoptera**		Pompilidae	7
Carabidae	20	Phalacridae	1	Rhyparochromidae	1	Dryomyzidae	1	Thysanoptera	1	Platygastridae	1
Cerambycidae	2	Cleridae	1	Thyreocoridae	1	Simuliidae	1	**Microcoryphia**		Proctotrupidae	3
Colydidae	2	Endomychidae	5	Tingidae	1	Tabanidae	1	Machilidae	1	Halictidae	1
Curculionidae	41	Derodontidae	1	Pentatomidae	2	Sciomyzidae	1	**Mecoptera**		Vespidae	6
Cryptophagidae	4	Dytiscidae	2	Aphididae	1	Anthomyiidae	1	Mecoptera	1	UNK Hymenoptera	18
Tetratomidae	1	Coccinellidae	1	Issidae	1	Scathophagidae	1	**Mantodea**		**Collembola**	
Chrysomelidae	5	Catharidae	1	Largidae	2	Sphaeroceridae	4	Mantidae	1	Smithuridae	1
Dermestidae	4	Nitidulidae	5	Ceratocombidae	1	Tachnidae	1	**Hymenoptera**		Isotomidae	7
Elateridae	18	Anobiidae	1	UNK Hemiptera	7	Fannidae	2	Apidae	3	UNK Collembola	11
Eucinetidae	1	Leiodidae	5	**Orthoptera**		Dolichopodidae	3	Bethylidae	4	**Lepidoptera**	
Melandryidae	1	Hydrophilidae	1	Gryllidae	1	Sciaridae	6	Chalcoidea	1	Geometridae	1
Histeridae	7	Ptilodactylidae	1	Gryllinae	1	Empididae	1	Chalcidoidea	4	UNK Lepidoptera	21
Geotrupidae	3	Ptinidae	1	Rhaphidophoridae	1	Chloropidae	1	Diapriidae	6	**Blattodea**	
Scarabaeidae	6	Anthicidae	1	UNK Orthoptera	1	Drosophilidae	4	Braconidae	6	Rhinotermitidae	1
Scarabaeoidae	15	Latridiidae	4	**Trichoptera**		Phoridae	6	Formicidae	32	Blatellidae	2
Scirtidae	2	Lycidae	2	Psychomyiidae	2	Syrphidae	1	Sircidae	1	**Neuroptera**	
Silphidae	2	UNK Coleoptera	45	Calamoceratidae	2	Asilidae	3	Sphecidae	4	Hemerobiidae	1
Staphylinidae	49	**Hemiptera**		Hydroptilidae	1	Sarcophagidae	1	Evaniidae	1	Chrysopidae	2
Erotylidae	1	Achilidae	1	UNK Trichoptera	7	Culicidae	5	Scoliidae	1	**UNKNOWN**	
Passandridae	1	Aradidae	4	**Psocodea**		Chaoboridae	1	Crabronidae	4	UNK	1
Laemophloeidae	1	Cicadellidae	14	Lepidopsocidae	1	Mycetophilidae	1	Ichneumonidae	3		
Mordellidae	6	Cercopidae	2	Psocidae	1	Elasminae	1	Mutilidae	6		

*UNK stands for unknown family.

Thysanoptera, 1; and unknown, 1.

Diversity was described in the form of species richness, defined as the number of morphospecies, the indices of Shannon and Simpson, and Sørensen's coefficient where applicable. Morphospecies captures were averaged for twig-based pitfalls and separated by year (Fig. 2.4), but species richness was only significant between years ($p \leq 0.0001$; Table 2.3). Similarly, antifreeze-based pitfall captures were averaged for the one year they were out (Fig. 2.5). However, results on total morphospecies were marginally significant among treatments ($p=0.0632$; Table 2.4). Simpson and Shannon indices for antifreeze-based pitfalls were calculated for pre- (Table 2.5) and post-inoculation periods (Table 2.6) by season and treatment, yet these transformed values were not significant among treatment for the year they were in place (Shannon, $p=0.0918$; Simpson, $p=0.2057$).

Averaged morphospecies for panel traps (Fig. 2.6) were also separated by year and totals were significant between years ($p=<0.0001$; Table 2.7). In the interest of comparing trends, separating total morphospecies caught per trap for year 1 (Fig. 2.7) and year 2 (Fig. 2.8) revealed seasonal variation. Shannon and Simpson index values for each panel trap were averaged according to season and for pre- (Table 2.8) and post-inoculation periods (Table 2.9) and transformed values supported initial significant results between years (Table 2.12; Table 2.13). Sørensen values were also averaged by season, showing – despite similar capture totals – most traps had less than 50% similarity of morphospecies for pre- (Table 2.10) and post-inoculation (Table 2.11). The three orders of insects that made up over 80% of all the morphospecies documented – Coleoptera, Diptera, and Hymenoptera – were also separated by year in panel traps, revealing trends (Fig. 2.9, 2.10, 2.11) similar to the overall total (Fig. 2.6).

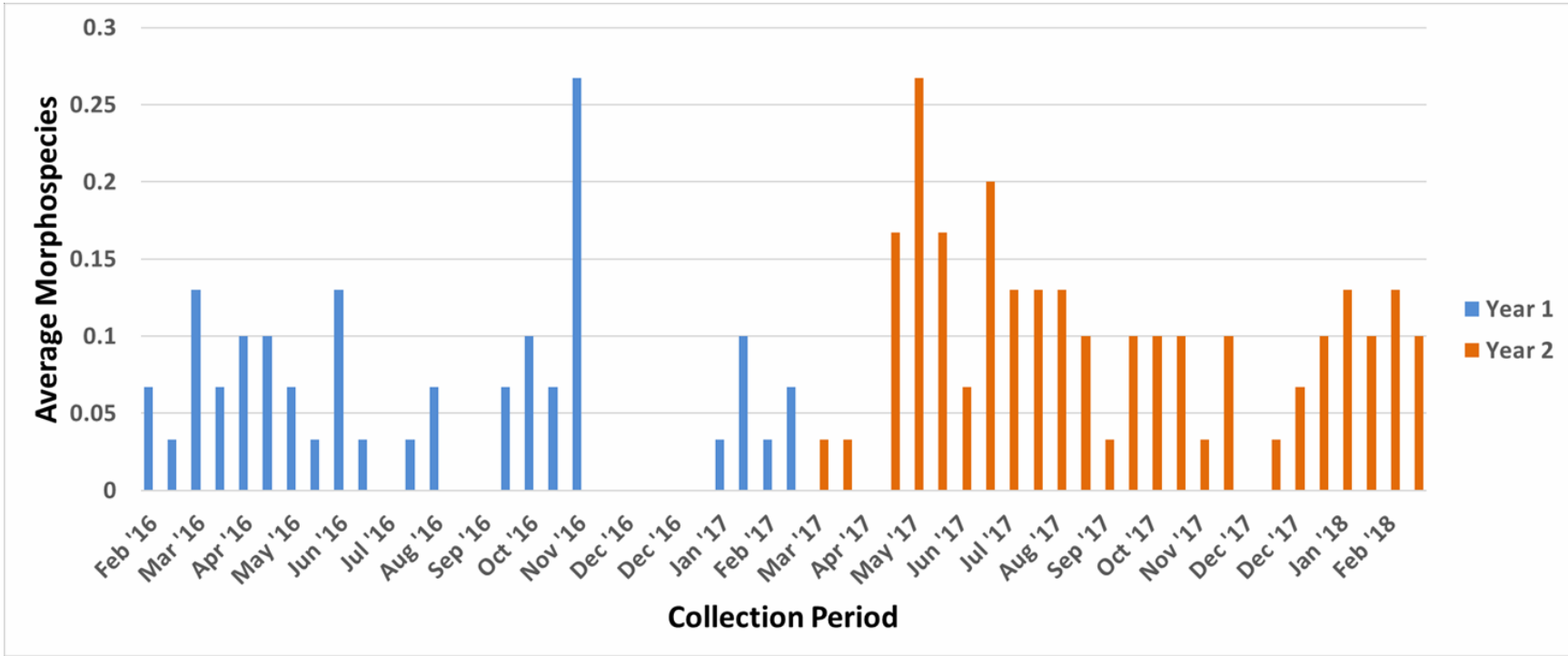

Figure 2.4. Average number of morphospecies collected in twig-based pitfall traps for both sampling years, with two-week sampling periods between February 26, 2016 to March 10, 2018.

Table 2.3. Repeated measures ANOVA results for twig-based pitfall traps with years 1 and 2 from February 26, 2016 to March 9, 2017 and March 23, 2017 to March 10, 2018, respectively.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	4	815	0.33	0.8609
Year	1	815	22.10	<.0001
Treatment*Year	4	815	1.04	0.3846

Species of concern (Table 2.1) by taxonomic group for the duration of the study were totaled (Fig. 2.9) and broken down further by trap type (Fig. 2.13). Panel traps captured overwhelmingly more ambrosia beetles, but accounted for few other species of concern. In contrast, pitfall traps captured most Hylobiini but few ambrosia beetles. After breaking down ambrosia beetles by species (Fig. 2.14), it can be seen that that *Xylosandrus germanus* Blandford, an introduced species originally from Asia, made up over sevenfold the next highest individual collected of a species. Indeed, even combining all species of concern caught, including ambrosia beetles, this species still made up over half of the total individuals of concern collected (Fig. 2.15).

Finally, species accumulation curves were created according to Colwell et al. (2012) for each trap type, divided by season, inoculation period where relevant, and extrapolated twofold. For twig-based pitfall traps (Fig. 2.16), morphospecies counts remained low and extrapolation revealed that additional traps of this type would gain few new species to warrant their placement. Antifreeze-based pitfalls (Fig. 2.17) revealed a similar trend, supporting the idea that enough traps are in place. Panel traps (Fig. 2.18) revealed sharper rates of increase, showing that, at least in warmer months, additional traps have the potential to catch two to four more species per trap.

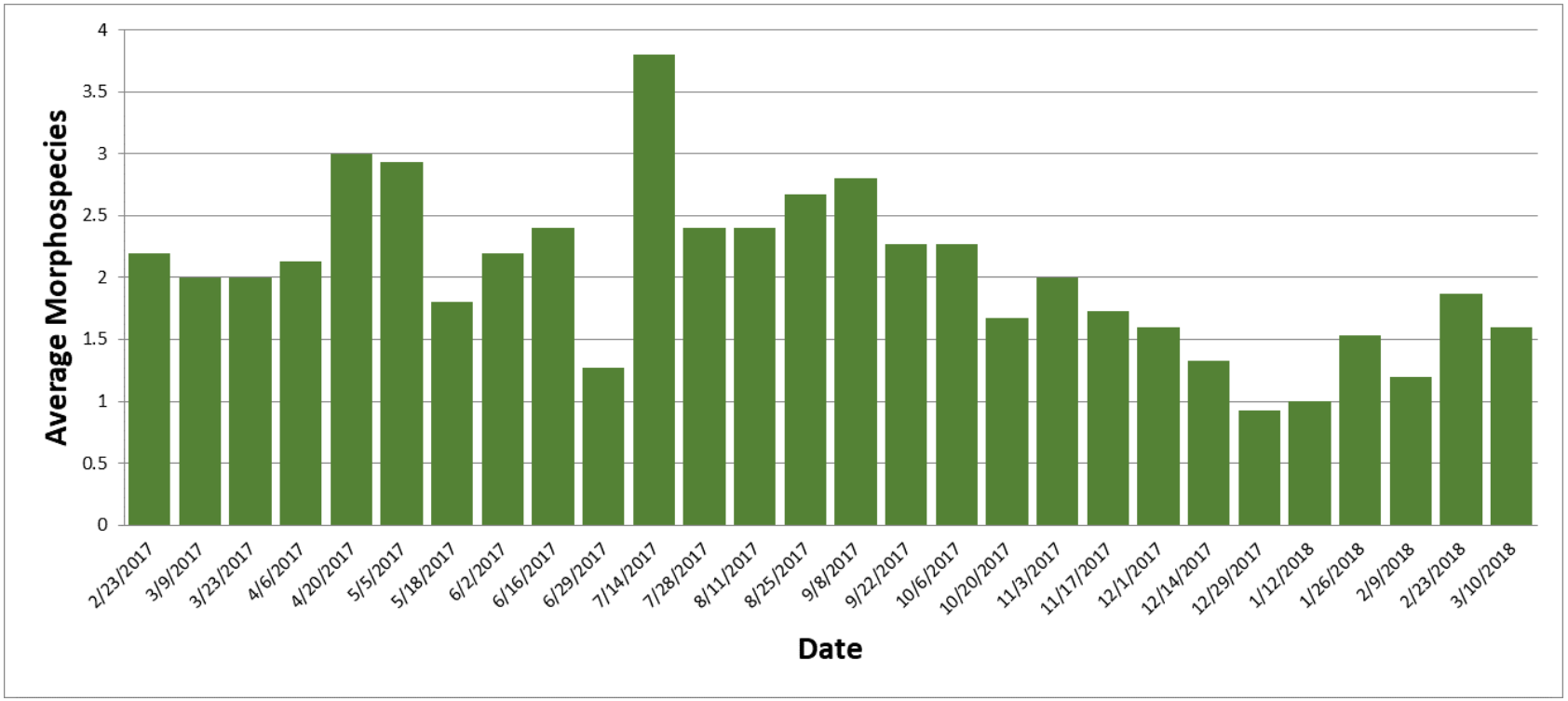

Figure 2.5. Average number of morphospecies collected in antifreeze-based pitfall traps according to sampling period.

Table 2.4. Repeated measures ANOVA results for antifreeze-based pitfall traps for one year from February 23, 2017 to March 10, 2018.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	4	413	2.25	0.0632

Table 2.5. Pre-inoculation averages for diversity indices, Shannon's and Simpson's Inverse, for antifreeze-based pitfall traps according to imminent treatment type and season, where n corresponds to the number bimonthly sampling periods used for each season.

	Winter 2 n = 3		**Spring 2** n = 3	
	Shannon	Simpson	Shannon	Simpson
Control	1.73	4.83	1.43	3.82
Wound	1.27	3.17	1.2	2.76
Low	1.63	4.34	0.95	2.59
Medium	1.56	4.02	1.01	3.11
High	1.88	5.8	1.22	3.22

Table 2.6. Post-inoculation averages for diversity indices, Shannon's and Simpson's Inverse, for antifreeze-based pitfall traps according to imminent treatment type and season, where n corresponds to the number of bimonthly sampling periods used for each season.

	Spring 2 n = 18		**Summer 2** n = 18		**Fall 2** n = 21		**Winter 3** n =18		**Spring 3** n = 18	
	Shannon	Simpson	Shannon	Simpson	Shannon	Simpson	Shannon	Simpson	Shannon	Simpson
Control	1.78	5.28	1.75	5.13	1.41	3.76	1.07	2.94	0.92	2.05
Wound	1.62	4.58	1.66	4.67	1.33	3.81	0.81	2.35	0.7	2.17
Low	1.45	4.1	1.6	4.48	1.38	4.01	0.99	2.81	0.92	2.3
Medium	1.23	3.41	1.61	4.32	1.37	3.31	0.95	2.71	0.68	1.98
High	1.77	5.13	1.71	4.9	1.33	3.69	0.82	2.33	1.3	4.05

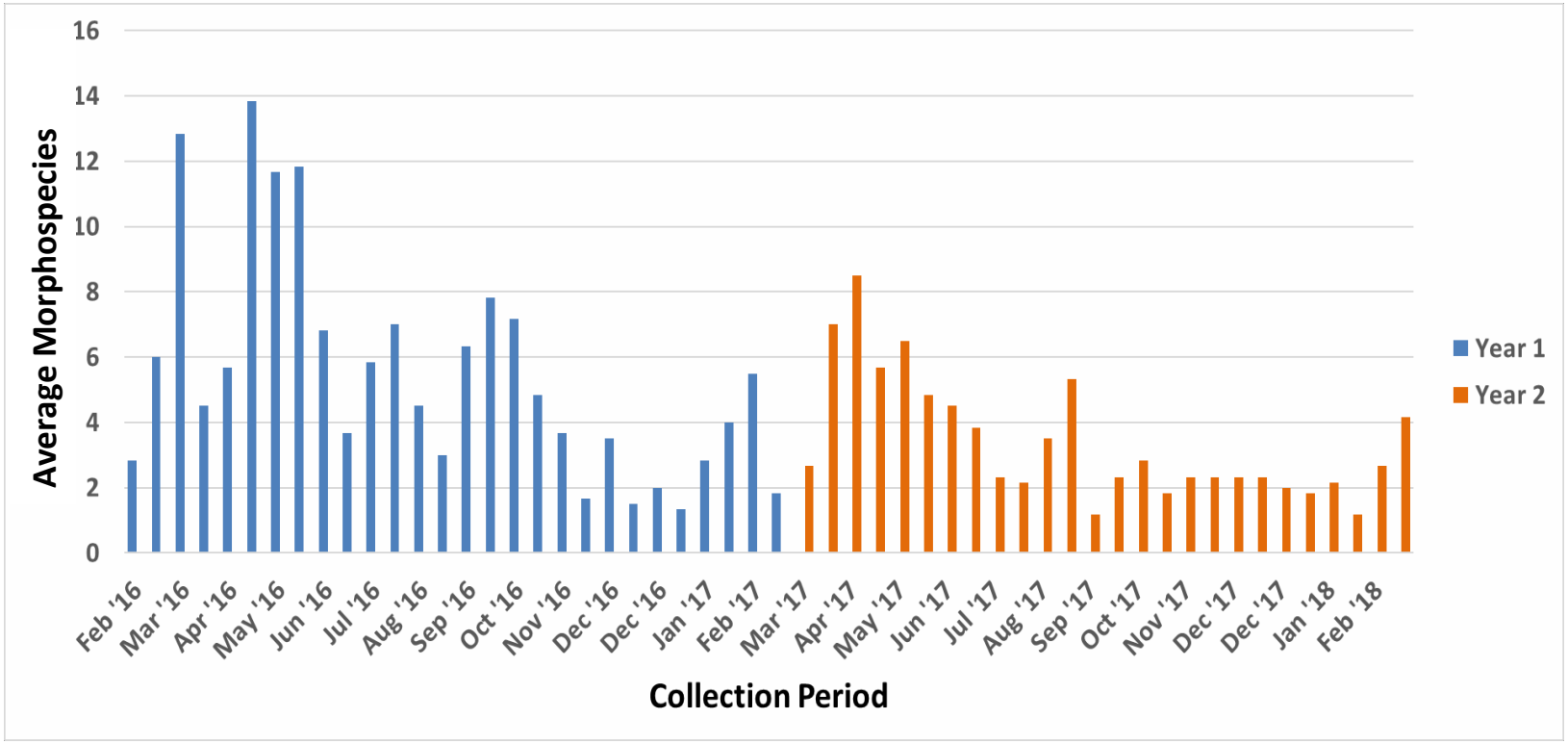

Figure 2.6. Average number of morphospecies collected in panel traps for both sampling years, with two-week sampling periods between February 26, 2016 to March 10, 2018.

Table 2.7. Repeated measures ANOVA results for panel trap species richness for Year 1 and Year 2.

Effect	Num DF	Den DF	F Value	Pr > F
Trap	5	312	1.04	0.3964
Year	1	312	32.90	<.0001
Trap*Year	5	312	0.43	0.8300

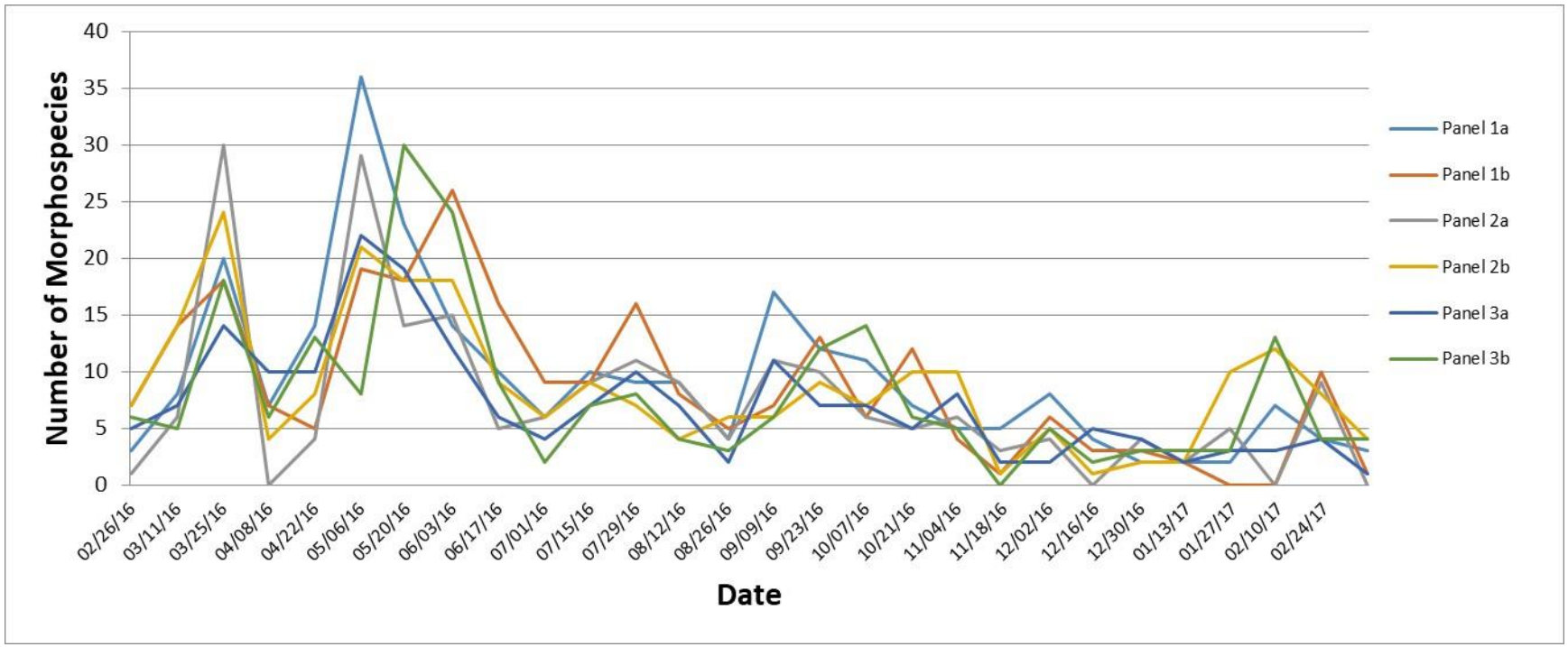

Figure 2.7. Number of morphospecies caught per panel trap according to sampling period for year 1.

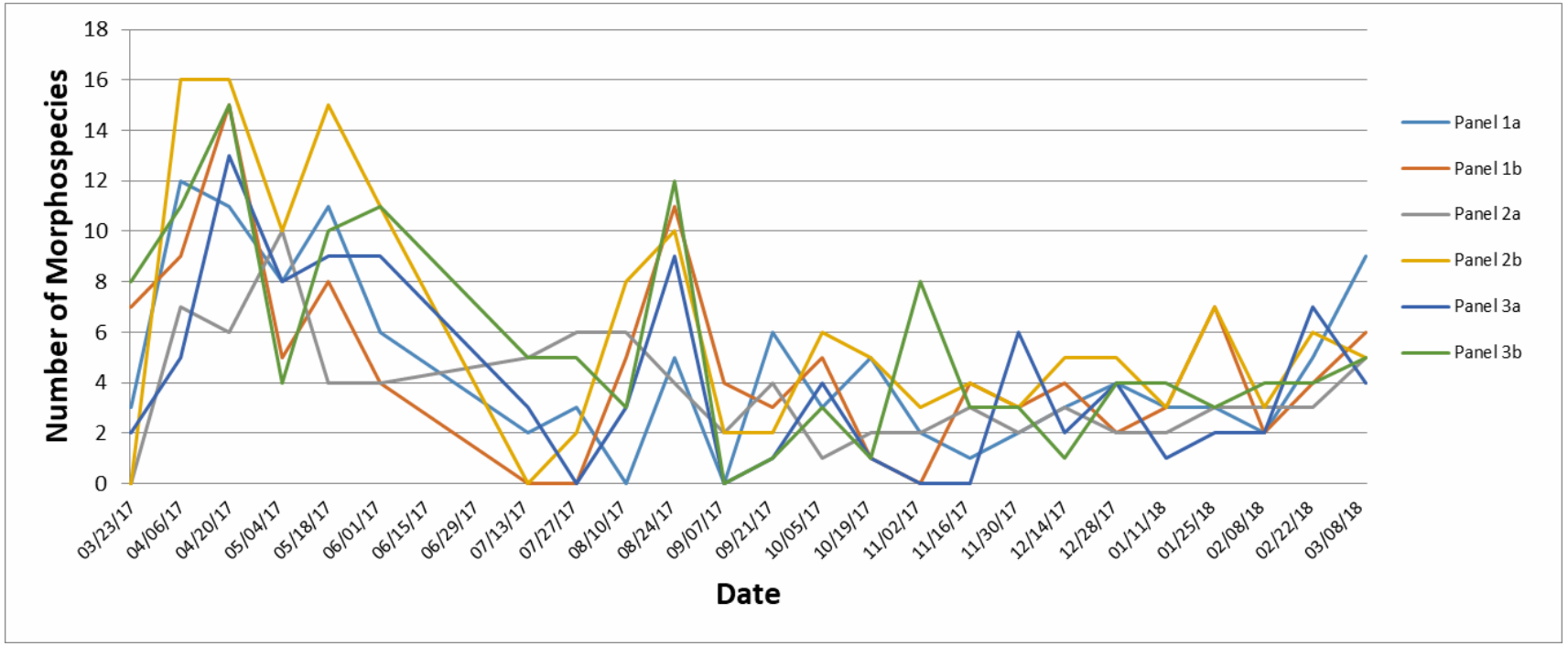

Figure 2.8. Number of morphospecies caught per panel trap according to sampling period for year 2.

Table 2.8. Pre-inoculation averages for diversity indices, Shannon's and Simpson's Inverse, according to panel trap and season, where n corresponds to the number of sampling events.

	Winter 1 n = 1		**Spring 1** n = 6		**Summer 1** n = 6		**Fall 1** n = 7		**Winter 2** n = 6		**Spring 2** n = 1	
	Shannon	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**
1A	*	*	2.39	9.64	1.8	5.5	1.78	4.98	0.9	2.51	1.099	3
1B	*	*	2.26	9.43	2.1	7.28	1.67	5.97	0.71	2.46	0	1
2A	*	*	1.88	7.56	1.82	5.73	1.18	2.66	0.69	1.58	*	*
2B	1.46	3.31	2.38	9.3	1.81	6.18	1.57	4.95	1.16	3.92	1.386	4
3A	1.47	3.76	2.31	9.04	1.62	5.07	1.34	3.98	0.97	2.63	0	1
3B	1.63	4.5	2.27	9.54	1.53	5.12	1.29	3.77	0.98	2.54	1.386	4

* Traps captured no insects

Table 2.9. Post-inoculation averages for diversity indices, Shannon's and Simpson's Inverse, according to panel trap and season, where n corresponds to the number of sampling events.

	Spring 2 n = 6		**Summer 2** n = 6		**Fall 2** n = 7		**Winter 3** n = 6		**Spring 3** n = 1	
	Shannon	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**	**Shannon**	**Simpson**
1A	1.88	6.14	1.07	3.25	0.82	2.53	0.98	2.54	2.16	8.33
1B	1.9	6.59	1.09	3.62	0.92	2.74	0.74	1.95	1.79	5.99
2A	1.44	4.86	1.45	4.31	0.72	2.12	0.75	2.05	1.61	5
2B	1.94	7.47	1.41	4.78	1.08	2.92	1.19	3.21	1.61	5
3A	1.81	6.63	1.34	4.09	0.43	1.19	0.81	2.71	1.39	4
3B	2.03	6.86	1.78	6.39	0.75	2.54	0.76	2.14	1.61	5

Table 2.10. Pre-inoculation averages of the Sørensen coefficient per panel trap pair according to season, where n corresponds to the number of sampling events.

	Winter 1 n = 1	**Spring 1** n = 7	**Summer 1** n = 6	**Fall 1** n = 7	**Winter 2** n = 6	**Spring 2** n = 1
1A - 1B	0.222	0.275	0.26	0.112	0.269	0
1A - 2A	0.5	0.237	0.099	0.283	0.216	0
1A - 2B	0.4	0.258	0.188	0.193	0.337	0
1A - 3A	0.5	0.231	0.133	0.219	0.309	0
1A - 3B	0.444	0.272	0.117	0.225	0.292	0
1B - 2A	0.285	0.205	0.116	0.103	0.15	0
1B - 2B	0.153	0.311	0.228	0.133	0.233	0
1B - 3A	0.363	0.23	0.272	0.235	0.2	0
1B - 3B	0.166	0.233	0.145	0.439	0.255	0.4
2A - 2B	0.25	0.231	0.131	0.134	0.185	0
2A - 3A	0.333	0.197	0.207	0.308	0.197	0
2A - 3B	0.285	0.191	0.234	0.307	0.197	0
2B - 3A	0.5	0.193	0.259	0.161	0.246	0
2B - 3B	0.461	0.244	0.164	0.192	0.272	0.25
3A - 3B	0.545	0.26	0.286	0.155	0.193	0

Table 2.11. Post-inoculation averages of the Sørensen coefficient per panel trap pair according to season, where n corresponds to the number of sampling events.

	Spring 2 n = 6	**Summer 2** n = 6	**Fall 2** n = 7	**Winter 3** n = 6	**Spring 3** n = 1
1A - 1B	0.224	0.03	0.067	0.401	0
1A - 2A	0.153	0.163	0.129	0.408	0.285
1A - 2B	0.245	0.085	0.086	0.368	0.142
1A - 3A	0.118	0.084	0.036	0.369	0
1A - 3B	0.216	0.096	0	0.447	0.142
1B - 2A	0.144	0.13	0.088	0.331	0
1B - 2B	0.215	0.032	0.052	0.439	0.181
1B - 3A	0.104	0.077	0.032	0.181	0
1B - 3B	0.226	0.091	0.048	0.314	0.363
2A - 2B	0.158	0.098	0.179	0.321	0.4
2A - 3A	0.121	0.133	0.036	0.255	0.222
2A - 3B	0.144	0.073	0	0.365	0.2
2B - 3A	0.144	0.063	0.079	0.229	0
2B - 3B	0.229	0.141	0.105	0.305	0
3A - 3B	0.193	0.22	0	0.224	0

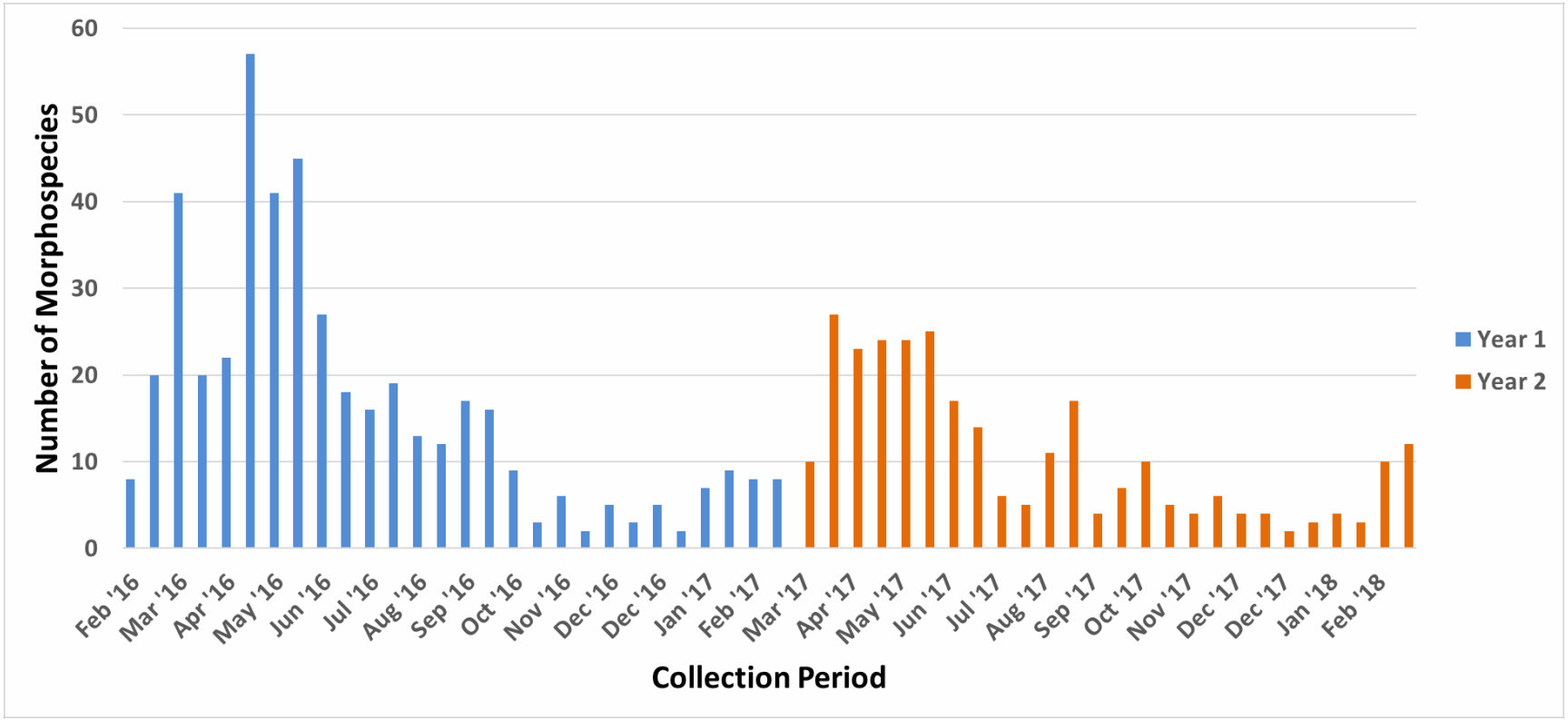

Figure 2.9. Total morphospecies of Coleoptera caught in panel traps during the study period.

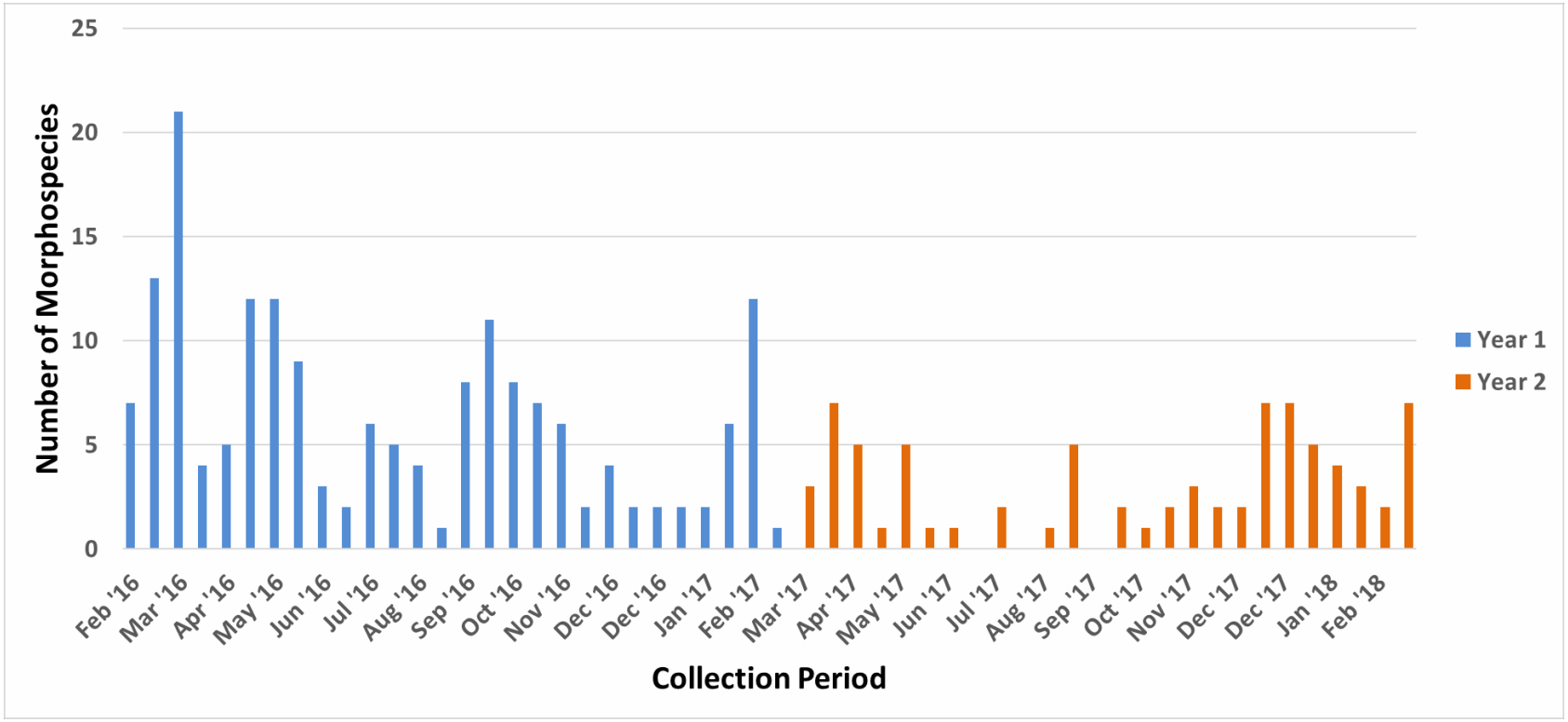

Figure 2.10. Total morphospecies of Diptera caught in panel traps during the study period.

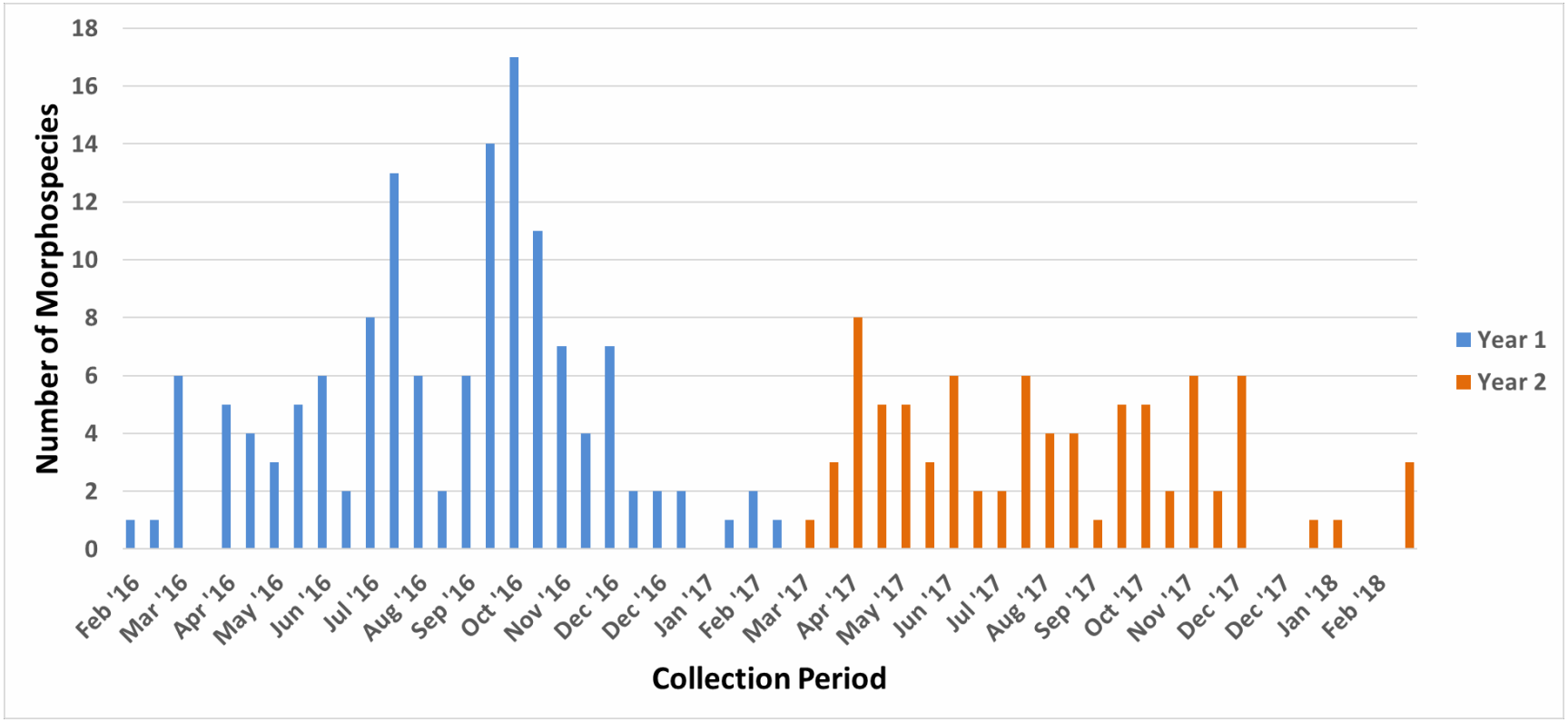

Figure 2.11. Total number of morphospecies of Hymenoptera caught in panel traps during the study period.

Table 2.12. Repeated measures ANOVA results for log transformed Shannon index values for panel traps for the two year collection period.

Effect	Num DF	Den DF	F Value	Pr > F
Trap	5	277	1.08	0.3728
Year_character	1	277	5.84	0.0164

Table 2.13. Repeated measures ANOVA results for Arcsine squared transformed Simpson index values for panel traps for the two year collection period.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Year_character	1	0.43560882	0.43560882	4.42	0.0364
Trap	5	0.30511971	0.06102394	0.62	0.6855

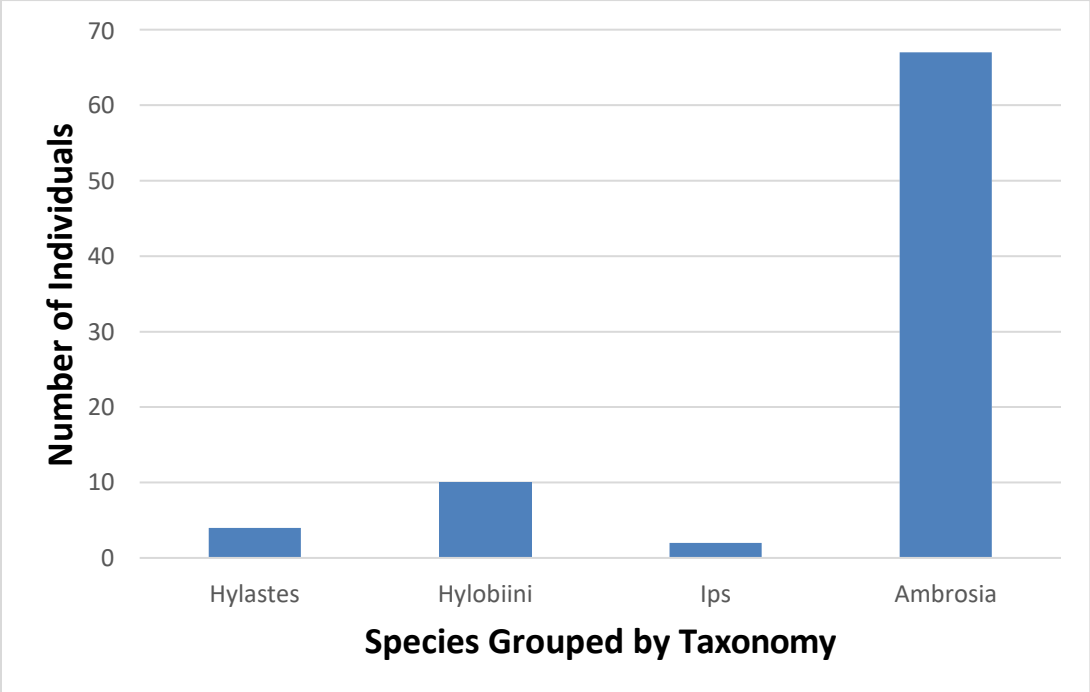

Figure 2.12. Total individuals of concern captured, as sorted by taxonomy.

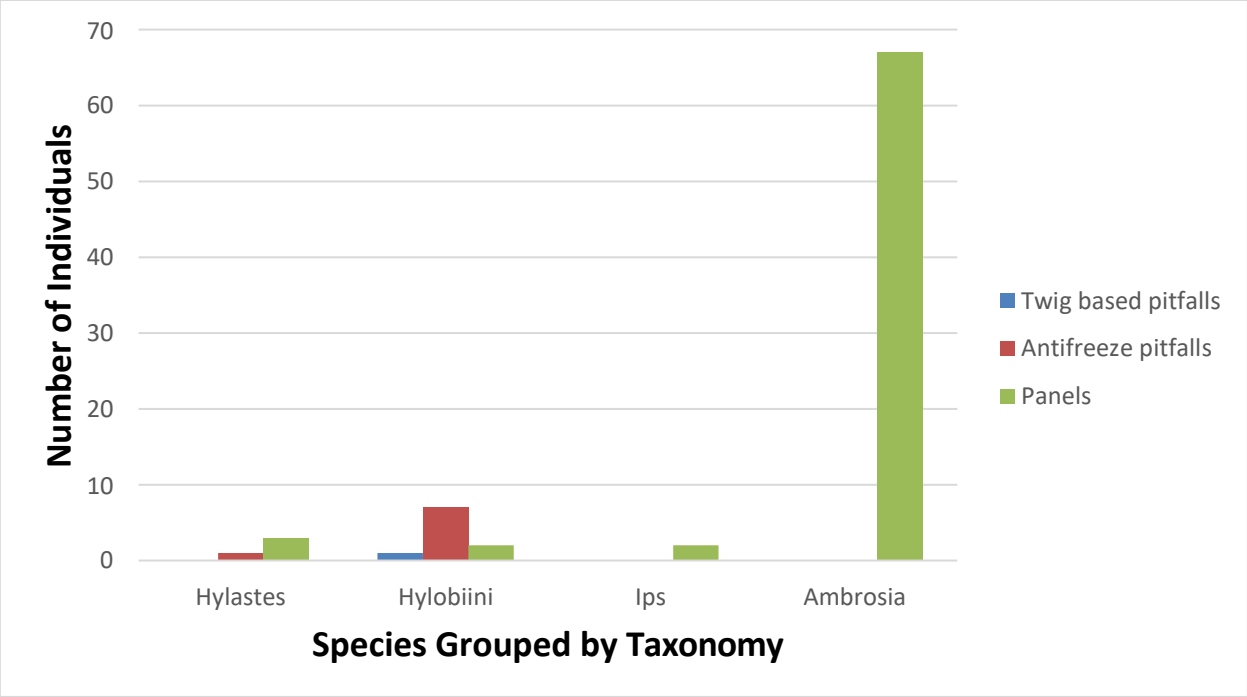

Figure 2.13. Total number of individuals of species of concern captured per trap type.

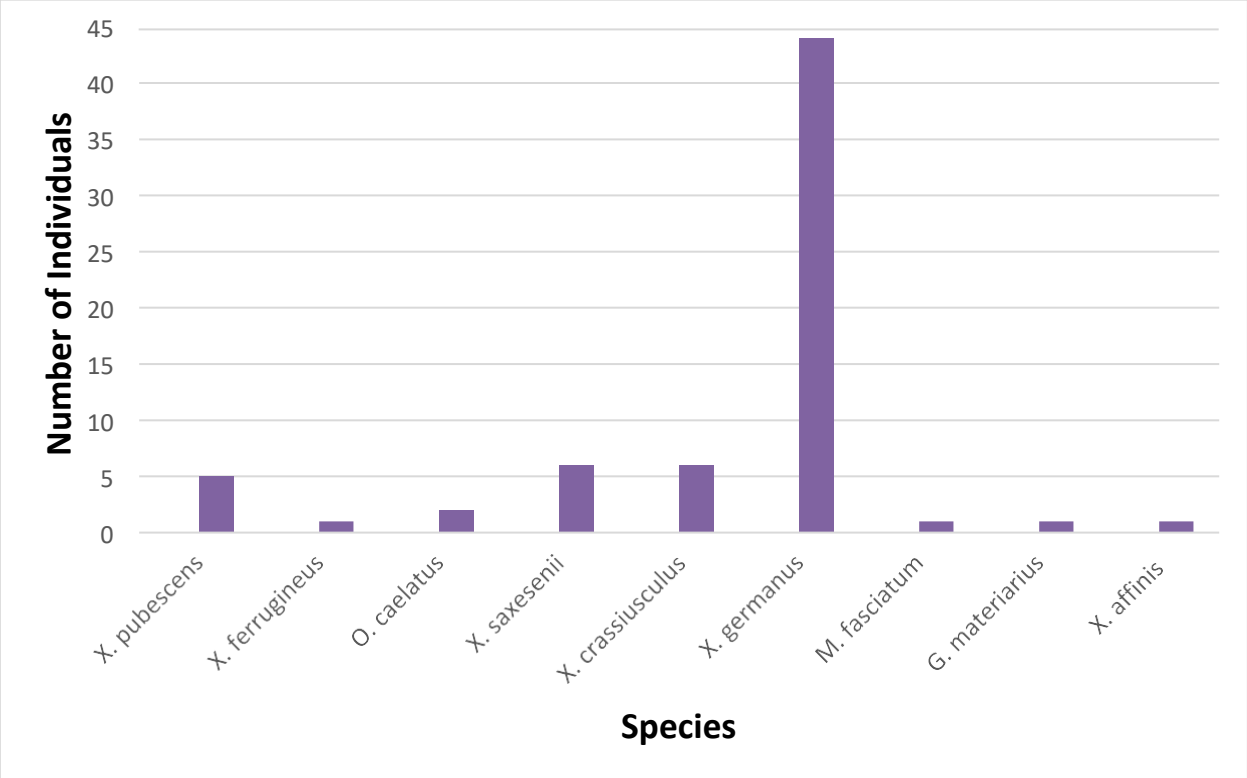

Figure 2.14. Total individuals of ambrosia (Scolytinae) beetle captured by species.

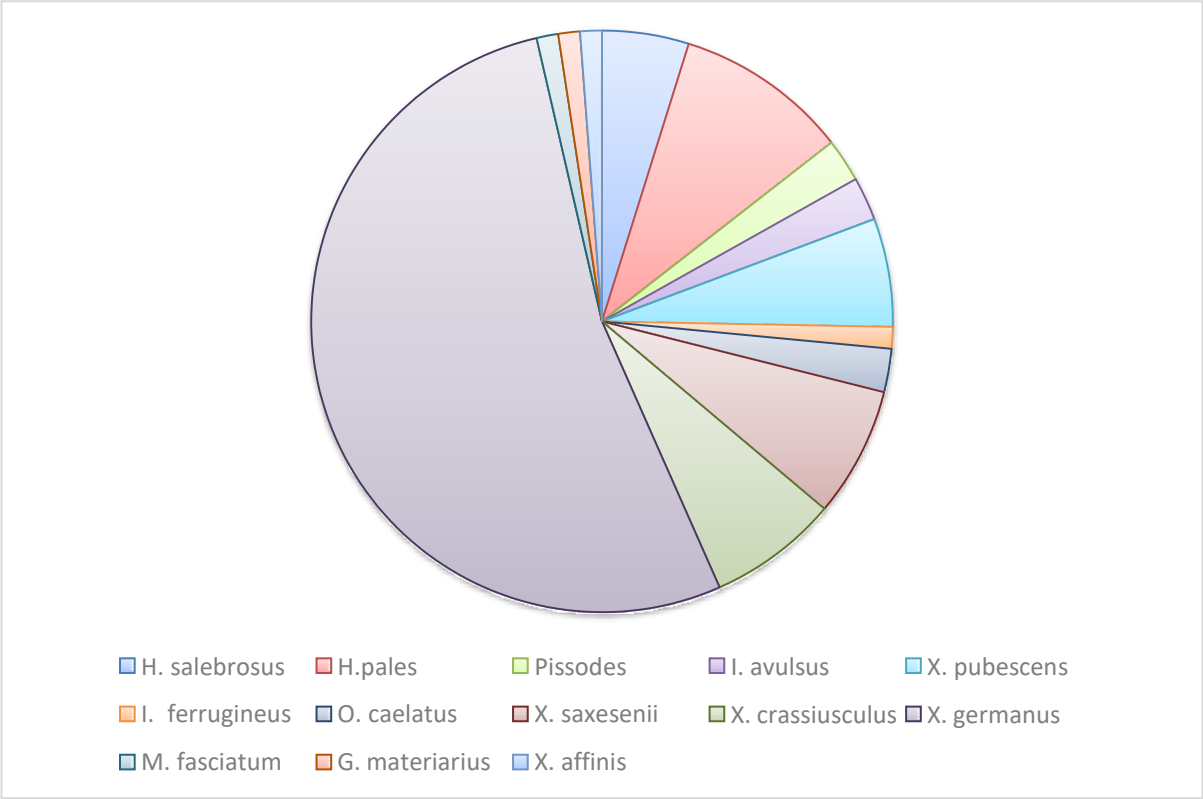

Figure 2.15. Pie chart representing the proportion of individuals caught by species.

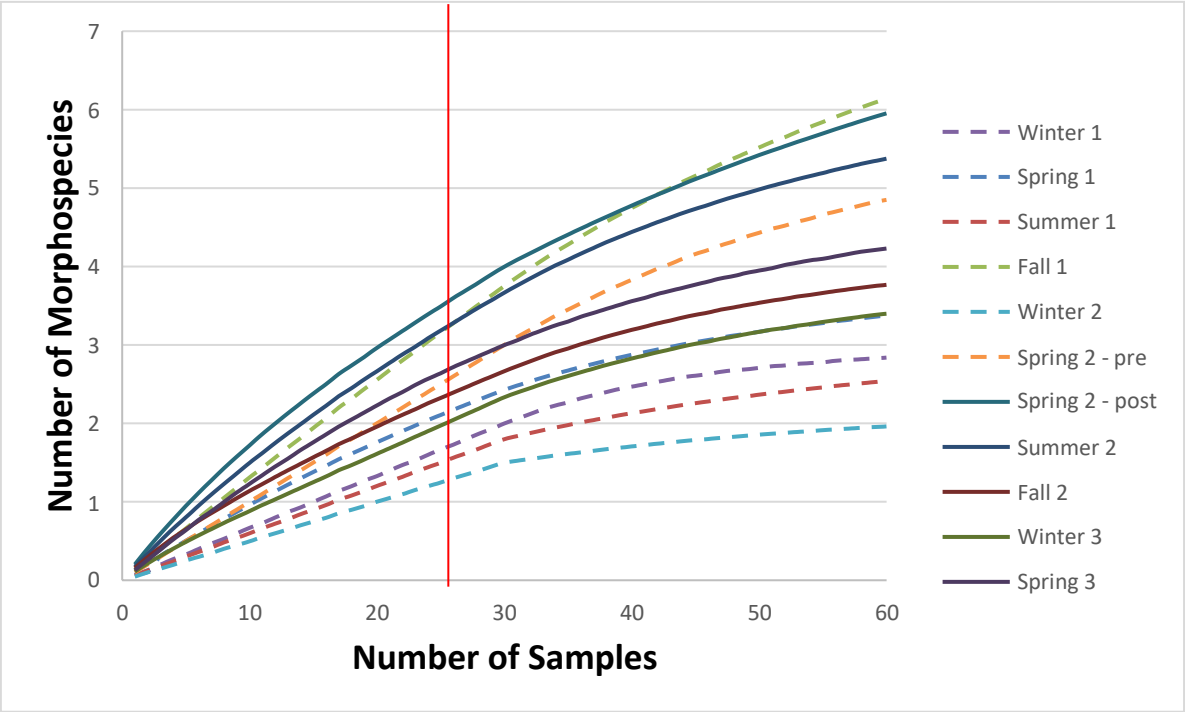

Figure 2.16. Species accumulation curves for twig-based pitfall traps based on season, with dotted lines denoting the pre-inoculation period and full lines corresponding to the post-inoculation period, each extrapolated past the number of samples by two-fold as defined by the vertical line.

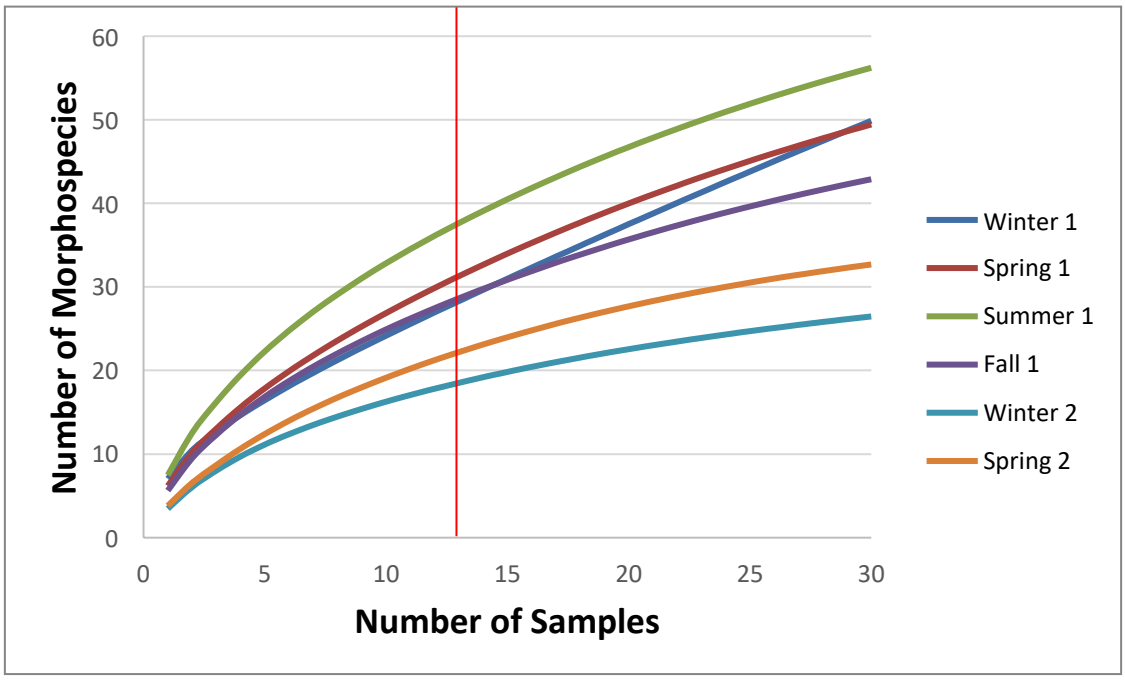

Figure 2.17. Species accumulation curve for antifreeze-based pitfall traps extrapolated past the number of samples by two-fold as defined by the vertical line.

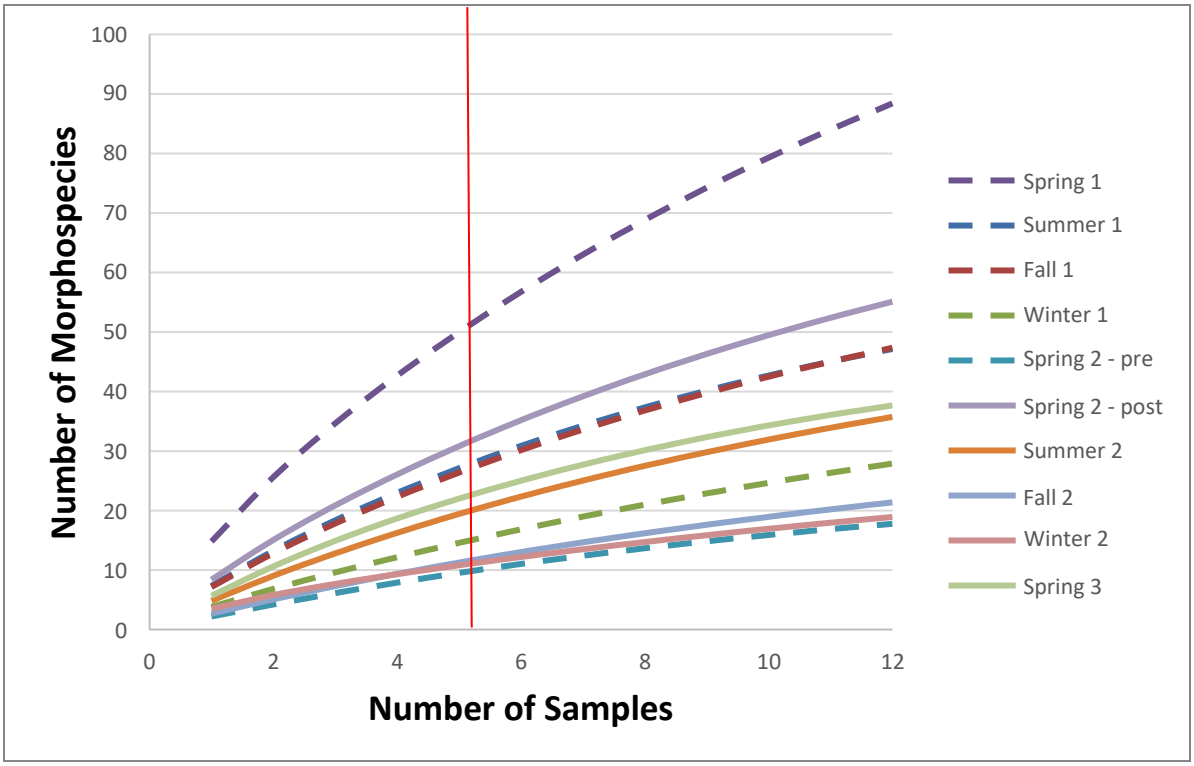

Figure 2.18. Species curves for panel traps based on season, with dotted lines denoting the pre-inoculation period and full lines corresponding to the post-inoculation period, each extrapolated past the number of samples by two-fold as defined by the vertical line.

2.6 Discussion

In this 25 month period of insect collection, we compared insect species richness of plots infected with varying levels of *L. terebrantis* inoculum in a commercial loblolly pine stand. Species richness only varied significantly between years among twig-based pitfall and panel traps. Due to the shorter period of duration for antifreeze-based pitfalls but given the marginal significance of the period they were in place, these traps show promise for recording results in future years. As for the panel and twig-based traps, the fact that years differed but treatment does not warrant further monitoring. A drought that occurred during the winter of the first year could be an influencing factor between years (Table 2.14). While these events can stress plants, leading to more insect attacks due to the attracting semiochemicals trees emit (Mattson and Haack 1987; Ferrell 1996), prolonged periods can also affect insect survival (Schowalter et al. 1981). Insects

Table 2.14 Average temperature in Fahrenheit and total rainfall in inches for each month of the study, separated by year.

Year	Month	Average Temperature (°F)	Precipitation (Inches)
2016	February	49.49	0.16
	March	60.56	0.14
	April	63.64	0.22
	May	70.32	0.04
	June	78.64	0.18
	July	80.87	0.08
	August	80.62	0.13
	September	77.02	0.01
	October	66.06	0.00
	November	54.67	0.05
	December	51.84	0.17
2017	January	52.03	0.31
	February	55.27	0.20
	March	58.17	0.06
	April	66.37	0.11
	May	70.37	0.18
	June	75.91	0.18
	July	79.77	0.16
	August	78.34	0.18
	September	74.02	0.14
	October	65.82	0.10
	November	54.22	0.03
	December	48.23	0.09
2018	January	43.80	0.09
	February	61.95	0.17
	March	57.55	0.09

in dormancy have both morphological and physiological methods to conserve water, but they are not invulnerable (Danks 2000). While insect diversity can be positively correlated with plant diversity (Haddad et al. 2001), decreasing pest populations (Power 1987), studies have suggested moisture can be a stronger influencing factor during certain parts of the year (Janzen 1972; Wenninger and Inouye 2008). Wood-boring beetles and hemipterans in particular have been shown to benefit from drought, taking advantage of lowered plant defenses (Mattson and Haack

1987; Ferrell 1996), concentrated nutrient content of tissues (Barras and Hodges 1969; Mattson and Haack 1987), elevated temperatures for development, and decreased parasite loads (Mattson and Haack 1987).

Panel traps, despite their differing placements around the study area, caught similar amounts of morphospecies, even across seasons (Fig. 2.7; Fig. 2.8), despite catching less than 50% of similar species the majority of the time (Table 2.10). However, this lower similarity of traps supports their separation and importance in recording diversity for the entire study area. Likewise, Shannon and Simpson index values supported individual trap's consistency in diversity and evenness (Table 2.8; Table 2.9). These same values showed higher diversity in the first year, supporting a significant find in terms of species richness between years (Table 2.7). Seasonal trends shown by panel traps (Fig. 2.6) were similar in both pitfall based traps (Fig. 2.4; Fig. 2.5) with peaks in spring and early summer and decreases in winter. This suggests that perhaps, despite lower catch numbers in twig-based traps, insect species captured were a reflection of the larger community trends. Other studies have shown flight intercept traps, such as our panel traps, to be more efficient at capturing total species, though pitfall traps catch insects that flight intercept traps do not, supporting their use (Hyvärinen et al. 2006). Insects can often be attracted to certain colors, especially flower visiting insects, influencing captures of certain groups (Campbell and Hanula 2007) and some insect groups, despite being observed, may be excluded from traps due to sampling bias, reinforcing the need for multiple trap types (Su and Woods 2001).

Our species accumulation curves for both twig- and antifreeze-based pitfall traps provided low rates of new species discovered per additional trap. Williams et al. (2007) previously suggested a new species discovery rate of more than two for additional sampling to be

considered worth the cost of time and effort. Both curves for pitfall traps provide support for the determination that sufficient traps are in place for the remaining duration of the study (Fig. 2.16; Fig. 2.17). However, in contrast to pitfall based traps, panel traps showed a possible greater collection rate of new species if more traps were deployed in the future (Fig. 2.18), especially during the warmer months. However, it is worth noting that the curve shows no near sign of leveling off as an asymptote, and therefore suggests sampling has not been sufficient (Williams et al. 2007). Nevertheless, with poorly described taxon such as in some insect groups, this result is not unexpected (Gaston et al. 1995).

Ambrosia beetles far outnumbered root-feeding bark beetles in this study (Fig. 2.12). Panel traps were by far the most efficient at recording these species, but ground based pitfalls were more efficient at capturing Hylobiini, possibly due to their root-feeding tendencies (Fig. 2.13). Pitfall traps have traditionally been used to sample ground-dwelling arthropods, especially those active at ground level (Prasifka et al. 2007) while ambrosia beetles, such as *X.germanus* or *Xyleborus glabratus,* have been shown to have better capture rates with airborne traps 0.5 and 1.5 meters off the ground (Reding et al. 2010; Hanula et al. 2011). Supporting this, more than one study has noted higher Hylobiini and *Hylastes* populations in areas of root mortality (Klepzig et al. 1991; Erbilgin and Raffa 2002; Eckhardt et al. 2007). The large number of invasive *X. germanus* (Fig. 2.14) may be an indicator that plant diversity is lacking (Power 1987; Knops et al. 1999) and may indicate a need for an increase in genetic diversity of plants currently present (Johnson et al. 2006). Invasive pests such as this can introduce and spread pathogens, their movements aided by increased spatial connectivity and concentration of resources (Ratnadass et al. 2012) and can outcompete native insects and disrupt pollination services (Kenis et al. 2009). Increasing plant diversity as a method to disrupt the spread of this species, dilute

available resources, and facilitate predators and competitors may be an option (Johnson et al. 2006). As a polyphagous pest of hardwoods such as elm, oak, hickory, maple, and walnut, and even conifers such as red, white, and scotch pine (Buchanan 1941; Weber and McPherson 1983), this species has a wide variety of hosts and is a possible vector of *Ophiostoma ulmi*, the pathogen that causes Dutch elm disease, a forest health threat (Hoffmann 1941).

Chapter 3

Identifying Fungal Spores on Coleoptera with Hyperspectral Interferometry

3.1 Abstract

Previous work with hyperspectral interferometry on bark beetles revealed oscillatory signals from specific surface features. Best resembling an interference pattern as from a Fabry-Perot model, this instance is unique in that the etalon is produced outside of the microscope measuring system and is instead on the surface feature being observed. Setae and spore-like objects on the beetle were described, with further work here being given to spores. Here, *Hylastes salebrosus* beetles were used as surfaces to detect spores of three different species. For us, the reflecting surface was the spore, outside the microscope measuring system, which had two or more surfaces. This pilot study determined whether interferometry could differentiate spore type on a beetle surface. Sizes and power spectra were obtained from spores, and maps of spores as they laid out on the beetles' surface were created with the use of a computer program in ImageJ. Statistical analysis revealed that two out of three fungal species could be differentiated with hyperspectral interferometry. However, two species had close average means and are likely not capable of being distinguished from one another in this manner.

3.2 Introduction

Previous work with hyperspectral imaging has centered on fields in medicine, pharmacy, agriculture, and astronomy (Hege et al. 2003; Rodionova et al. 2005; Del Fiore et al. 2010; More et al. 2016). While previous work by Beach et al. (2015) showed a capability of observing and

sizing spores on bark beetles, further research is needed to determine if it is possible to speciate spores using this method. Current methods of fungal identification involve culturing samples on media plates, often utilizing time periods of three weeks or more, and using molecular analyses such as PCR and DNA fingerprinting (Six et al. 2003). Additional studies could provide a quicker and more cost efficient alternative to fungal identification, quickening management implementation.

As phytophagous insects, *H. salebrosus* feed on pine roots and stumps, and have even been documented on green seedlings (Wood 1982). These beetles are known carriers of ophiostomatoid fungi such as *Leptographium* and *Grosmannia* (Klepzig et al. 1991, Jacobs and Wingfield 2001; Matusick et al. 2013) that cause blue staining and vascular occlusion of wood tissues (Eckhardt et al. 2007). These effects can be detrimental to the forestry industry, as they devalue wood and affect tree growth (Rane and Tattar, 1987; Eckhardt et al. 2004a). Other fungal species, including yeasts and *Graphium*, have also been found associated with *Hylastes* species (Dowding 1973; Wingfield and Gibbs 1991) and add to the ecology of microflora found on these beetles.

In bark beetles, including *Hylastes*, fungi are often vectored externally on beetle surfaces, internally through the digestive system, and sometimes in specialized body structures called mycangia (Harrington 1993). These ophiostomatoid fungi have spores that are coated and sticky, allowing for adherence to the insect body. Whitney and Blauel (1972) suggested this was to prevent washing off by rain and instead allows for detachment in resin filled galleries of new dispersal trees. In addition to this, the mucilage allows for spores to travel as discrete masses and keeps them moist (Dowding 1969) and in some species, can allow for safe gastrointestinal travel (Francke-Grosmann 1963). Furthermore, fungal spores have been collected by rolling *Hylastes*

salebrosus beetles over media plates, lending additional support to the idea that these spores are vectored externally in this species (Eckhardt et al. 2007).

Here, we observed spores of three species in an attempt to differentiate them: *Grosmannia alacris* T.A. Duong, Z.W. de beer & M.J. Wingf., *Leptographium procerum*, and a *Graphium* species. *Grosmannia alacris* has spores that are hyaline, aseptate, and oblong with rounded apices and truncate bases. The fungi are reported to have spores in the size range of (4.5-)5.1-6.1(-6.8) X (1.8-)2.1-2.4(-2.6) µm, depending on genetics and environmental conditions (Duong et al. 2012). Likewise, *L. procerum* has spores with a similar shape, ranging from hyaline, aseptate, obovoid to broadly ellipsoid with rounded apices and truncate bases. Their sizes range from (3 – 5) x (1 – 3) µm (Jacobs and Wingfield 2001). In addition to these known pathogenic species, we also looked at an unknown *Graphium* species that is also carried by *Hylastes* as a comparison. Spores from this fungus measured between (0.6 – 1.1) x (0.7 – 1.2) µm within the mucilage covering, as observed within this study.

3.3 Methods and Materials

3.3.1 Site Location

Spore preparation was done in the laboratory at the Forest Health Cooperative at Auburn University in Auburn, AL. Hyperspectral imaging of both spores and beetles was done at CytoViva, Inc. in Auburn, AL.

3.3.2 Fungal Spore Cultures and Observation

Fungal cultures each of *L. procerum* and *G. alacris* were used from the existing culture collection at the Forest Health Cooperative Laboratory at Auburn University. Fungi were cultured on media plates composed of twice sterilized loblolly pine (*Pinus taeda*) twigs and 1% malt extract agar. Plates of oatmeal agar were used to encourage sporulation of difficult or slow

to sporulate species such as *L. procerum*. Cultures were allowed to grow for 14 days inside an incubator at 25 °C.

Fungal smears on microscope slides were initially obtained by sterile technique onto empty 75x25x1 mm microscope slides (VWR VistaVision). Microscope slides were fitted with an o-ring (Danco, #15, 1x0.75x0.125 in.) sealed in place with clear nail polish (Sally Hansen, Hard as Nails, 0.45 fl. oz.)(Fig. 3.1). Spores were smeared inside the o-ring, next to an adjacent drop of distilled water and covered with an 18x18 mm, 1 oz. cover glass (Fisher Scientific, Pittsburgh, PA) to preserve humidity until observation. Prepared slides were then placed onto a napkin damp with distilled water inside a sterilized empty 28.5 oz. plastic container with a lid (Breyers Ice Cream) for transport to CytoViva, Inc.

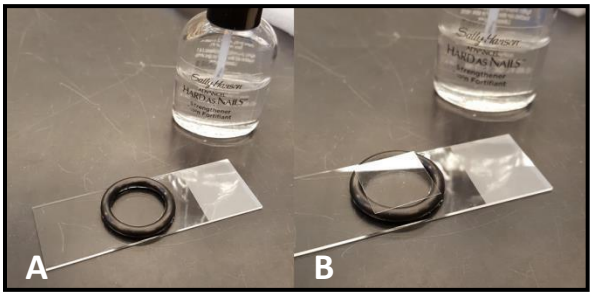

Figure 3.1. Microscope slides fitted with o-rings, (A) without a cover slip and (B) with a coverslip.

However, as our ultimate goal was to obtain readings directly from beetle surfaces, we sought another object similar glass slides as an alternative. The reflective nature of clear glass proved to be an interference in initial results, so to reduce interference from strong light reflections produced by the clear microscope slide, fungal species were subsequently observed on black glass seed beads (Cousin, 1.41. oz.)(Fig. 3.2). This was done by sterilizing beads in 95% alcohol for 30 seconds and allowing them to dry before rolling beads on sporulating

Figure 3.2. Glass seed beads (Cousin, 1.41 oz.) obtained for rolling onto spores.

plates of fungi. Beads were then placed onto sterilized 3" x 1" concavity microscope slides (Ted Pella, Inc., 1-1.2 mm wide) for observation.

3.3.3 Beetle Trapping and Preparation

Hylastes salebrosus beetles were captured with the use of both ground based panel traps and hanging panel traps placed at Auburn University's Mary Olive Thomas Demonstration Forest and the Louise Kreher Forest Ecology Preserve in Auburn, Alabama from February through June 2018. Pitfalls consisted of a 20-cm length of a 10-cm diameter polyvinyl chloride plastic pipe with eight holes spaced equally around the circumference (Klepzig et al. 1991)(Fig. 3.3). Both ends of the pipe had caps that served as removable lids, with holes drilled into the bottom cap for drainage. Traps were buried into the soil and leaf litter until the entrance holes lined up at ground level. These traps' interiors were coated with a layer of liquid Teflon™ (Northern Products Woonsockets, RI) to prevent insects from climbing out once inside. Panel traps (APTIV Company, Portland, Oregon) made of black corrugated plastic and Lindgren were funnels hung on metal poles one meter above the ground and had a plastic cup attached to the bottom to capture flying beetles (Fig. 3.4).

Figure 3.3. A pitfall trap baited with hanging vials of 3-carene and 95% ethanol.

Figure 3.4. A panel trap to be baited with hanging vials of 3-carene and 95% ethanol.

Both trap types included two 8 mL glass vials hung and filled with 90% 3-carene (Sigma-Aldrich) and 95% ethanol (1:1), respectively. These acted as an attractant to obtain more *Hylastes* over other bark beetles (Kelsey and Westlind 2018). Traps were checked and vials refilled every three days during the collection period. After

identification, captured *Hylastes salebrosus* were put into the cooler at 4 ºC to keep fresh until use and were utilized within 30 days of capture.

Beetle preparation involved washing *H. salebrosus* in 95% ethanol (Beach et al. 2015) for 30 seconds and allowing them to dry. Specimens were then rolled onto sporulating fungal plates with the use of sterilized forceps. Beetles were then transported to CytoViva inside of sterilized 2 oz. condiment cups (Dixie), Inc. for microscopic imaging on concavity microscope slides.

3.3.4 Reflected Light Microscopy

An upright microscope (Olympus BX-43) was used to observe beetles at 10X magnification with bright-field reflected light. Focus was used to examine areas and to obtain discernable, quality images of surface features on the beetles. Illumination was provided by a lamp using a halogen source and aluminized reflectors to provide a spectral range of visible and near infrared wavelengths. To direct light at the specimen and provide illumination, a half-silver mirror was used, passing reflected light to a detector (Beach et al. 2015).

3.3.5 Hyperspectral Imaging

The CytoViva hyperspectral imaging system uses a motorized stage to move a sample over a set scale of small distances, allowing for a camera to take pictures every time the stage stops. The camera is spectrographic, capable of capturing visible near-infrared (VIS-NIR) images; it comprises of an imaging spectrograph and a CCD camera and mounted to the imaging port of the microscope. Two spatial dimensions, X and Y, are used in a grid-coordinate system. A motion algorithm guides the movement of the stage, making it equivalent to the pixel spacing along X and projecting correct image geometry. The spatial resolution for the microscope at 10X objective magnification is 1.29 μm.

3.3.6 Size Estimation and Spectral Analysis of Spore Size

Oscillations of spore spectra were taken from spectra in hyperspectral images using Z axis profiles of associated pixels. The separation between adjacent intensity maxima or minima in the oscillations on single pixels were used to determine oscillation frequencies. Sizes were determined based on the assumption that oscillation waveforms came from the interference caused by multiple surface reflections. The surface oscillations were obtained in the form of maps with the use of ImageJ using the hyperspectral image.

The usage of the "Analyze Particles" feature in ImageJ also provided rough estimate of initial spore assessments, allowing for non-conforming pixels to be excluded out of these sizing procedures. The feature gave an output of average area in the form of image pixels; knowing pixel size, we were able to calculate average area. Detectable by a map later developed in ImageJ, these same objects allowed us to analyze the spectral oscillations and we compared these average area outputs to the sizes in the model. The closer both values were to each other, then the more supportive the results.

3.3.7 Etalon Modeling

As oscillations arise from interference produced by multiple surfaces, a semitransparent object like a spore provides a path for light between back and front surfaces, using the change in the refractive index to produce a reflection. A fraction of the light gets reflected back into the object, and a standing wave pattern is produced as light goes back and forth between surfaces. This effect is similar to the Fabry-Perot etalon model which uses two parallel surfaces. Beach et al. (2015) observed these spectra as a simple sine wave-like pattern, similar to the one produced by etalons in back-illuminated CCD image sensors. They explained the pattern as a product of

the beetle spores' non-parallel surfaces. In addition to this, the exoskeleton scatters and absorbs light due to its lack of uniformity, adding to the less than optimum etalon production.

These method utilizes the space between pulses to determine size, and this space varies from point to point. Due to this, some averaging is required to utilize the etalon model (Beach et al. 2015). However, since we are only concerned with discovering the origin of the oscillations, the etalon model is fitting as it can determine the size of adhering spores.

Reflections straight off the beetle's surface are off a higher refractive index and return 180° degrees out-of-phase with the original light (Beach et al. 2015). The internal path is through a higher refractive index, so reflections are from a high to low index and are therefore in phase. This final phase of reflection is determined as direct or indirect depending on the internal length of the light pathway and the optical wavelength. These reflections stay out of phase with each other, causing destructive interference and therefore reducing the light intensity, if there are a whole number of wavelengths to fill the pathway of the round trip. If the optical path difference is half an odd multiple of the wavelength then the reflections come into phase and increase the intensity. As a result, broadband illumination reflects as a function of wavelength, causing the reflected light spectrum' oscillations to appear between minimum and maximum values. This process is similar when the light passes through an object of lower refractive index, where the phase relationship is reversed but the oscillation period of the reflection spectrum is the same. The etalon free spectral range, defined by the separation between maxima, is equivalent to twice the number of wavelengths that go between the surfaces. The necessary number of optical waves, *n*, is determined by wavelengths of the two successive maxima by equating the conditions:

$$(n + 1)\lambda_{i-1} = n\lambda_i$$

$$= d$$

Here, λ_{i-1} is the shorter wavelength maximum and λ_i is the next longer maximum. This function *i* defines a series of maxima with lengthening periods. As the wavelength increases, the oscillation frequencies decreases, following an inverse relationship. While this reflects an ideal situation, in actuality, the refractive index of the object is not constant across the wavelength and is obvious only if the range of the wavelengths is large. The number of roundtrip wavelengths with respect to wavelength λ_i is found by:

$$n = \lambda_{i-1}(\lambda_i - \lambda_{i-1})^{-1}$$

$$= \lambda_{i-1}\, \nu_i$$

Here the oscillation frequency, ν_i takes the place of the reciprocal of the wavelength interval. The roundtrip distance through the object is also defined as:

$$d = 2\, \eta\, D \cos(\Theta)$$

Here, η is the refractive index, D is the distance between surfaces, and Θ is the angle of the light as it hits the surface. With regards to the nm range of 600 and 750, η is equivalent to 1.53 for fungal spores (Hart and Leski 2006) and 1.6 for chitin, the latter of which makes up the composition of insect exoskeletons (Azofeifa et al. 2012). Combining our first equation and this latest one for distance, we get:

$$D = n\, \lambda_i\, (2\, \eta \cos(\Theta))^{-1}$$

$$= \lambda_{i-1}\, \nu_i\, \lambda_i\, (2\, \eta \cos(\Theta))^{-1}$$

Assuming the cosine value is 1 (Beach et al. 2015), the latest equation represents the object dimension, defining it as proportional to the oscillation frequency between the two successive maxima. Given this, as long as η is known, this formula is valid over a wide range of wavelengths. High frequencies correspond to large dimensions, while lower frequencies correspond to smaller dimensions. While the input of our model is the separation (nm) between

adjacent peaks, the output is the value *D*. After converting to microns, this value serves as an estimate for the size of surface features.

Sampling error and background noise can affect results. Therefore, best values for maxima were determined by removing random noise with the use of a three-point moving average filter. The resulting wave then produced with frequency representing a linear function of wavelength.

3.3.8 Object Mapping by Fourier Analysis

Spore mapping was done with the use of the scientific image analysis program ImageJ. Similar to Beach et al. (2015), a code was generated for use after examining amplitudes of spores on slides and beads to map possible regions on spore loaded beetles while excluding non-spore features.

3.4 Data Analysis

Microscope captured images were analyzed with the ENVI 4.8 (Exelis Visual Information Solutions, Boulder, CO) program with features customized for microscopy (CytoViva, Auburn, AL). ImageJ was used to process data cube files and locate coordinates of interest. Spore sizes were calculated with the use of Excel 2016. SAS 9.4 was then used on compiled species' sizes to determine an analysis of variance. Paired species were then subsequently used in a T-test to determine if they could be differentiated.

3.5 Results

Initial fungal smears on glass microscope slides produced observable spores at 100X magnification (Fig. 3.5) and associated wavelengths at certain coordinates on the edge of spores (Fig. 3.6). In Figure 3.5, the size of the selected spore at that coordinate was determined to be

1.583 µm when considering the two valleys in the wavelength, 449.75 and 495.11 nm, as per the model equation (Beach et al. 2015).

However, despite these results, additional reflectance from the clear glass slide used provided less than optimal waves, even if sizes were consistent. As the alternative, we turned to using glass seed beads which had the benefit of being black while fitting inside the microscope view piece. Spores also adhered easily to the sterilized bead surface and these were visible under the lens (Fig. 3.7). Similar to methods before, coordinates were located, wavelengths obtained, and sizes measured. In this way, we looked at spores of *Grosmannia alacris* and obtained wavelengths (Fig. 3.8).

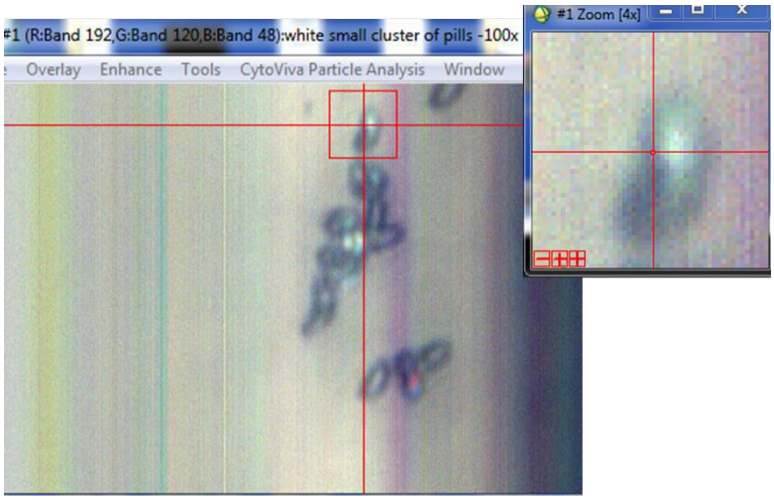

Figure 3.5. Spore smears of *Grosmannia alacris* on glass, viewed at 100X with a coordinate selected for the edge of a spore.

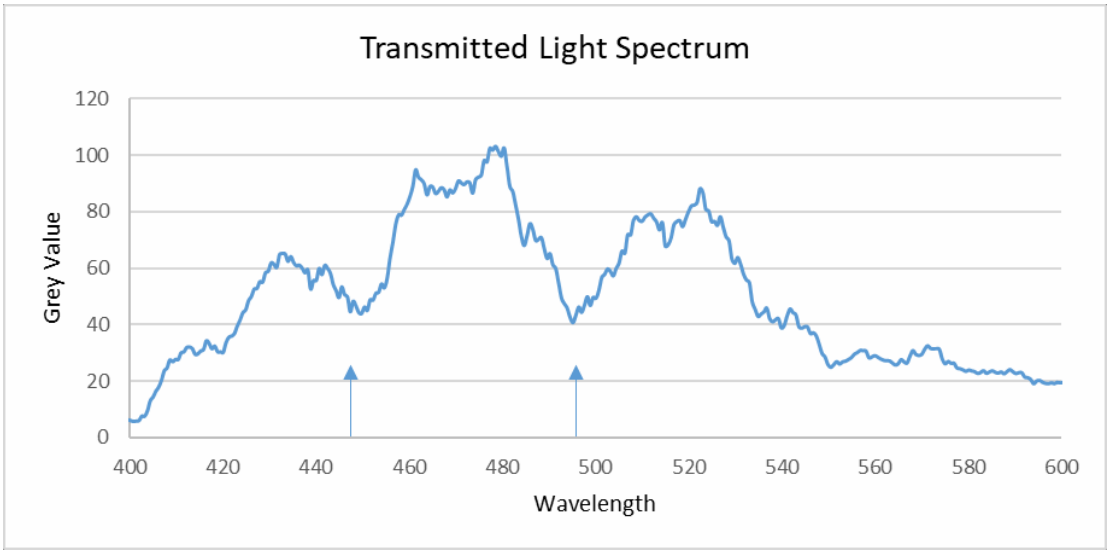

Figure 3.6. The associated wavelength obtained from the coordinate outline in Figure 3.4. Arrows pointing to the valleys are used in size calculation, each at 449.75 and 495.11 nm, respectively.

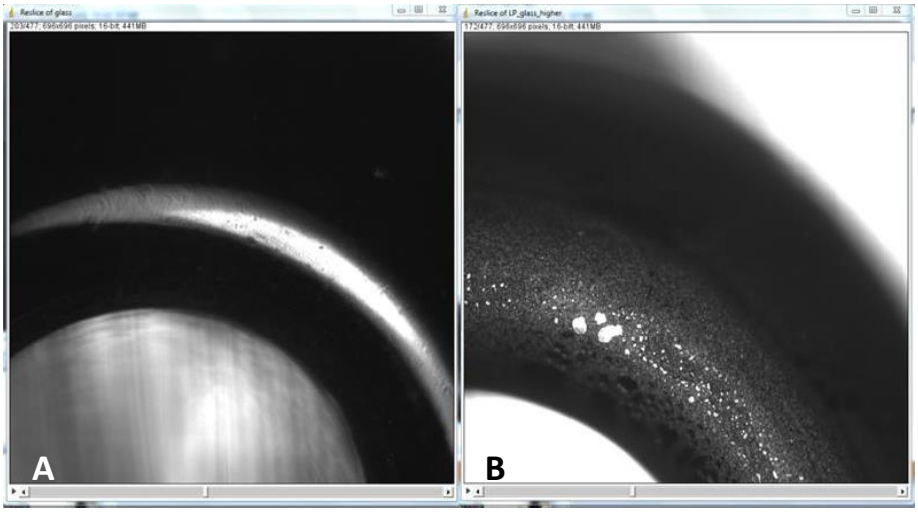

Figure 3.7. Glass seed beads (A) without spores and (B) with spores coating the surface.

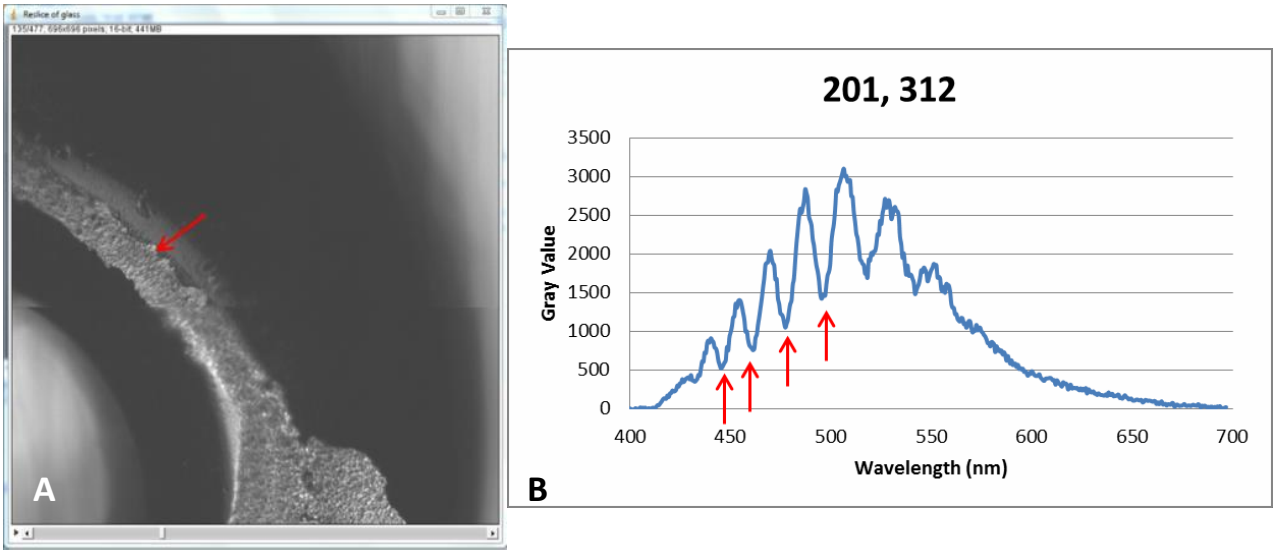

Figure 3.8. (A) A black glass seed bead spore loaded with *Grosmannia alacris* spores and a point associated with (B) the wavelength obtained for sizing, with red arrows denoting minima used in size determination.

To quicken spore region identification, we sought to develop a universal tool for use on images of beetle surfaces. Using initial amplitudes for spores we found, a code was generated for use in ImageJ to aid in locating spore areas on beetle surfaces. Named “FFT map” for its origin in the fast fourier transform method, ensuing images showed areas of potential for spores (Fig. 3.9). In addition to these images, our map code also produced an associated histogram and power spectrum for the overall image, the latter of which provided a relative measure through an index of frequencies, and which may provide an area for future study.

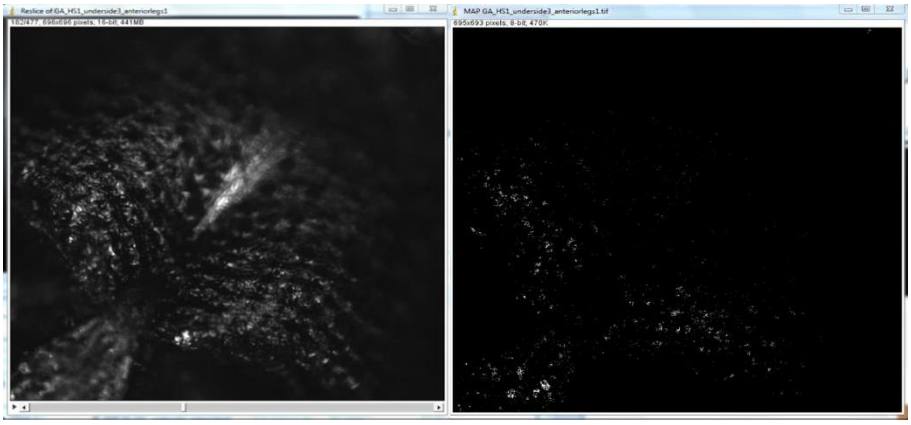

Figure 3.9. (A) An elytral surface of a *H. salebrosus* beetle rolled in spores and (B) regions identified as potential spore locations according to the FFT map code.

Beetles were spore loaded the same as beads and placed under the microscope. Values for each sample are listed in Table 3.1. The average results and p values from the one-tailed T tests are shown in Table 3.2. Two spore species – *G. alacris* and *L. procerum* – were significantly different in size from the others (F=7.07; P=0.0020; df=2; ANOVA; Fig. 3.10).

3.6 Discussion

Oscillations in the spectrum of reflected light carries information about features on the surface of an object. Beach et al. (2015) showed that these features could be defined as diameters of clinging spores. We were able to observe spores in the visible and near-infrared range and deduce their size in methods replicable to the Fabry-Perot etalon model of their previous work. This work served as a pilot study for determining spore species based on hyperspectral

Table 3.1. Individual spore sizes recorded for each species using the hyperspectral method[a,b]

Species	*G. alacris*	*L. procerum*	*Graphium* sp.
n	8	22	22
Sizes (µm)	2.7	3.86	1.97
	2.34	3.85	1.7
	2.2	3.12	3.22
	3.8	3	1.62
	2.68	3.2	3.19
	4.01	2.98	3.29
	3.34	2.67	1.92
	4.1	2.37	2.69
		2.56	1.88
		2.83	2.24
		3.69	1.53
		3.32	2.17
		2.85	2.5
		1.78	2.31
		1.9	2.84
		2.25	1.99
		3.06	1.77
		3.82	2.53
		1.78	2.22
		4.36	2.7
		4.56	1.84
		2.13	2.8

[a]Samples from single image pixels.
[b]Refractive index 1.53 is assumed.

Table 3.2. Fungal species *Grosmannia alacris* (GA), *Leptographium procerum* (LP), and the *Graphium* species (Gr), their documented sizes according to non-hyperspectral methods, our average size findings, and p values of t test results.

Species	Documented sizes (µm)	N	Our Average Size (µm)	GA	LP	Gr
GA	(4.5-6.8) x (1.8-2.6)	8	3.15	-		0.0011*
LP	(3-5) x (1-3)	22	3	0.325	-	0.00089*
Gr	(0.6-1.1) x (0.7-1.2)	22	2.31	0.0011*	0.00089*	-

*Significance.

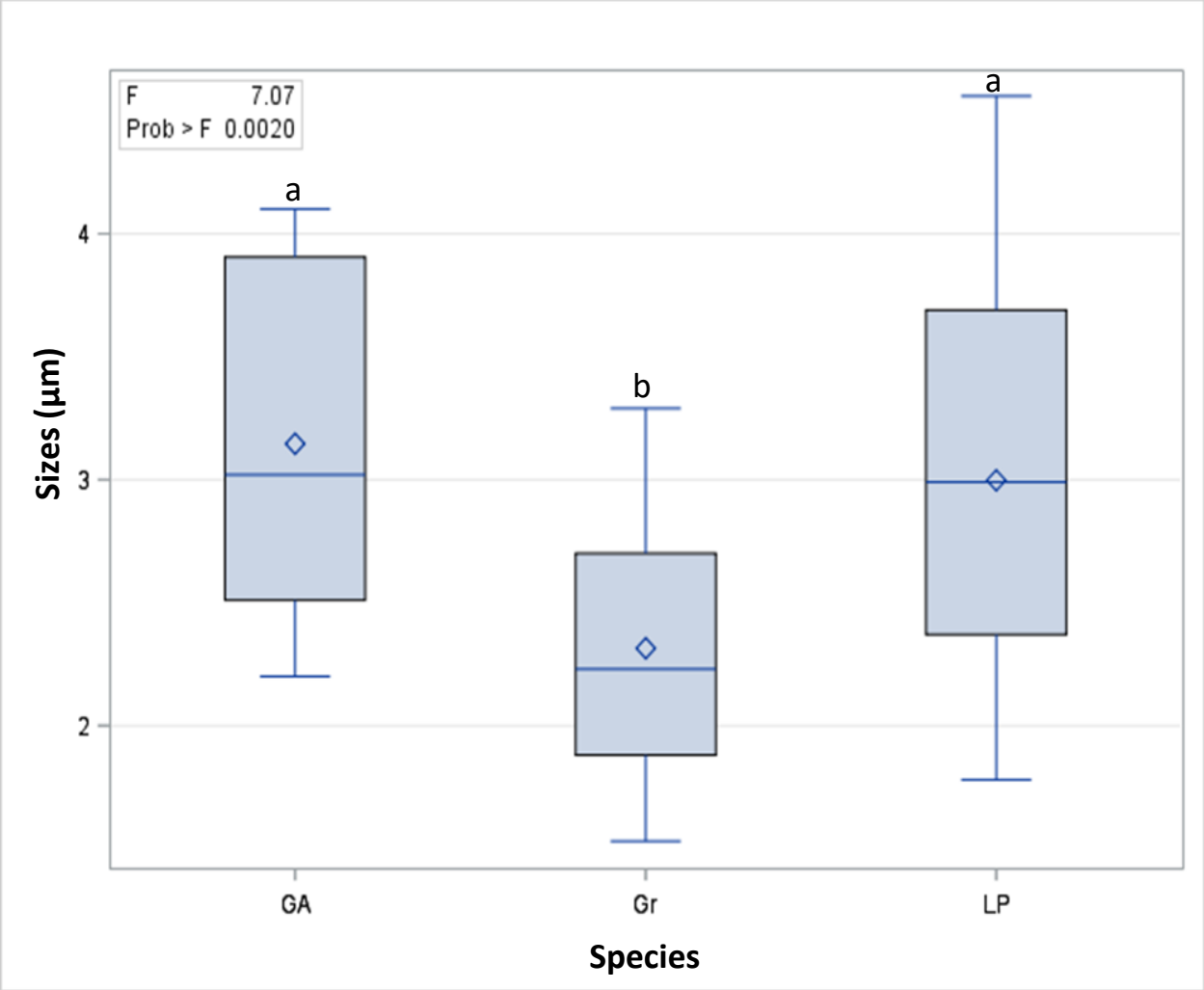

Figure 3.10. Maximum, average, and minimum sizes for each *Grosmannia alacris* (GA), *Graphium* sp. (Gr), and *Leptographium procerum* (LP), where means associated with a different lowercase letter are significant.

interferometry. It was the first time interferometry has been used with the focus on spores and not only surface features of the beetle (Parker and Lawrence 2001; Galusha et al. 2008). Previous research has investigated insect colors as an impact of light reflection on body surfaces (Ghiradella 1991; Seago et al. 2009; Sharma et al. 2009) and the potential impacts on visual signaling (Shevtsova et al. 2011). Hyperspectral interferometry can identify fungal spores on the body of bark beetles to an extent, and our FFT map shows potential in identifying areas where

spores can reside, however it needs much more automation. Bright spots for potential spore locations only showed up when the threshold value was just below the peak amplitude value. In addition to this, surface features such as setae can create etalons that are picked up on. A more universal, autonomous map should be developed, as this challenge is made more difficult by the fact that a beetle's surface provides more interference than a glass slide. This method could provide a simpler, optical technology for determining spore loads of beetles, aiding in an even quicker identification.

While we were able to observe spores on glass slides, getting consistent signatures from black, glass seed beads proved to be more successful, providing a concrete foundation for obtaining spore data on beetle surfaces. Our ANOVA and t tests results showed that we could differentiate two out of our three spore species with hyperspectral interferometry. However, *L. procerum* and *G. alacris* have close average mean sizes and likely cannot be discriminated on this alone. Data from our hyperspectral work (Fig. 3.10) supported previous measurements of *L. procerum* by Beach et al. (2015) and provided new data for *G. alacris* and the *Graphium* species through the use of hyperspectral interferometry. Our *Graphium* species' spores were shown to be smaller than the other two species, both in hyperspectral and manual methods. Differences in regards to this species' manual and hyperspectral methods may have resulted from the mucilage covering. Manual measurements were conservative where masses on beetles can be composed of many spores and mucilage. Additional areas of future research may focus on discerning differences in these coverings.

Considering the impact fungal carrying beetles can have on commercial forestation (Eckhardt et al., 2004a), these findings carry interest. Fungal identification can take weeks for samples to be collected and grown and for analyses such as DNA fingerprinting or PCR to be

completed (Six et al., 2003). Further development in optical modeling and in hyperspectral techniques presented here and by Beach et al. (2015) may help to specify fungal spore identification and may have implications for other industries.

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