IN THE EYE OF A HURRICANE: THE EFFECTS OF PRESCRIBED BURNS AND SALVAGE LOGGING ON LOWER STEM AND ROOT FEEDING BEETLES AND THEIR SYMBIOTIC BLUE-STAIN FUNGI

by

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(Under the Direction of Caterina Villari and Kier Klepzig)

ABSTRACT

Hurricane Michael caused immense damage in Southwest Georgia passing directly over the Jones Center at Ichauway on October 2018. This area includes 11,400 ha of longleaf pine (*Pinus palustris* Mill.) forest, which is a threatened ecosystem across the southeastern USA. After wind damaging events, the stress endured by the forests can make them highly susceptible to secondary agents such as bark beetles and blue-stain fungi, which overall exacerbate their already compromised health. With this thesis, we seek to better understand the effects of post-storm management practices such as salvage logging and subsequent prescribed burns have on longleaf pine associated lower stem and root-feeding beetles and their symbiotic blue-stain fungi. In our first study, we analyzed the abundance of six bark beetle species for two years following Hurricane Michael and that of blue-stain propagules on *Hylobius pales* and *Pachylobius picivorus* after different three management possibilities and found that time, and not the treatments, was the main driver of beetle abundance in the year following the hurricane. We did see a delayed effect on the first-year management activities. Abundance of *Dendroctonus terebrans, Hylastes porculus,* and *H. tenuis* increased in the second year of collection, the year following the management activities. We also

found that there was no significant difference in the number of blue-stain propagules across management treatments. In the second study, we identified the species of blue-stain fungi phoretically carried by three target beetles and within longleaf pine roots. We found *Leptographium profanum* and *Ophiostoma ips* on root-feeding beetles, *L. terebrantis* on *Dendroctonus terebrans,* and *L. profanum* in one longleaf pine root. In our system, post-hurricane management strategies did not affect the abundance of lower stem and root feeding beetles and their symbiotic blue-stain fungi in a longleaf pine ecosystem. We also found *L. profanum*, which had been previously isolated only from hardwoods, to be abundant in the longleaf pine-root feeding insect system.

INDEX WORDS: Longleaf pine, *Pinus palustris*, bark beetles, *Hylastes*, *Hylobius*, *Pachylobius*, *Dendroctonus,* blue-stain fungi, *Ophiostoma*, *Leptographium,* Hurricane Michael, wind damage.

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DEDICATION

This is dedicated to my parents; I appreciate all their support and encouragement to continue my path.

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CHAPTER 1

THESIS INTRODUCTION AND LITERATURE REVIEW

1.1 The Longleaf Pine Ecosystem

Longleaf pine (*Pinus palustris*) was well-established in the southeastern United States since pre-Columbian times. In the past, longleaf pine was maintained by Native American tribes through purposeful burning to manage their environment. By reducing wildfire fuel loads and enhancing desired wildlife habitats, they improved edible plant habitat and wildlife populations, thereby increasing their food supplies (Van Lear et al. 2005).

By the time that Europeans began to colonize North America, longleaf pine covered the southeastern United States from southeastern Virginia to central Florida and west to eastern Texas (Brockway and Outcalt 1998), encompassing the geographical locations of Piedmont, Blue Ridge, Ridge, and Valley, and Cumberland Plateau regions (Van Lear et al. 2005). Longleaf pine dominated about 30 million ha and was also commonly found in smaller pockets of approximately 7 million ha within mixed stands (Van Lear et al. 2005). At present, longleaf pine has lost approximately 97% of its original habitat and in 1995 was ranked as one of the top three most endangered ecosystems in the United States (Van Lear et al. 2005). This massive reduction of longleaf pine coverage is the result of clearing land for agriculture, pastures, development, and conversion to other more lucrative pine species such as loblolly pine (*Pinus taeda*) and slash pine (*Pinus elliottii*) (Brockway and Outcalt 1998). Further, the most impactful contribution to the reductions of longleaf pine coverage was the containment of natural wildfires which lead to a regime shift to other pines or hardwoods (Brockway and Outcalt 1998). However, beginning in the 1940s, the United States Forest Service reintroduced fire to reduce fuel loads in the historical longleaf pine range (Van Lear et al. 2005). At the present, resource

managers use prescribed fire to prepare sites for planting, improve site quality, plant diversity, wildlife habitat, carbon sequestering, and enhance forest aesthetics, resulting in 3.2 million ha being burned annually in the South (Alavalapati et al. 2002, Van Lear et al. 2005).

Longleaf pine is an important forest resource in the Southeast and is known to be more resistant to natural disasters such as windstorms and to have lower mortality rates from southern pine beetle (Susaeta and Gong 2019). These two benefits are becoming increasingly important in the future because of an increase in storm intensity associated with climate change (Susaeta and Gong 2019). Although its coverage has been greatly diminished, longleaf pine can still be found throughout the southeastern United States (Oswalt et al. 2012). From the historical area, the remaining 1.3 million ha is spread out through the Florida Panhandle, southern Alabama, Georgia, and Mississippi, within mostly private ownership (Susaeta and Gong 2019). The importance of longleaf pine ecosystems is highlighted by the fact that they are among the most species-rich in North America. Although the canopy is dominated by a single species, the understory is home to an incredible diversity of herbaceous plants, encouraging higher biodiversity in the lower strata of the forest (Frost 2006). For example, longleaf pine is associated with wiregrass in the eastern part of the coastal plain. Longleaf pine-wiregrass systems are characterized by open savanna conditions and lack of shrubs and hardwoods. Wiregrass also contributes to this fire dependent ecosystem by enhancing fire ignition and spread, being thus a keystone species (Brockway and W. Outcalt 1998). In addition, longleaf pine ecosystems provide habitat for endangered species like the red-cockaded woodpecker, the gopher tortoise, the fox squirrel, the Florida pine snake, and the gopher frog (Kirkman and Jack 2017). Longleaf pine ecosystems can also include other micro-habitats such as upland pine grasslands, rare habitats such as sinkholes, depressional wetlands, hammocks, and wetland-slash

upland ecotones (Van Lear, et al. 2005). The longleaf pine ecosystem is characterized by frequent low to moderate intensity fires that do not cause large scale changes in forest structure. Since these fires are typically not lethal to the coevolved vegetation found within the longleaf pine ecosystems, they instead function as a release disturbance. The intervals between fires are short, usually 2-5 years, thus preventing a large accumulation of fuel between burns. This promotes a moderately intense fire that longleaf pine is adapted to and thrives in as is seen in the fire-resistant grass stage (Van Lear et al. 2005). After a period of time in the grass stage, longleaf pine attains rapid height growth and becomes very fire tolerant.

In addition to being highly fire resistant, longleaf pine regeneration is driven by its extremely high shade intolerance such that it can usually only be found in canopy gaps. Longleaf pine is among the most light demanding species in North America (Ashton and Kelty 2018). Moreover, it is also been documented that competitive influences from surrounding mature longleaf pine trees encourage further growth of saplings (Brockway and Outcalt 1998). Along with fire disturbances, longleaf pine is exposed to other types of disturbance such as lightning strikes, which can cause pockets of tree mortality and result in gaps and wildfires, and hurricanes (Landers et al. 1995). Due to its importance, the restoration of longleaf pine to its historical coverage has long been promoted by several organizations such as the USDA Forest Service, Longleaf Alliance, and the Longleaf Pine Initiative (Georgia Prescribed Fire Council). The main tool to accomplish this restoration to date has been prescribed fire, resulting in approximately 1.5 million ha of longleaf pine in Georgia being burned annually (Oswalt, et al. 2012). However, it is expected that with proper management longleaf pine can be restored even further, adding diversity to the southeastern United States ecosystems and landscapes (Oswalt, et al. 2012).

1.2 Hurricane Michael

From a forest management perspective, windstorms are among the most important and damaging events. The degree of damage sustained is influenced by wind speed, wind direction, and local topographic features (Miller 1985), resulting in defoliation through wind shear, broken stems, snags, downed trees, and timber loss (Landers et al. 1995). Not only are individual trees affected by intense wind, but windthrow can also cause changes to soil structure and influence tree regeneration (Landers et al. 1995). While this is a natural disturbance, the intensity and frequency of hurricanes are incredibly important to the longleaf pine ecosystem as a large portion of the native range of longleaf pine is within 150 miles of the Atlantic Ocean and subject to damaging tropical storms and hurricanes (Landers et al. 1995). Longleaf pine is considered to be more resistant than other pines to wind damage, due to its stem properties like elasticity, wood strength, and structural differences like the crown and branching patterns (Rutledge et al. 2021). However, hurricane damage is still a concern, especially due to anthropogenic driven climate change during the 21st century, and the fact that the number of global hurricanes attaining at least category four is projected to increase (Cole et al. 2021). Although most models predict little change in global frequencies of hurricanes, it is possible that maximum wind speed and precipitation rates will increase for future hurricanes (Landers et al. 1995). To put this in perspective, it was estimated that the amount of wind damage to forest resources caused by hurricanes Katrina and Rita was between $2-3 billion, affecting 5.5 million acres of timberland between Texas, Louisiana, Mississippi, and Alabama (Cole et al. 2021).

In October 2018, Hurricane Michael impacted the southeastern US causing severe damage to timber stands, forests, agriculture fields, and structures. Upon making landfall, Hurricane Michael produced a total of 16 confirmed tornadoes and widespread rainfall ranging

from 3 to 6 inches, but as much as 10 inches in some areas. This category four storm at the time of landfall in Florida was the strongest hurricane to impact the state within the last 50 years (Georgia Forestry Commission 2018). It then impacted Georgia as a category three hurricane before moving northward through Virginia. Although it weakened as it passed, the hurricane caused a tremendous amount of damage in southern Georgia, with wind speeds up to 240 kph (150 mph) and heavy rains, leaving snapped stems and coarse woody debris in its wake throughout agricultural land, urban landscapes, plantations and natural forests (Georgia Forestry Commission 2018). In Georgia, it resulted in nearly $3 billion of damage to the state's agricultural industry, and $1 billion in timber alone was destroyed (Brett 2018). Additionally, approximately 1 million acres of small and private landowners' properties were devastated (Brett 2018). However, this was only the immediate damage done. Hurricanes can cause long lasting effects not only due to sudden massive tree mortality but also because they initiate complex patterns of additional mortality, which include delayed mortality and altered patterns of forest regeneration (Dale et al. 2001). Resultant effects can include species shifts, increased landscape heterogeneity, faster biomass and nutrient turnover, and overall lower levels of mature vegetative biomass (Dale et al. 2001). It can also amplify the effects of other disturbances like fire or herbivory (Rutledge et al. 2021). These compounding factors can affect the balance of the longleaf pine ecosystem and increase stress allowing for secondary agents of disturbance to flourish.

1.3 Root and Lower Stem Feeding Beetles

Insects, including beetles, fill important niches within forest ecology, ranging from beneficial to destructive, and are the most abundant animal life form on earth (Baker 1972). They have been around for approximately 350 million years, occupying various habitats and filling

crucial ecological niches. However, some plant feeding insects, when conditions arise that favor their explosive growth, can become agents of destruction that can devastate ecosystems (Baker 1972). Economic losses can also occur when important commercial resources are affected by insect damage. This damage ranges from superficial wounding to major damage which can severely stunt or kill trees. (Baker 1972).

For instance, after wind disturbance events such as hurricanes, a primary disturbance agent which routinely affects longleaf pine forests, trees are stressed and secondary agents, such as tree colonizing beetles, may take advantage of these conditions and attack affected trees. Bark beetles attack and colonize phloem containing parts of a pine tree, and particular beetles, known as lower stem and root feeding beetles, only colonize the lower and below ground portions of the stem (Baker 1972). These beetles feed on and reproduce within the phloem of stressed or otherwise unhealthy trees. Additionally, they are known vectors of blue-stain fungi, some of which are pathogenic and may play a compounding role in the decline of the health of pine trees (Zanzot, et al. 2010).

Beetles in the genera *Hylastes*, *Hylobius*, *Pachylobius,* and *Dendroctonus* are subcortical feeders found in the roots and stems of trees (Baker 1972) (Figure 1.1). These beetles are in the family *Curculionidae,* which contains approximately 50,000 species worldwide (Ohsawa 2005). Several agriculturally destructive pests belong to this family, such as the cotton boll weevil (*Anthonomus grandis*), the alfalfa weevil (*Hypera postica*), and the grain weevil (*Sitophilus granarius*) (Baker 1972). Within the forests of the southeastern region of North America, the pitch-eating weevil (*Pachylobius picivorus*), the pales weevil (*Hylobius pales*), and beetles in the genus *Dendroctonus,* such as southern pine beetle (*Dendroctonus frontalis*) and mountain pine beetle (*Dendroctonus ponderosae*) are pests of note (Baker 1972), particularly on loblolly and

longleaf pine due to their impacts on Christmas tree farms and damage to pine regeneration (Baker 1972). Both *P. picivorus* and *H. pales* are in the subfamily *Molytinae* and the tribe *Hylobiini* and are generally characterized by short, broad snouts, and an oval depression on the face of the mandibles marked by the position of pupal cusps (Baker 1972). Larvae are found underground, where they feed on roots and pupate (Baker 1972).

Hylobius pales is considered the most serious insect pest of pine regeneration, feeding and ovipositing through the growing season into winter in the warm southern climate. The new adults emerge in the spring, with especially high trap rates in May and June (Peirson 1921, Salom et al. 1994), and begin feeding on pine seedlings. The feeding pattern of adult *H. pales* is to strip the bark from the stem and side branches in an irregular fashion leaving numerous crisscross ridges (Peirson 1921). These beetles typically occur in low numbers throughout forested areas but seem to be attracted to trees that have pitch or sap exposed, such as the trees adjacent or within lumbering operations or in areas with downed trees (Peirson 1921). The adult beetle is dark reddish-brown to black, 7 to 12 mm long, and has patches or lines of yellow-white scales on its head with irregular patches throughout the elytra (Baker 1972).

Pachylobius picivorus is found within the same geographic areas as *H. pales* and is known for its ability to injure and kill pine seedlings (Franklin and Taylor 2012). Adults are robust, dark brown, beetles with yellowish or reddish-brown hairs, and their tibia are thick and have a flared base, while their tarsi are densely covered in hair (Baker 1972). *Pachylobius picivorus* is common in the South and attacks several species of pine. The adults feed on the bark of small twigs and the stems of seedlings, and they are attracted to recently damaged pines and where new seedlings are established (Baker 1972). *Pachylobius picivorus* is similar to *H. pales* in that they both feed on the roots of pine trees and can inhibit seedling regeneration (Dixon and

Foltz 1990). They also have overlapping generations where they overwinter as adults, emerge in the spring, and oviposition in recently cut or stressed trees; however, larval development takes place during different seasons. *Pachylobius picivorus* complete its development in midsummer and is a well-established pest in southern forests, whereas *H. pales* completes its development in spring (Rieske and Raffa 1990). *Pachylobius picivorus* has been observed to have peaks during July and August, which is later than that of *H. pales,* which occurs from May to June (Franklin and Taylor 2012, Peirson 1921). Both *P. picivorus and H. pales* are capable of prolonged flight, but most short distance movement is through walking (Rieske and Raffa 1990).

Bark beetles (*Scolytinae*), also found in the family *Curculionidae*, cause major disturbances in conifer forests, affecting tens of millions of ha, specifically in western North America (Hicke et al. 2012). Within the United States, bark beetles cause the destruction of an average 25,500,000 m^3 of timber and pulpwood annually, which accounts for 90% of insect caused tree mortality (Baker 1972). While secondary bark beetle attacks occur in dead or dying trees, many primary bark beetle species can attack and kill healthy trees through a strategy called mass attack. This is initiated by a pioneer beetle which, if it can successfully enter a host tree, will start producing a pheromone attracting other bark beetles. As new individuals arrive, they continue to produce an aggregation of pheromones that will eventually attract a sufficient number of bark beetles to overwhelm the host tree's defenses, resulting in the death of a healthy host tree (Baker 1972). Finally, not only do bark beetle outbreaks affect timber production, but the increase in tree mortality may also increase fire fuels and wildfire behavior (Hicke et al. 2012).

The most damaging bark beetles tend to be found in the genera *Dendroctonus*, *Ips*, and *Scolytus* (Baker 1972). One of these beetles, *Dendroctonus terebrans*, the black turpentine beetle

(BTB), is one of two beetles in the *Dendroctonus* genus found in North America that occupies the lower 2 m of living host trees and is often found to be cohabiting the same tree as other species of bark beetles (Payne et al. 1987). The other species, *D. valens*, the red turpentine beetle, does as well but is found further north (Munro et al. 2019). The black turpentine beetle is described as dark reddish-brown to black and 5 to 10 mm long, its pronotum and elytra are coarsely and shallowly punctuated, and its head is densely granulated and roughly punctuate (Baker 1972).

Due to the nature of BTB, they rarely kill pines, and their associated blue-stain fungi may not be aggressively virulent in healthy trees, though they are capable of causing extensive tree defense reactions (Munro et al. 2019). BTB is known to be attracted to the volatiles of pines and is attracted to forested areas that have recently been logged, burned, or attacked by other bark beetle species (Payne et al. 1987). This beetle feeds on all species of southern pines and red spruce (*Picea rubens*) but is found to cause the most damage to loblolly and slash pine. Once a female BTB locates a suitable host tree, she excavates a gallery along the sapwood of the basal 90 cm of the trunk or on large roots, and after she is joined by a male, they clear the gallery of frass and the female will lay eggs into elongated pockets along the gallery, packing them tightly with frass (Baker 1972, Munro et al. 2019). The larvae feed on the phloem of pine trees while enlarging their chambers, where they will pupate. These larvae will pupate for two weeks and then move to the larval feeding chamber and emerge as adults either through holes formed by the parent beetles or by chewing new exit holes (Munro et al. 2019). All life stages can generally be found throughout the year in the South and typically 2 to 3 generations occur each year (Baker 1972). Signs of a tree colonized by BTB are whitish pitch and bark pellets at the base of the tree. Another sign is also the presence of piles of fine white sawdust at the base of trees, which are

produced by ambrosia beetles, secondary colonizers that frequently attack BTB colonized trees (Baker 1972).

A lesser-known genus of bark beetles, *Hylastes*, is in the subfamily Hylesininae. There are 15 *Hylastes* species found in North America and three of those are found within the eastern United States, *H. porculus*, *H. salebrosus*, and *H. tenuis* (Baker 1972). *Hylastes* primarily feed on the bases and roots of declining, wounded, dead, or dying pines and spruces and can occasionally kill young plantation trees through feeding (Baker 1972, Eckhardt et al. 2004).

1.4 Blue-stain fungi

The literature on the association of fungi with bark beetles is extensive and mature (Seifert et al 2013). Some of these fungi stain wood blue as they colonize it and are consistently vectored by bark and root beetles. These blue-stain fungi are mostly weakly virulent and belong to the Ascomycota order *Ophiostomatales*, in the genera *Ophiostoma*, *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia*, and related anamorphs such as *Pesotum* and *Leptographium* (Harrington 2005, Jankowiak 2013, Kirisits 2004, Six and Wingfield 2011).

Blue-stain fungi form close symbiotic relationships with their insect vectors, who benefit from the fact that fungi can detoxify tree defense metabolites, creating a suitable feeding environment for them. Fungi, on the other hand, benefit from the association with beetles because they provide means of dispersal for these otherwise sessile species (Biedermann and Vega 2020). Bark beetles bore into the bark of pines to access and feed upon phloem tissue and lay eggs. Some species of bark beetles have unique structures called mycangia that harbor symbiotic fungi which can be inoculated directly into the phloem (Six 2003); other bark beetles carry fungal spores phoretically on their exoskeleton (Six and Wingfield 2011). For example, the southern pine beetle has mycangia that may inoculate the tree with the nutritional mutualists

Entomocorticium cobbi and *Cranaculosus* that act as sources of nutrition. However, the beetle also phoretically carries *O. minus*, a blue-stain fungus that is antagonistic to the nutritional fungi but may help to decrease the vigor of the tree and detoxify defense metabolites (Diguistini et al. 2011, Klepzig et al. 2001). In addition to being pathogenic, blue-stain fungi can also diminish economic returns by reducing the value of pine logs through staining (Loeffler and Anderson 2018).

In the early 2000s, Eckhardt et al. (2004) observed declining or unhealthy stands of loblolly pine in the Southeast. Later termed Southern Pine Decline, they defined this syndrome as being exemplified by trees with short chlorotic needles, sparse crowns, reduced radial growth, and premature mortality (Eckhardt et al. 2004). They also noted being able to consistently isolate blue-stain fungi (specifically *L. terebrantis*, *G. huntii*, *L. procerum*, and *G. alacris*) from pine roots (Eckhardt et al. 2007). These associations, and seedling inoculations producing resinous lesions, led some to conclude that these fungi are virulent pathogens that actively affect the health of pine trees (Devkota and Eckhardt 2019). However, research is insufficient to fully understand the effects of blue-stain fungi in this system (Otrosina et al. 1999). While blue-stain fungi may be found where pines are in decline, it is unknown if they are primary agents or just consequences of disturbance (Coyle et al. 2015).

When compared to loblolly pine, little literature is available on the importance of blue-stain fungi in longleaf pine ecosystems. More research is needed to elucidate the relationship among longleaf pine, lower stem, and root-feeding beetles and their associated blue-stain fungi. While these beetles individually cause physical damage to pine trees, the addition of blue-stain fungi may enhance damage done and reduce the economic value of attacked trees.

1.5 Management of longleaf pine ecosystems

Longleaf pine ecosystems benefit from prescribed low-intensity fires and provide high biodiversity and increased resilience to natural disturbances, improving the general resilience of forests in North America (Susaeta and Gong 2019). Currently, there are several efforts to restore longleaf pine in its native range. The longleaf alliance was established in 1995 to coordinate partnerships of landowners, industries, public agencies, conservation groups and researchers, America's Longleaf (Hertz and Jones 2022), and the Million Acre Challenge (Matthews et al. 2020) in restoring longleaf pine forests (Susaeta and Gong 2019). However, longleaf pine is generally less profitable for timber production than other timber pine species and there is a lack of information on how economical it is to manage longleaf pine (Stainback and Alavalapati 2004). There are programs specific to longleaf investment that promote economic incentives for carbon sequestration, wildlife habitats, silvopasture, and other non-timber incentives (Susaeta and Gong 2019). However, there are also areas like Tall Timbers and the Jones Center at Ichauway that manage for longleaf pine. The Jones Center at Ichauway is located in Baker County, Georgia (31.22N, 84.48W), and is an 11,400 ha restored longleaf pine forest. The area has a humid subtropical climate that is characterized by a mean annual temperature of 19 °C and long hot summers with short, moderate cooler winters. It has an annual precipitation of approximately 1,310 mm per year that is evenly distributed throughout the region (Wagner, et al. 2019). Occupying approximately 60 to 90% of the canopy basal area, longleaf pine is the dominant canopy tree (Wagner, et al. 2019). The forested areas average between 6 to 20 m^2 ha of basal area of longleaf pine that are mostly 80 to 100-year-old second growth mature pine trees and is sparse in shrubs and midstory but encourages Wiregrass as a robust understory (Wagner, et al. 2019). The Jones Center at Ichauway manages for longleaf pine with a 2-to-3-year fire rotation, changing based on the ability to safely burn in the spring. Prescribed burning is affected

by the weather and wind direction to avoid smoke on nearby highways. During the growing season, approximately 60 percent of the forested areas at Ichauway are burned annually (The Jones Center at Ichauway 2022). While other silvicultural practices are designed to conserve biological diversity, water resources and maintain ecosystem functions, the Jones Center at Ichauway has the primary objectives of reducing fire fuels, wildlife management, research, and restoration of the longleaf pine ecosystem (The Jones Center at Ichauway 2022, Mitchell et al. 2006). The longleaf pine forest is managed using a modified Stoddard – Neal approach where some level of canopy cover is maintained, and harvests are conservative. Individual tree selection is utilized to maintain the proper distribution of fine fire fuels and to minimize impacts to wildlife habitats (The Jones Center at Ichauway 2022). Timber resources are carefully targeted and salvage operations are performed as needed along with on-site salvage operation to remove hazardous trees near roads and buildings (The Jones Center at Ichauway 2022).

1.6 **Thesis objectives**

Even though there is an increased interest by private landowners and government programs, there are still gaps in the knowledge of longleaf pine ecology and management practices, especially concerning wind disturbance events such as hurricanes. The overall goal of this thesis is to better understand how post hurricane management practices affect lower stem and root-feeding beetle abundance, and the blue-stain fungi carried by these insects. To accomplish this, we conducted two experiments at the Jones Center at Ichauway, a longleaf pine ecosystem heavily damaged by Hurricane Michael in October of 2018. The first experiment, described in **Chapter 2**, aims at comparing longleaf pine associated root and lower stem feeding beetle abundance and flight path across three management treatments: i) wind-disturbed (no active treatment in the year following the hurricane, but prescribed burning in spring of 2020);

(ii) wind-disturbed with prescribed burning in spring of 2019 and salvage logging in winter 2018 to spring 2019; and (iii) wind-disturbed with prescribed burning in spring of 2019 and no salvage logging in winter of 2018 to spring of 2019. We collected beetles in two, one-year periods that extended over a three-year time frame using a baited Lindgren funnel trap and identified two target groups of lower stem and root feeding beetles species, *H. salebrosus*, *H. porculus*, *H. tenuis*, *H. pales*, *P. picivorus,* and *D. terebrans* based on their morphological features. In addition, we cultured and counted blue-stain fungi CFUs from live collected *H. pales* and *P. picivorus* utilizing billet traps within the previously described plots. Results of this study will help to elucidate the relationship between removing coarse woody debris and prescribed burns to lower stem and root feeding beetles' abundance and blue-stain fungi.

In **Chapter 3** we characterized the blue-stain fungi associated with the insects colonizing roots and lower stems within the same management plots mentioned in the previous chapter. We collected live beetles during their peak of flight period and isolated their associated fungi by washing the beetles and plating different dilutions of the washing solution onto selective media. We then identified fungal colonies to the specie level by a combination of morphological observations and molecular approaches. In addition, we collected root samples from healthy and symptomatic trees and assessed the presence of the fungi in the tree hosts.

The impacts of damage sustained during Hurricane Michael could have further effects through trophic cascades impacting other organisms such as bark beetles and their associated fungal species. By further investigating the relationship between silvicultural practices such as salvage logging and prescribed burning to lower stem and root feeding beetles and the blue-stain fungi they carry, we can better inform managers on what treatments to follow in the years after major storm events like hurricanes. With this information, we aimed to provide a starting point

for future improvements to recommendations for managers of longleaf pine forests that have been affected by catastrophic wind events.

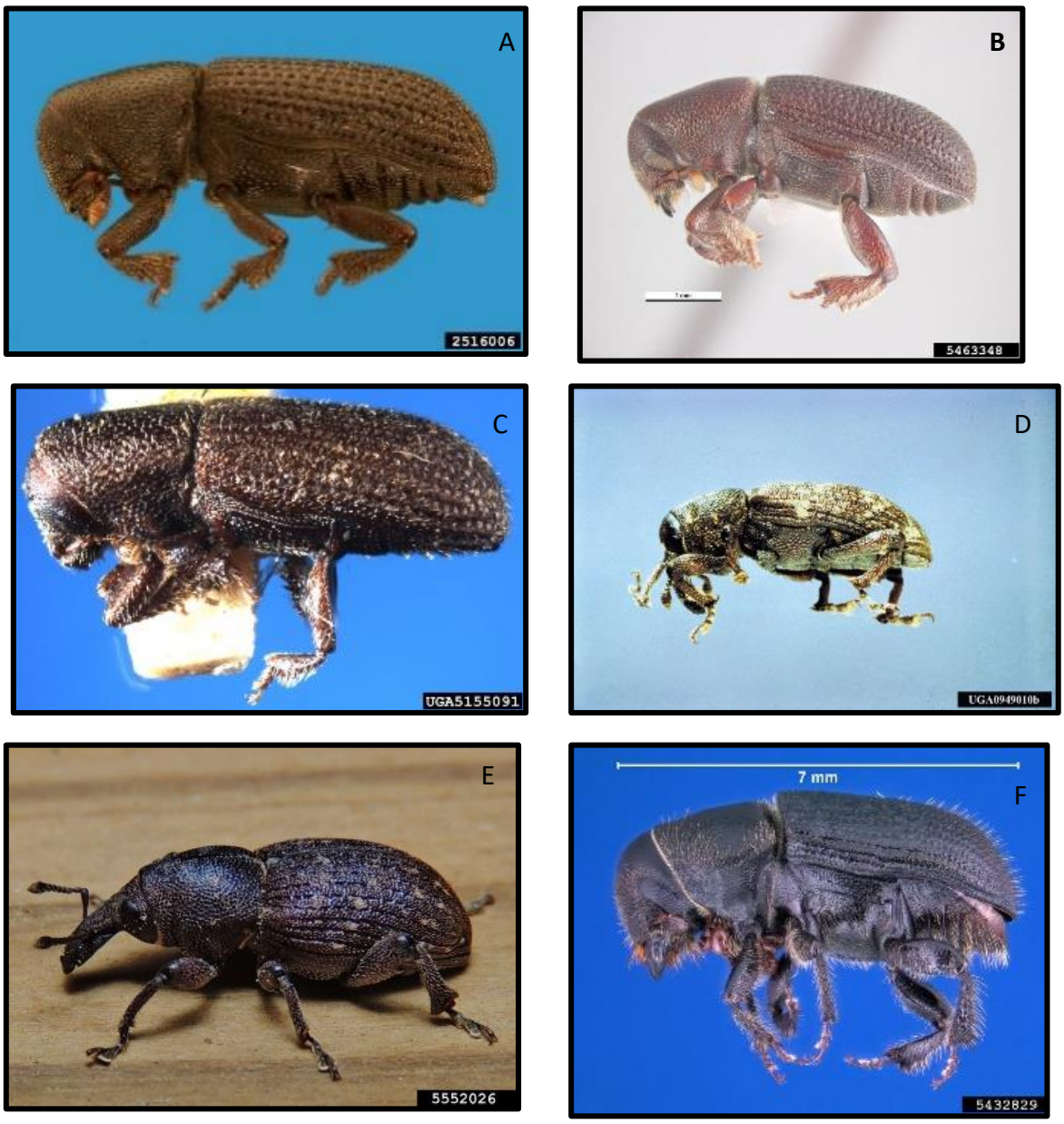

Figure 1.1 Lower stem and root feeding beetles *Hylastes porculus*, *Hylastes salebrosus*, *Hylastes tenuis*, *Hylobius pales*, *Pachylobius picivorus*, and *Dendroctonus terebrans.* Image credit: (A) David T Almquist, bugwood.org; (B) Pest and Disease image library, bugwood.org; (C) J. R. Baker and Bambara, bugwood.org; (D) Wayne N. Dixon, bugwood.org; (E) Royal Tyler, bugwood.org; (F) Erich G. Valley; bugwood.org

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CHAPTER 2

RESPONSE OF LOWER STEM AND ROOT FEEDING BEETLES AND THEIR SYMBIOTIC BLUE-STAIN FUNGI TO WIND DISTURBANCE MANAGEMENT PRACTICES WITHIN A LONGLEAF PINE WOODLAND IN SOUTHWEST GEORGIA[1]

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Abstract

In October 2018, Hurricane Michael made landfall in the Florida Panhandle as a category four hurricane and devastated timber stands, forests, agriculture fields, and infrastructures in its path, as it moved northeastward across the southeastern USA. In addition, the incredible amount of damaged trees and coarse woody debris Michael left behind created an optimal environment for secondary forest pests, such as lower stem and root feeding beetles. To better understand the impacts of post-hurricane forest management on secondary pests of longleaf pine ecosystems, we established a research project at the Jones Center at Ichauway, a restored longleaf pine ecosystem in southwest Georgia that was directly impacted by Hurricane Michael. Following silvicultural management options in response to catastrophic wind disturbance events, we established 5 replicates within sites managed using three approaches: (i) no salvage logging and no post-hurricane prescribed burning in the year following Michael, (ii) no salvage logging, but prescribed burning the spring following Michael, and (iii) both salvage logging and prescribed burning in the spring after Michael. Within each plot, we characterized the seasonal patterns of flight activity of beetles within the genera *Dendroctonus*, *Hylastes*, *Hylobius*, and *Pachylobius* for two years following the hurricane event. We found that time of the year is a significant factor for determining beetle abundance in both years while treatment or the interaction between treatment and time were only significant for three beetle species, *D. terebrans*, *H. porculus*, and *H. tenuis*, in the second year. We also calculated the beetle abundance during peak flight for each species and found that in year one only *H. salebrosus* and in year two only *H. porculus* were significantly affected by the treatment. We then quantified the number of propagules of blue-stain fungi vectored by *H. pales* and *P. picivorus* during their flight peak in Fall 2020 and found that treatment did not affect the number of blue-stain propagules. This information suggests that management to reduce coarse woody debris affects species *D. terebrans*, *H.*

porculus, and *H. tenuis* two years after hurricanes, but does not affect *H. pales*, *P. picivorus*, or their symbiotic blue-stain fungi.

2.1 Introduction

Longleaf pine (*Pinus palustris)*, is an important forest resource that once covered about 30 million ha in the southeastern United States (Van Lear et al. 2005). The longleaf pine ecosystem is the most species diverse in North America (Sharma et al. 2020). These ecosystems are characterized by a canopy dominated by longleaf pine, a sparse mid-story, and a diverse understory of wiregrass, hundreds of species of herbaceous plants, and habitats for other endangered species (Brockway and Outcalt 1998, Frost 2006, Knapp et al. 2018). This species is seen as being more resistant to natural disasters and disturbances, such as windstorms and pest outbreaks, than other southern pines (Susaeta and Gong 2019). Longleaf pine once dominated its range due in part to its evolution with wildfires, which can inhibit other pines and hardwoods that could compete with longleaf pine (Knapp et al. 2018). Unlike other species that are not fire-adapted, longleaf has a grass stage that protects it from low-intensity fires that could kill the saplings of other pine species (Knapp et al. 2018). Other disturbances, such as lightning strikes and wind disturbances, also promote longleaf reproduction, by creating gaps that encourage new growth of this very shade-intolerant species (Brockway and Outcalt 1998). Yet, currently, about 97% of its original range has been reduced to the point that the species is ranked as one of the top three most endangered ecosystems in the world (Van Lear et al. 2005). While hurricanes are a natural occurrence in the southeastern United States, they appear to be increasing in number and intensity (Cole et al. 2021). For example, in October 2018, Hurricane Michael made landfall in Florida as a category four hurricane and was the fourth-strongest hurricane to hit the United States (EPA 2019). It was also the strongest hurricane in the 30 years since Hurricane Andrew in

1992 (EPA 2019). Although it weakened as it passed over land, it caused enormous amounts of damage in southern Georgia, with heavy rainfall, and wind speeds up to 240 kph (150 mph) (Georgia Forestry Commission 2018). This caused immense damage and affected 5.5 million acres of timberland, agricultural fields, cities, and forests, also producing over 16 tornadoes and 3 to 6 inches of widespread rainfall (Cole et al. 2021). Hurricane Michael resulted in approximately $3 billion in damage to Georgia's agriculture industry and another $1 billion in cost to timber production (Brett 2018). In addition, 1 million acres of private lands were negatively impacted (Brett 2018).

While the initial damage caused by this hurricane was already massive, additional long-term effects were also of concern due to a large amount of weakened plants and coarse woody debris left behind. Coarse woody debris is an integral part of a functioning ecosystem because it influences energy flow, nutrient cycling, and provides habitats for a diversity of insects (Harmon et al. 1986). However, an increase in hurricane-driven coarse woody debris accumulation may attract bark beetles, due to increased release of pine volatiles (Munro et al. 2019). Tree colonizing beetles, such as bark beetles (*Coleoptera: Curculionidae: Scolytinae*) and pine root weevils (*Coleoptera: Curculionidae*: *Molytinae*), feed on and reproduce in the phloem layers of pine trees and play a compounding role in the decline of the health of pine trees after hurricanes (Zanzot et al. 2010). The black turpentine beetle (*Dendroctonus terebrans*), the pales weevil (*Hylobius pales*), the pitch-eating weevil (*Pachylobius picivorus*), and *Hylastes* root beetles, are pests of conifers and found in forests of the southeastern region of North America (Baker 1972). *Dendroctonus terebrans* feed on the lower 2 m of all pines and co-habits with other bark beetle species (Payne et al. 1987). Female beetles initiate the attack and bore egg chambers where the larvae will feed and grow until they pupate and emerge from the host tree (Munro et al. 2019). In

the South, they can usually be found year-round, having 2-3 generations per year (Baker 1972). *Hylobius pales* are a serious pest of conifer regeneration; they emerge in the spring and feed primarily on pine seedlings (Peirson 1921). *Pachylobius picivorus* is similar to *H. pales* in life cycle, with the variation of a later emergence during mid-summer (Rieske and Raffa 1990). *Hylastes* are a lesser-known species and feed on the bases and roots of dead or dying pines and spruces (Baker 1972, Eckhardt et al. 2004). Three of the 8 species in this genus are found in the eastern United States, *H. porculus*, *H. salebrosus*, and *H. tenuis* (Baker 1972). While lower stem and root feeding beetles are important insects and well-studied in other pine systems, their response to hurricane damage within the longleaf pine system is poorly understood.

These beetles are also associated with blue-stain fungi, which are mostly weakly virulent fungi in the Ascomycota order *Ophiostomatales*, in the genera *Ophiostoma*, *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia*, and asexual forms, such as *Pesotum* and *Leptographium* (Harrington 2005, Jankowiak 2013, Kirisits 2004, Six and Wingfield 2011). These fungi have a close symbiotic relationship with these beetles, benefitting from being inoculated into host pine trees by their vectors, which in turn obtain a more suitable feeding environment due to the fungi's ability to detoxify pine defense metabolites (Biedermann and Vega 2020). *Hylastes* species have been associated with declining pine stands and the presence of *Leptographium* spp, blue-stain fungi that affects many pine trees (Eckhardt et al. 2004). All beetles of the previously mentioned genera are associated with one or more *Ophistomatoid* species (Baker 1972, Eckhardt et al. 2004, Jankowiak et al. 2018, Linnakoski et al. 2012). For example, *H. pales* is a vector of *L. procerum*, and together with the beetle's feeding habits and the colonization area of this fungus within the root collar phloem of pines, these two agents can cause significant damage and

mortality (Jankowiak and Bilański 2013). However, there is little research on blue-stain fungi specifically within the longleaf pine ecosystem.

In response to site-wide wind disturbance from Hurricane Michael, forest managers of the Jones Center at Ichauway, an 11,400-ha restored longleaf pine forest in southwest Georgia, implemented combinations of silvicultural management practices such as prescribed burning to maintain longleaf pine dominance and salvage logging to reduce fuel loads. All areas of the property experienced significant damage, as the eye of the hurricane passed approximately 10 km away from the site: blocks dominated by longleaf pine suffered averages of 12.5% treefall and 14.8% tree damage (Rutledge et al. 2021). To better understand the impacts of post-hurricane forest management on secondary pests of longleaf pine ecosystems, we established a research project at the Jones Center at Ichauway to investigate the responses of lower stem and root feeding beetles in relation to the different management strategies implemented after hurricane damage. We hypothesized that areas with a higher amount of coarse woody debris (i.e. stumps, downed trees, and snags) will attract a higher abundance of lower stem and root feeding beetles, which will also carry a higher blue-stain propagule count, and continue so in the subsequent years post hurricane. Our specific objectives were (i) to characterize the seasonal patterns of flight activity for *D. terebrans*, *H. porculus*, *H. salebrosus*, *H. tenuis*, *H. pales*, and *P. picivorus* over two, one-year periods, (ii) to determine if peak beetle trap catches of all the above mentioned species (as measured by response to baited traps) are significantly different between treatments, and (iii) to determine if the number of propagules of blue-stain fungi vectored by *H. pales* and *P. picivorus* are significantly different across management treatments.

2.2 Materials and Methods

2.2.1 Study Site and Experimental Design

Our study site, The Jones Center at Ichauway, is located in Baker County, Georgia (31.22N, 84.48W). The site climate is humid-subtropical, characterized by a mean annual temperature of 19 °C, long, hot summers, and short, moderate, cool winters, with an annual precipitation of 1,310 mm per year, evenly distributed (Wagner et al. 2019). Longleaf pine is the dominant canopy tree, occupying approximately 60-90% of the canopy basal area (Wagner et al. 2019). The managed stands at the site average between 6 to 20m^2 ha^{-1} basal area of longleaf pine and are comprised mostly of mature 80 to 100-year-old second-growth trees that are prescription burned on a two-year interval (Wagner et al. 2019). The field site is sparse in shrubs and midstory but has a robust understory dominated mainly by wiregrass. The soil at the Jones Center at Ichauway is mostly comprised of siliceous, loamy, thermic Arenic Paleudults with poorly separated horizons (Hendricks et al. 2006).

In 2019, we established plots in three different management treatments within longleaf pine-dominated stands. All treatments were disturbed by wind in October 2018. The three treatments included (i) salvage logging following the hurricane in winter of 2018 to spring of 2019 and prescribed burning in the spring of 2019; (ii) no salvage logging but prescribed burning in spring of 2019; and (iii) no salvage logging and delayed prescribed burning in spring of 2020, 17 to 19 months after the hurricane. We replicated each treatment five times for a total of 15 plots, but due to spring weather conditions, two of the 15 plots could not be prescription burned in the correct time frame and were dropped from the study. This resulted in only four replicate treatments for both the (i) salvage logging and prescribed burning and (iii) no salvage logging and delayed prescribed burning treatments.

2.2.2 Lower stem and root feeding beetle trapping and identification

To assess lower stem and root feeding beetle abundance associated with the different treatments and characterize the seasonal patterns of flight activity, we established a transect of traps across each of the treatment plots. In each plot, we placed one Lindgren funnel trap fastened to a ~2 m pole, positioned at least 50 m away from a road or fire break. We baited each trap with two semi-permeable polyvials (West Green Global Technologies Inc., Langley, BC, Canada) containing 95% EtOH and 100% turpentine (Sunnyside Corporation, Wheeling, Illinois, USA), respectively, plus a packet of *exo*-brevicomin lure at a release rate of approximately 1.5 mg per day (Synergy Semiochemicals, Delta, British Columbia, Canada) (Flechtmann et al. 1999, Munro et al. 2019). We refilled the polyvials as needed and the *exo*-brevicomin packets were replaced every 6 weeks. We attached all lures to the sixth funnel from the top and the collection cup was filled with propylene glycol (Tractor Supply Co., Brentwood, Tennessee, USA) to preserve beetles until collection. We collected the content of funnel traps every two weeks for two collection phases between May 2019 to April 2020 (year one) and August 2020 to August 2021 (year two).

In addition, to capture *P. picivorus* and *H. pales,* which usually travel in or on the soil (Erbilgin et al. 2001), we used four pitfall traps spaced 10 m away from each other in a straight line transect and 10 m adjacent to the Lindgren funnel trap. The traps were made from 10 cm diameter polyvinyl chloride plastic drainpipes cut to be approximately 20 cm in length with eight holes drilled into the midsection approximately 5 cm in diameter. Each pitfall was capped at both ends with removable plastic covers (PVC S&D Cap, Home Depot), following the design described in Raffa & Hunt (1988) and Klepzig et al. (1991), and had the interior surface coated

with sprayable Teflon-based dry lube (Blaster Corporation, Valley View, Ohio, USA). To enhance drainage, we drilled three 2 mm holes in the bottom plastic cover. We also hung a plastic deli cup ~473 mL in volume (Webstaurant Store, Litz, Pennsylvania, USA) with a thin wire handle inside each pitfall to facilitate the collection and removal of insects and debris that fell into them. As with the Lindgren funnel traps, we baited each pitfall trap with two semi-permeable polyvials with 95 % EtOH and 100% turpentine, respectively, which were attached to the top cover with twist ties. Traps were then buried at a depth of 10 cm. We collected the contents of pitfall traps three times per week from May to August of 2019, but due to the low capture rates compared to the Lindgren funnel traps, we decided to interrupt the pitfall trap collection approach. Previous studies using these traps have also shown low numbers of *P. picivorus* captures in Georgia (Helbig et al. 2016). These capture rates stand in stark contrast to those seen in the Lake States in red pine plantations (Klepzig et al. 1991) and Christmas tree farms (Rieske and Raffa 1990).

We grouped and labeled all collected beetles by trap, plot, and date of collection, and identified those specimens belonging to the target genera in the lower stem and root feeding beetle groups (i.e., *Dendroctonus*, *Hylastes*, *Hylobius*, and *Pachylobius*) to the species level based on morphological features (Arnett 2002, Baker 1972, Wood 1982).

2.2.3 Live beetle collection

To assess if the number of propagules of blue-stain fungi phoretically carried by root feeding beetles are affected by specific management treatments, we collected live specimens of the target species *H. pales* and *P. picivorus* using billet traps. We dug a shallow hole approximately 100 cm into the ground and placed three small logs inside which we then covered with dirt and branches. The logs were approximately 30 cm long and with a diameter of

approximately 13 cm, with four 2 cm² patches of bark removed before being placed into the hole (Flechtmann et al. 1999). We set up one billet trap per plot, ~10 m away from any other trap (i.e., pitfall and Lindgren funnel trap) from September 2020 to October 2020, which was the assumed peak flight of root feeding beetles. We collected beetles from all traps every three days until five live beetles per target species per treatment were collected or until the end of the assumed peak flight period. We stored collected live beetles individually in 1.5 ml centrifuge vials labeled by trap, plot, and date of collection and transported them on ice to a laboratory at the Jones Center at Ichauway, where they were stored at 4 °C for up to one week, until transportation to the University of Georgia. During all transportation steps, to avoid direct contact of the live specimens with ice, we stored the vials in plastic deli cups. At the University of Georgia, we again stored the beetles at 4 °C until further processing, which occurred within four weeks after arrival.

2.2.4 Quantification of Propagules of Blue-Stain Fungi

To quantify the colony forming units (CFUs) of blue-stain fungi vectored by *H. pales* and *P. picivorus*, we used a serial dilution plating technique modified from Harrington and Fraedrich (2010). To minimize the presence of contaminants and insect decomposers, we only used live or recently dead insect specimens (within 3 days of death). We removed the specimens from the centrifuge vials with sterile forceps and placed them in a new 2 ml centrifuge vial along with 1 mL of sterile 1% Tween 80 (Sigma-Aldrich, Burlington, Massachusetts, USA) solution. We vortexed insects at 30 HZ for 60 seconds (Battisti et al. 1999) and used 100 µL of the washing solution to make serial dilutions in sterile DI water of 10 X and 100 X. We added 100 µL of each dilution solution to the surface of 90 mm petri plates of Cycloheximide-Streptomycin Malt Agar (2% malt agar media amended with 200 ppm of cycloheximide and 300 ppm of

streptomycin) (CSMA) which inhibit bacteria and non-blue-stain fungi (Zhou et al. 2007) and evenly spread them using a sterile spreader. We replicated each dilution plating three times and before incubation, we sealed plates with Parafilm® (Bemis Company, Inc., Sheboygan Falls, Wisconsin, USA) and stacked them three high. We incubated the plates at 19 °C in the dark for 7 to 10 days. On the seventh day of incubation, for each sample we selected the dilution that yielded greater than 20 but less than 200 colonies per plate and counted and organized all colonies into morphotypes based on size, color, shape, yeasty consistency, and mycelial patterns. We isolated subcultures of each morphotype for each beetle from the original plate, placed the colony onto a 90 mm 2% malt agar plate, and incubated it at room temperature. We assessed whether different morphotypes classified as blue-stain fungi based on the morphological traits of their subcultures, such as the presence of mononematous or synnematous darkly pigmented conidiophores, which are asexual fruiting structures that produce conidia in slimy masses (Jankowiak et al. 2018, Roets et al. 2010).

2.2.5 Statistical Analyses

Beetle abundance

We analyzed each beetle species separately in addition to each year alone, due to the different time intervals between sampling periods. To structure the data, we divided the total number of collected beetle into 12-month time periods. For both years, we developed a model that included time and treatment, and the interactions between the two, as factors. The parameters of the model are reported in Table 2.1. Due to the count and repeated-measure nature of the beetle abundance data, we utilized a generalized estimating equations (GEE) regression model with Poisson distribution to compare the data of beetle species abundance among each treatment. We performed all calculations in SAS (version 9.4, SAS Institute Inc, Cary, North

Carolina, 2013) (Sas 2013). In addition, for each year we totaled the peak beetle flight, determined by selecting the months with the highest number of beetles, for each species, and utilized a general linear model GLM regression model with a negative binomial distribution to compare peak beetle flight abundance to each treatment. These calculations were performed in R (R Core Team 2020).

Propagules of Blue-stain Fungi

We expressed CFUs of blue-stain fungi for each beetle as the average of the three plates of the same dilution that were counted per beetle and considered this to be one replicate. The distribution of blue-stain fungi CFU counts was not normally distributed for either *H. pales* and *P. picivorus* (Shapiro Wilkes, $p<0.0001$), so we chose to use a generalized linear model (GLM) to compare CFU counts among the three treatments. All calculations were performed in R (version 4.1.2) with package "nlme" (R Core Team 2020, Wickham et al. 2017).

2.3 Results

2.3.1 Beetle Abundance

In the first year, we collected a total of 9,349 beetles of all species combined (Table 2.2). Species abundance peaked at different times of the year for each species (Figure 2.1). Analysis of the GEE model revealed that there were no significant responses detected from any beetle species to any treatment in the first year. However, time was a significant factor for four of the six species surveyed: *D. terebrans* (p-value = 0.0015), *H. porculus* (p-value = 0.0323), *H. salebrosus* (p-value = 0.0275), *and P. picivorus* (p-value =0.0018), indicating that these species' populations fluctuated through time (Tables 2.3 to 2.8). We did not detect any significant interaction effects between time and treatment during year one (Tables 2.3 to 2.8).

In the second year, we collected a total of 8,922 beetles of all species combined (Table 2.2), and as in the first year, each beetle species peaked at different times throughout the year (Figure 2.2). Analysis of the GEE model showed that again time was a significant factor, with all six species responding significantly. However, during year two, treatment alone appeared to influence the abundance of two beetles, *H. tenuis* and *H. salebrosus*. *Hylobius tenuis* had a higher abundance in the no prescribed burning and no salvage logging treatment (p-value = 0.0248). *Hylastes. salebrosus* abundance was statistically lower in the prescribed burning and salvage logging treatments (p-value = 0.0466). When looking at the interaction between time and treatment, our model indicated that *D. terebrans, H. porculus* and *H. tenuis* increased in abundance within the no prescribed burning no salvage logging treatment only in some of the months (p-value = 0.0065 and p-value <0.0001, respectively) (Table 2.9 and 2.10) (Figure 2.2).

The numbers of beetles caught in our traps peaked as follows for year one: *D. terebrans* from June to August of 2019, *H. porculus* from January to March of 2020, *H. salebrosus* from January to March of 2020, *H. tenuis* from October to December of 2019, *H. pales* from January of 2020, and *P. picivorus* from June to August of 2019 (Figure 2.1). For year two, beetle response peaked as follows: *D. terebrans* from October to November of 2020, *H. porculus* from February to March of 2021, *H. salebrosus* from March to April of 2021, *H. tenuis* from March to April of 2021, *H. pales* from September to October of 2020 and *P. picivorus* in March of 2021 (Figure 2.2). In the first year, only the peak of *H. salebrosus* differed between the prescribed burning and no salvage logging treatment and the no prescribed burning and no salvage logging one (p-value = 0.0166), with the latter having a higher number of beetles (Figure 2.3). For the second year, only the peak of *H. porculus* differed between the prescribed burning and no salvage logging treatment and the no prescribed burning and no salvage logging one (p-value =

0.0308), with a lower abundance in the no prescribed burning, no salvage logging treatment (Figure 2.4).

2.3.2 Propagules of Blue-Stain Fungi

We recovered blue-stain propagules on 52% (24/46) of all beetles from respective treatments. For *H. pales*, 61% (17/28) of the plates specimens yielded blue-stain fungi, for an overall average of 413 CFUs/beetle, while *P. picivorus* had a lower recovery rate of 39% (7/18), and an overall an average of 206 CFUs/beetle. The GLM revealed that for both beetle species, treatment had no effect on the number of CFUs (p-value > 0.05). Averages (± standard error) for *H. pales* were 688 (+/-173) for prescribed burning and no salvage logging, 440 (+/-229) for prescribed burning and salvage logging, and 543 (+/-239) for no prescribed burning and no salvage logging; and for *P. picivorus* were 128 (+/-58) for prescribed burning and no salvage logging, 84 (+/-59) for prescribed burning and salvage logging, and 360 (+/-251) for no prescribed burning and no salvage logging.

2.4 Discussion

The longleaf pine ecosystem is biologically diverse, resilient and rich in ecosystem services, but it is an endangered habitat due to reduction of wildfires and human encroachment via settlement and historic agriculture (Van Lear et al. 2005). Longleaf pine is also more resistant to natural disasters, like wildfires and pest attacks, such as the southern pine beetle (Susaeta and Gong 2019). This system may also be more resistant to secondary agents such as lower stem and root feeding beetles that carry weakly virulent blue-stain fungi (Baker 1972, Devkota and Eckhardt 2019, Eckhardt et al. 2004, Matusick et al. 2012). Due to the decrease in its range, interest in understanding and conserving this ecosystem is growing. While there is wealth of literature on lower stem and root feeding beetles and their associated Ophiostomatoid fungi, most

research examines more economically beneficial pine species, such as loblolly pine (*Pinus taeda*). Here, we examined the effects of post hurricane management practices on lower stem and root feeding beetles and their symbiotic blue-stain fungi.

We hypothesized that lower stem and root feeding beetles would be more abundant in plots with more coarse woody debris. For instance, we expected that in the first year, root and lower stem feeding beetles would be less abundant in plots that were burned and logged. Presumably this pattern would result from lower levels of attractant volatiles due to lower levels of damaged tree material within a stand (Flechtmann et al. 1999, Munro et al. 2019, Payne et al. 1987). We also expected that in the second year following the hurricane, the plots without active management (i.e. the delayed prescribed burning and no salvage logging) would have a higher beetle abundance, due to more damaged host material being present in the stand and the effects of prescribed burnings (Flechtmann et al. 1999, Munro et al. 2019, Payne et al. 1987). However, the results of our study show no overall major effects of stand management activities on the numbers of lower stem and root feeding beetles caught in traps in the first year. Our results also show a similar effect of time for all species for the second year of collection, with the additional contribution of treatment or the interaction between time and treatment also having a statistical effect for four species during this year. Of those, three species, *H. porculus*, *H. salebrosus* and *D. terebrans*, had a higher abundance in the no prescribed burning and no salvage logging treatments, which indicates a delayed effect of beetle response two years after Hurricane Michael in treatments that delayed burning and had no salvage logging. Our results from peak analysis showed that in year one *H. salebrosus* had a significantly higher abundance in the no prescribed burning, no salvage logging treatment than the prescribed burning but no salvage logging one,

while *H. porculus* was in higher abundance in the prescribed burning, no salvage logging plots when compared to the no prescribed burning and no salvaged logging ones (Figures 2.3 and 2.4).

We hypothesized that treatments that resulted in higher amounts of coarse woody debris would result in higher levels of blue-stain fungal propagules on root feeding beetles as these areas would be more attractive to beetles carrying spores and have a higher chance of beetles contacting fruiting fungi. Yet, there were no significant differences between the number of blue-stain propagules per beetle between treatments. This implies that the blue-stain fungal associates of these beetles do not appear to be influenced by management activities. However, a future study to assess the fungal-beetle association and its interaction with disturbance events more thoroughly is warranted due to the low numbers of beetles collected. It would also be interesting to examine other root feeding beetles, such as *Hylastes* species.

Our results indicate that time of year is the most significant driver of beetle population dynamics for the first year, while there is a delayed effect of no management activities in the second year following Hurricane Michael for *D. terebrans*, *H. porculus* and *H. tenuis*. It is likely that *H. pales* and *P. picivorus* and their fungal community populations dynamics are not driven by management treatments aimed at lowering the amount of coarse wood debris. The results gathered here can inform land managers that are concerned about tree health and mortality following wind disturbance events, such as hurricanes. Longleaf pines are resilient to wind disturbance events and the increases in insects that follow them, even without management that aims to reduce the amount of downed timber or coarse woody debris through salvage logging or prescribed burning.

2.5 Literature Cited

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Table 2.1. Parameters of the generalize estimating equation regression model with Poisson distribution used on all beetle species per each year. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Parameter	**Estimate**	**Treatment**
PRM1	Intercept	
PRM2	Treatment	BS
PRM3	Treatment	NBNS
PRM 4	Treatment	BNS
PRM 5	Time	
PRM 6	Time*treatment	BS
PRM 7	Time*treatment	NBNS
PRM 8	Time*treatment	BNS
PRM 9	Time*time	
PRM 10	Time*time*treatment	BS
PRM 11	Time*time*treatment	NBNS
PRM 12	Time*time*treatment	BNS

Table 2.2. First and second year summary of all lower stem and root feeding beetles captured in Lindgren funnel traps.

Species	**Total Count**	
	Year 1	**Year 2**
Dendroctonus terebrans	3140	1557
Hylastes porculus	3260	1782
Hylastes salebrosus	1764	4519
Hylastes tenuis	592	305
Hylobius pales	119	361
Pachylobius picivorus	474	398
Grand total	9349	8922

Table 2.3. Results of the generalized estimating equation regression model with Poisson distribution for *Dendroctonus terebrans* collections in year one. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		1.0383	0.0690	0.9030	1.1735	15.05	<.0001
treatment	BS	-0.1342	0.2123	-0.5503	0.2818	-0.63	0.5272
treatment	NBNS	0.0611	0.1994	-0.3298	0.4519	0.31	0.7595
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.2070	0.0378	0.1330	0.2811	5.48	<.0001
time*treatment	BS	0.0511	0.0637	-0.0738	0.1760	0.80	0.4224
time*treatment	NBNS	-0.0227	0.0405	-0.1021	0.0566	-0.56	0.5746
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0073	0.0015	-0.0102	-0.0044	-4.94	<.0001
time*time*treatment	BS	-0.0024	0.0026	-0.0075	0.0028	-0.90	0.3695
time*time*treatment	NBNS	0.0010	0.0016	-0.0021	0.0041	0.63	0.5292
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.4. Year one: generalized estimating equation regression model with Poisson distribution p-values, for *Hylastes porculus* in year one. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-1.3546	0.9520	-3.2204	0.5113	-1.42	0.1548
treatment	BS	-1.4285	1.1805	-3.7424	0.8853	-1.21	0.2262
treatment	NBNS	-0.6256	1.5824	-3.7270	2.4758	-0.40	0.6926
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		-0.0920	0.1195	-0.3262	0.1421	-0.77	0.4411
time*treatment	BS	0.2298	0.1445	-0.0535	0.5131	1.59	0.1119
time*treatment	NBNS	0.1337	0.1611	-0.1821	0.4495	0.83	0.4067
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		0.0062	0.0027	0.0009	0.0114	2.29	0.0219
time*time*treatment	BS	-0.0053	0.0032	-0.0115	0.0009	-1.67	0.0948
time*time*treatment	NBNS	-0.0028	0.0033	-0.0094	0.0037	-0.85	0.3934
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.5. Year one: generalized estimating equation regression model with Poisson distribution p-values, for *Hylates salebrosus* in year one. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-6.9664	4.6732	-16.126	2.1929	-1.49	0.1360
treatment	BS	3.3387	5.0520	-6.5631	13.2405	0.66	0.5087
treatment	NBNS	3.3060	4.9677	-6.4305	13.0424	0.67	0.5057
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.2594	0.3515	-0.4294	0.9483	0.74	0.4604
time*treatment	BS	-0.1710	0.3929	-0.9411	0.5992	-0.44	0.6635
time*treatment	NBNS	-0.1329	0.3803	-0.8782	0.6124	-0.35	0.7267
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		0.0001	0.0062	-0.0120	0.0122	0.02	0.9879
time*time*treatment	BS	0.0026	0.0071	-0.0113	0.0164	0.36	0.7167
time*time*treatment	NBNS	0.0017	0.0068	-0.0116	0.0149	0.25	0.8064
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.6. Year one: generalized estimating equation regression model with Poisson distribution p-values, for *Hylastes tenuis* in year one. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-6.7350	1.8444	-10.350	-3.1201	-3.65	0.0003
treatment	BS	2.5455	2.3580	-2.0762	7.1671	1.08	0.2804
treatment	NBNS	4.4018	2.1035	0.2789	8.5247	2.09	0.0364
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.6031	0.1513	0.3066	0.8996	3.99	<.0001
time*treatment	BS	-0.2285	0.2013	-0.6231	0.1661	-1.13	0.2565
time*treatment	NBNS	-0.4009	0.1759	-0.7457	-0.0561	-2.28	0.0227
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0127	0.0032	-0.0190	-0.0064	-3.97	<.0001
time*time*treatment	BS	0.0050	0.0041	-0.0031	0.0130	1.20	0.2284
time*time*treatment	NBNS	0.0085	0.0038	0.0011	0.0159	2.25	0.0246
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.7. Year one: generalized estimating equation regression model with Poisson distribution p-values, for *Hylobius pales* in year one. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-0.6089	0.4450	-1.4811	0.2633	-1.37	0.1712
treatment	BS	-0.6601	0.5661	-1.7696	0.4495	-1.17	0.2436
treatment	NBNS	-1.3540	0.5967	-2.5234	-0.1845	-2.27	0.0233
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.2338	0.0412	0.1530	0.3146	5.67	<.0001
time*treatment	BS	0.0276	0.1184	-0.2043	0.2596	0.23	0.8154
time*treatment	NBNS	-0.0123	0.0753	-0.1600	0.1353	-0.16	0.8699
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0091	0.0015	-0.0120	-0.0061	-6.04	<.0001
time*time*treatment	BS	-0.0017	0.0051	-0.0117	0.0082	-0.34	0.7328
time*time*treatment	NBNS	0.0019	0.0028	-0.0037	0.0075	0.67	0.5010
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	

Table 2.8. Year one: generalized estimating equation regression model with Poisson distribution p-values, for *Pachylobius picivorus* in year one. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-1.9055	0.3151	-2.5231	-1.2879	-6.05	<.0001
treatment	BS	-2.1407	1.6027	-5.2820	1.0006	-1.34	0.1817
treatment	NBNS	-1.1901	2.5457	-6.1796	3.7995	-0.47	0.6402
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.1954	0.0266	0.1433	0.2476	7.34	<.0001
time*treatment	BS	0.2052	0.1503	-0.0893	0.4997	1.37	0.1721
time*treatment	NBNS	0.0628	0.2646	-0.4557	0.5813	0.24	0.8123
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0039	0.0007	-0.0053	-0.0026	-5.71	<.0001
time*time*treatment	BS	-0.0049	0.0032	-0.0112	0.0014	-1.51	0.1299
time*time*treatment	NBNS	-0.0009	0.0059	-0.0125	0.0107	-0.15	0.8780
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.9. Year two: generalized estimating equation regression model with Poisson distribution p-values, for *Dendroctonus terebrans* in year two. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		0.5485	0.1691	0.2170	0.8800	3.24	0.0012
treatment	BS	-0.3586	0.3774	-1.0984	0.3811	-0.95	0.3420
treatment	NBNS	0.8135	0.4973	-0.1611	1.7881	1.64	0.1018
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.0950	0.0134	0.0688	0.1212	7.11	<.0001
time*treatment	BS	0.0461	0.0764	-0.1037	0.1959	0.60	0.5462
time*treatment	NBNS	-0.1466	0.0266	-0.1987	-0.0945	-5.51	<.0001
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0021	0.0004	-0.0028	-0.0014	-5.96	<.0001
time*time*treatment	BS	-0.0025	0.0027	-0.0078	0.0027	-0.94	0.3458
time*time*treatment	NBNS	0.0045	0.0007	0.0031	0.0059	6.33	<.0001
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.10. Year two: generalized estimating equation regression model with Poisson distribution p-values, for *Hylastes porculus* in year two. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-1.7804	1.0494	-3.8372	0.2764	-1.70	0.0898
treatment	BS	-0.0742	1.2379	-2.5006	2.3521	-0.06	0.9522
treatment	NBNS	1.8018	1.2560	-0.6600	4.2636	1.43	0.1514
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.4138	0.0863	0.2446	0.5829	4.79	<.0001
time*treatment	BS	-0.0502	0.1042	-0.2544	0.1540	-0.48	0.6298
time*treatment	NBNS	-0.2001	0.0923	-0.3809	-0.0193	-2.17	0.0301
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0106	0.0017	-0.0140	-0.0072	-6.07	<.0001
time*time*treatment	BS	0.0017	0.0022	-0.0025	0.0060	0.79	0.4272
time*time*treatment	NBNS	0.0042	0.0018	0.0006	0.0078	2.30	0.0213
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.11. Year two: generalized estimating equation regression model with Poisson distribution p-values, for *Hylastes salebrosus* year two. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-5.2281	2.7514	-10.620	0.1645	-1.90	0.0574
treatment	BS	-2.7388	3.4362	-9.4737	3.9961	-0.80	0.4254
treatment	NBNS	1.6854	3.4648	-5.1055	8.4762	0.49	0.6267
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.7174	0.2382	0.2505	1.1843	3.01	0.0026
time*treatment	BS	0.2142	0.2940	-0.3621	0.7905	0.73	0.4663
time*treatment	NBNS	-0.1568	0.2895	-0.7242	0.4106	-0.54	0.5880
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0151	0.0049	-0.0248	-0.0055	-3.07	0.0022
time*time*treatment	BS	-0.0044	0.0060	-0.0162	0.0074	-0.73	0.4637
time*time*treatment	NBNS	0.0036	0.0060	-0.0081	0.0153	0.60	0.5485
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.12. Year two: generalized estimating equation regression model with Poisson distribution p-values, for *Hylastes tenuis* year two. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-6.7350	1.8444	-10.345	-3.1201	-3.65	0.0003
treatment	BS	2.5455	2.3580	-2.0762	7.1671	1.08	0.2804
treatment	NBNS	4.4018	2.1035	0.2789	8.5247	2.09	0.0364
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.6031	0.1513	0.3066	0.8996	3.99	<.0001
time*treatment	BS	-0.2285	0.2013	-0.6231	0.1661	-1.13	0.2565
time*treatment	NBNS	-0.4009	0.1759	-0.7457	-0.0561	-2.28	0.0227
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0127	0.0032	-0.0190	-0.0064	-3.97	<.0001
time*time*treatment	BS	0.0050	0.0041	-0.0031	0.0130	1.20	0.2284
time*time*treatment	NBNS	0.0085	0.0038	0.0011	0.0159	2.25	0.0246
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.13. Year two: generalized estimating equation regression model with Poisson distribution p-values, for *Hylobius pales* in year two. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-0.6089	0.4450	-1.4811	0.2633	-1.37	0.1712
treatment	BS	-0.6601	0.5661	-1.7696	0.4495	-1.17	0.2436
treatment	NBNS	-1.3540	0.5967	-2.5234	-0.1845	-2.27	0.0233
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.2338	0.0412	0.1530	0.3146	5.67	<.0001
time*treatment	BS	0.0276	0.1184	-0.2043	0.2596	0.23	0.8154
time*treatment	NBNS	-0.0123	0.0753	-0.1600	0.1353	-0.16	0.8699
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0091	0.0015	-0.0120	-0.0061	-6.04	<.0001
time*time*treatment	BS	-0.0017	0.0051	-0.0117	0.0082	-0.34	0.7328
time*time*treatment	NBNS	0.0019	0.0028	-0.0037	0.0075	0.67	0.5010
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Table 2.14. Year two: generalized estimating equation regression model with Poisson distribution p-values, for *Pachylobius picivorus* in year two. Beetle abundance was divided into 12 time periods corresponding to months of the year. Prescribed burning and no salvaged logging (BNS) was used as the reference to compare the other treatments, prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

Analysis Of GEE Parameter Estimates							
Parameter	**Treatment**	**Estimate**	**Standard Error**	**95% Confidence Limits**		**Z**	**P-value**
Intercept		-1.9055	0.3151	-2.5231	-1.2879	-6.05	<.0001
treatment	BS	-2.1407	1.6027	-5.2820	1.0006	-1.34	0.1817
treatment	NBNS	-1.1901	2.5457	-6.1796	3.7995	-0.47	0.6402
treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time		0.1954	0.0266	0.1433	0.2476	7.34	<.0001
time*treatment	BS	0.2052	0.1503	-0.0893	0.4997	1.37	0.1721
time*treatment	NBNS	0.0628	0.2646	-0.4557	0.5813	0.24	0.8123
time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.
time*time		-0.0039	0.0007	-0.0053	-0.0026	-5.71	<.0001
time*time*treatment	BS	-0.0049	0.0032	-0.0112	0.0014	-1.51	0.1299
time*time*treatment	NBNS	-0.0009	0.0059	-0.0125	0.0107	-0.15	0.8780
time*time*treatment	BNS	0.0000	0.0000	0.0000	0.0000	.	.

Figure 2.1. First year monthly mean beetle counts per treatment, beginning in April of 2019. X-axis is month of capture, y- axis is average of beetles caught and standard error bars are shown for each time point. Treatments are prescribed burning and no salvaged logging (BNS), prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

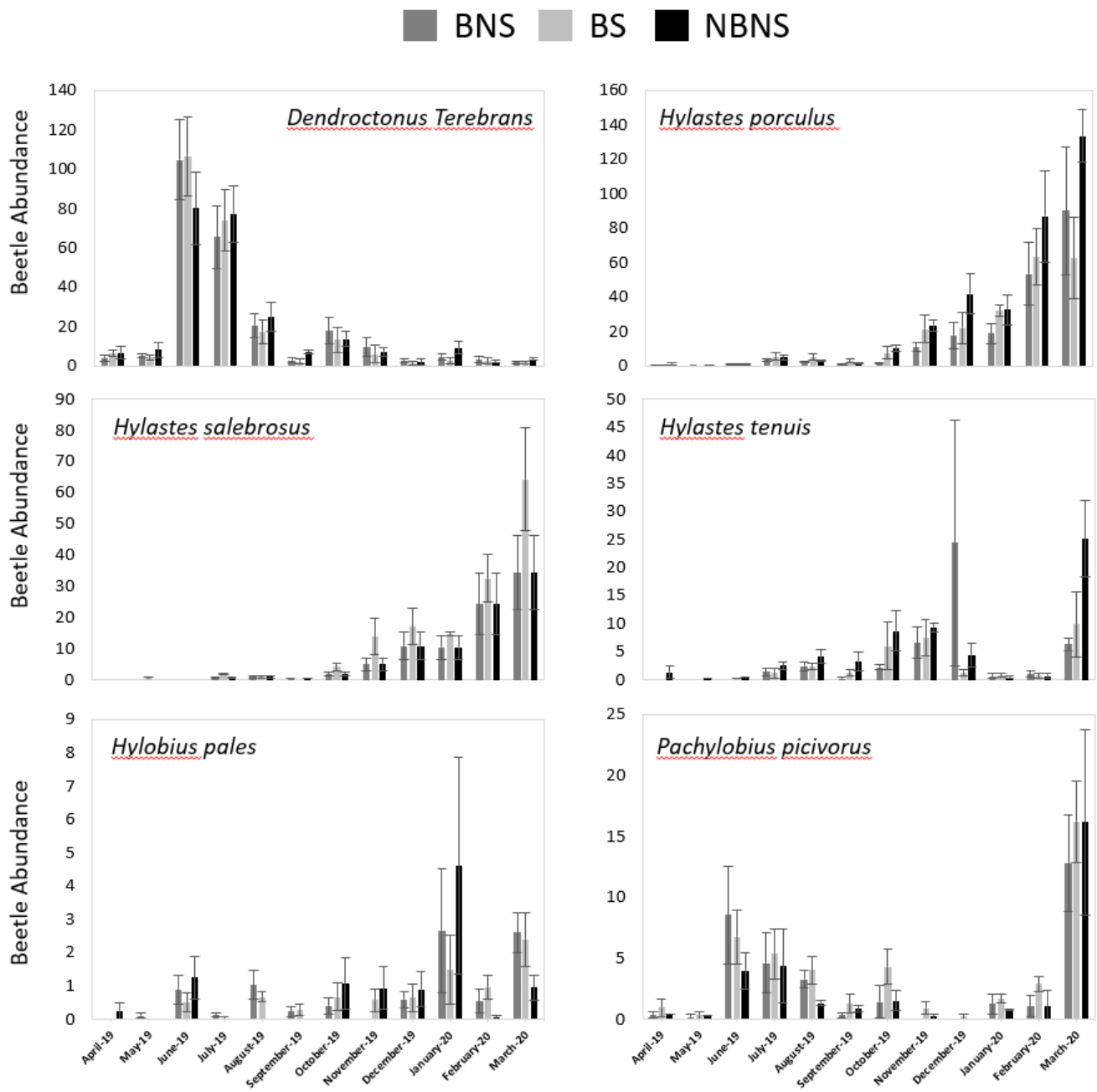


Figure 2.2. Second year monthly averages, beginning in August of 2020. X- axis is month of capture, y- axis is average of beetles caught and standard error bars are shown for each time point. Treatments are prescribed burning and no salvaged logging (BNS), prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS).

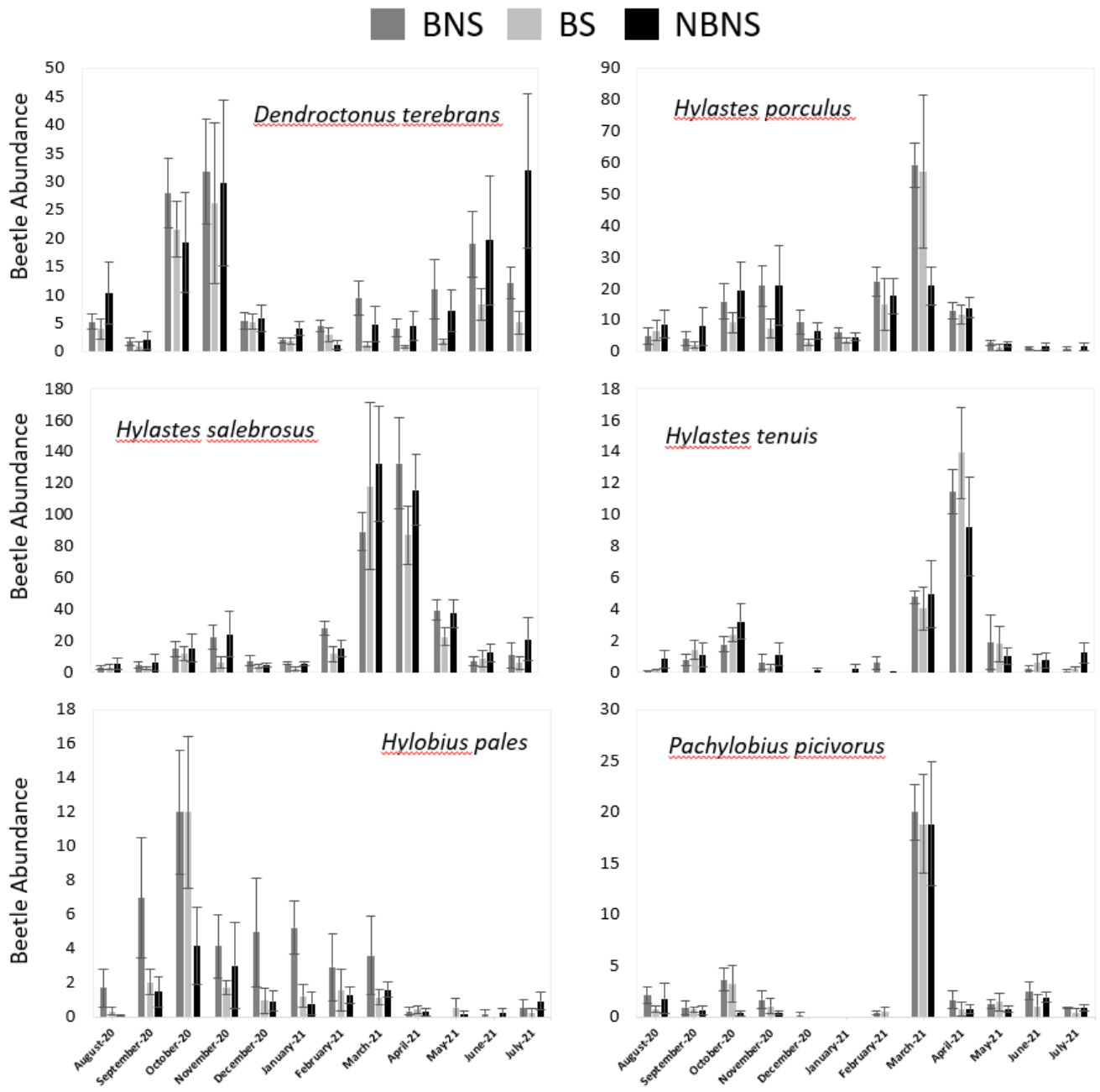


Figure 2.3. First year mean beetle counts per treatment for the peak of beetle abundance for each species. Standard error bars are shown for each time point. Treatments are prescribed burning and no salvaged logging (BNS), prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS). For each species, different letters above bars indicate a significant difference at $p<0.05$ according to the general linear model with negative binomial distribution.

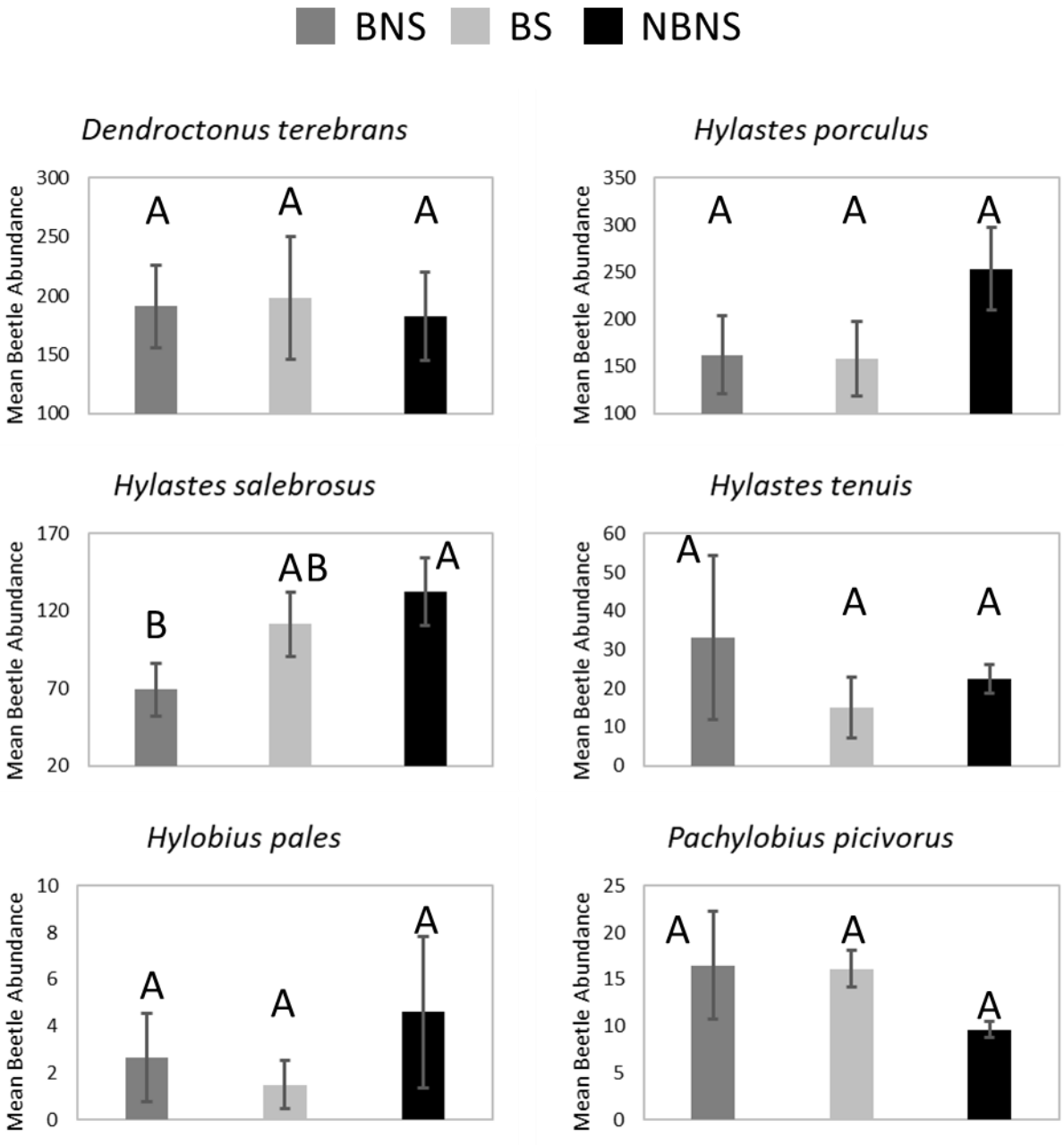
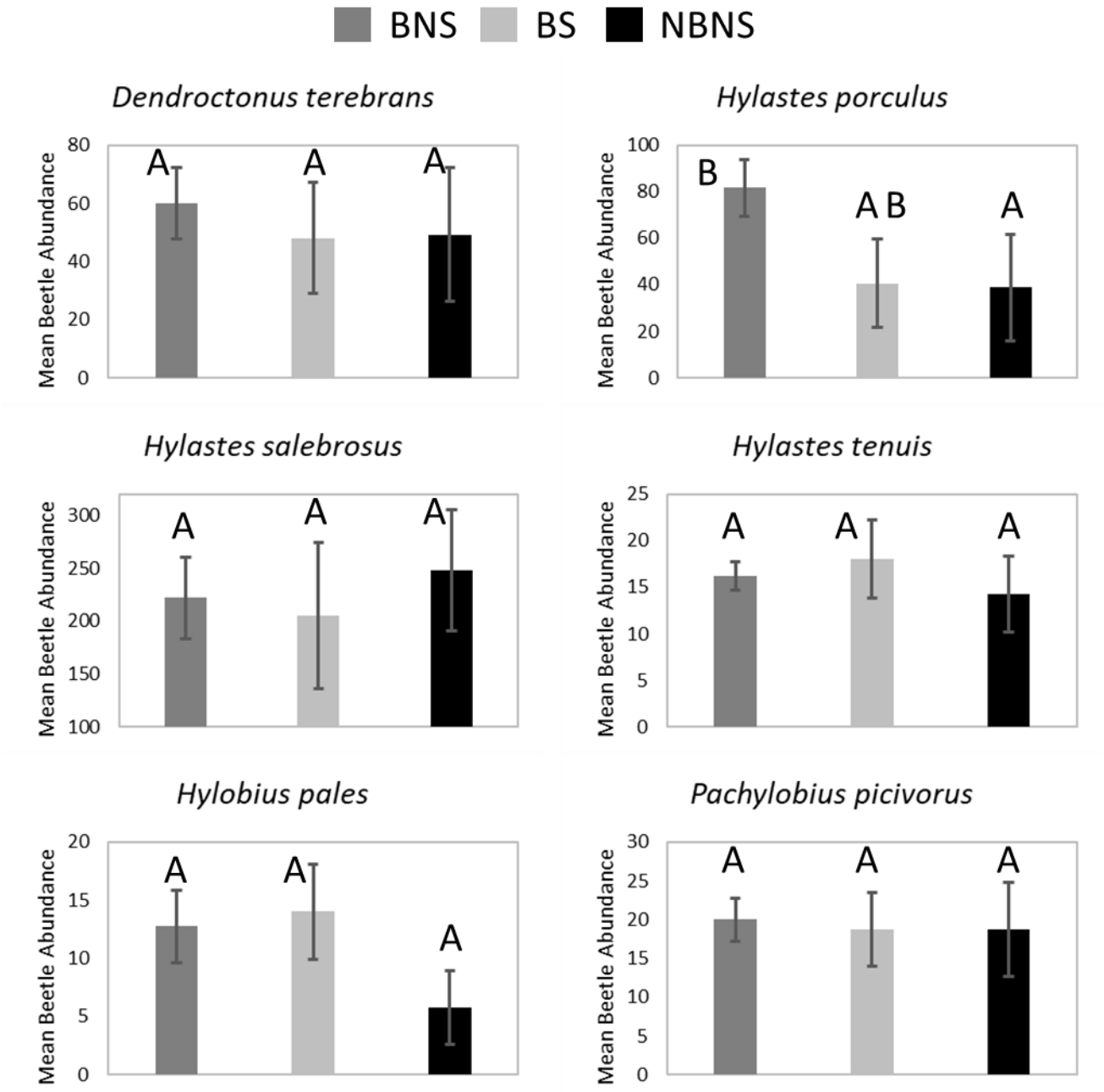


Figure 2.4. Second year mean beetle counts per treatment for the peak of beetle abundance for each species. Standard error bars are shown for each time point. Treatments are prescribed burning and no salvaged logging (BNS), prescribed burning and salvage logging (BS) and no prescribed burning and no salvage logging (NBNS). For each species, different letters above bars indicate a significant difference at $p<0.05$ according to the general linear model with a negative binomial model.



2.6 Appendix

SAS Code used to analyze treatments in relation to 12 time points.

```
/*---------- Year 1 - btb ----------*/


proc import out=work.data1btb
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year1_btb.xlsx"
                            DBMS=xlsx REPLACE;
          datarow = 2;
run;


proc sort data=data1btb;
 by treatment time replicate;
run;


proc sgscatter data = data1btb;
  plot btb_int * time;
  by treatment;
run;


proc genmod data = data1btb;
class treatment(ref="BNS") replicate(ref="1");
model btb_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;
```

```
/*---------- Year 1 - hpo ----------*/
proc import out=work.data1hpo
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year1_hpo.xlsx"
                        DBMS=xlsx REPLACE;
          datarow = 2;
run;

proc sort data=data1hpo;
 by treatment time replicate;
run;

proc sgscatter data = data1hpo;
  plot hpo_int * time;
  by treatment;
run;

proc genmod data = data1hpo;
class treatment(ref="BNS") replicate(ref="1");
model hpo_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;
```

```
/*---------- Year 1 - hsa ----------*/
proc import out=work.data1hsa
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year1_hsa.xlsx"
                        DBMS=xlsx REPLACE;
          datarow = 2;
run;

proc sort data=data1hsa;
 by treatment time replicate;
run;

proc sgscatter data = data1hsa;
  plot hsa_int * time;
  by treatment;
run;

proc genmod data = data1hsa;
class treatment(ref="BNS") replicate(ref="1");
model hsa_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;


/*---------- Year 1 - hte ----------*/
proc import out=work.data1hte
```

```
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_hte.xlsx"
                                DBMS=xlsx REPLACE;
            datarow = 2;
run;


proc sort data=data1hte;
 by treatment time replicate;
run;


proc sgscatter data = data1hte;
  plot hte_int * time;
  by treatment;
run;


proc genmod data = data1hte;
class treatment(ref="BNS") replicate(ref="1");
model hte_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;



/*---------- Year 1 - pales----------*/
proc import out=work.data1pales
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_pales.xlsx"
                                DBMS=xlsx REPLACE;
```

```
        datarow = 2;
run;

proc sort data=data1pales;
 by treatment time replicate;
run;

proc sgscatter data = data1pales;
  plot pales_int * time;
  by treatment;
run;

proc genmod data = data1pales;
class treatment(ref="BNS") replicate(ref="1");
model pales_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;

/*---------- Year 1 - pici----------*/
proc import out=work.data1pici
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_pici.xlsx"
                        DBMS=xlsx REPLACE;
        datarow = 2;
run;
```

```
proc sort data=data1pici;
 by treatment time replicate;
run;

proc sgscatter data = data1pici;
  plot pici_int * time;
  by treatment;
run;

proc genmod data = data1pici;
class treatment(ref="BNS") replicate(ref="1");
model pici_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;

/*---------- Year 2 - btb ----------*/

proc import out=work.data2btb
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_btb.xlsx"
                        DBMS=xlsx REPLACE;
          datarow = 2;
```

```
run;


proc sort data=data2btb;
 by treatment time replicate;
run;


proc sgscatter data = data2btb;
  plot btb_int * time;
  by treatment;
run;


proc genmod data = data2btb;
class treatment(ref="BNS") replicate(ref="1");
model btb_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;




/*---------- Year 2 - hpo ----------*/
proc import out=work.data2hpo
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_hpo.xlsx"
                            DBMS=xlsx REPLACE;
          datarow = 2;
run;
```

```
proc sort data=data2hpo;
 by treatment time replicate;
run;

proc sgscatter data = data2hpo;
  plot hpo_int * time;
  by treatment;
run;

proc genmod data = data2hpo;
class treatment(ref="BNS") replicate(ref="1");
model hpo_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;



/*---------- Year 2 - hsa ----------*/
proc import out=work.data2hsa
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_hsa.xlsx"
                              DBMS=xlsx REPLACE;
            datarow = 2;
run;

proc sort data=data2hsa;
```

```
 by treatment time replicate;
run;

proc sgscatter data = data2hsa;
  plot hsa_int * time;
  by treatment;
run;

proc genmod data = data2hsa;
class treatment(ref="BNS") replicate(ref="1");
model hsa_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;



/*---------- Year 2 - hte ----------*/
proc import out=work.data2hte
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_hte.xlsx"
                              DBMS=xlsx REPLACE;
            datarow = 2;
run;

proc sort data=data2hte;
 by treatment time replicate;
run;
```

```
proc sgscatter data = data2hte;
  plot hte_int * time;
  by treatment;
run;

proc genmod data = data2hte;
class treatment(ref="BNS") replicate(ref="1");
model hte_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;



/*---------- Year 2 - pales----------*/
proc import out=work.data2pales
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_pales.xlsx"
                              DBMS=xlsx REPLACE;
            datarow = 2;
run;

proc sort data=data2pales;
 by treatment time replicate;
run;

proc sgscatter data = data2pales;
```

```
  plot pales_int * time;
  by treatment;
run;

proc genmod data = data2pales;
class treatment(ref="BNS") replicate(ref="1");
model pales_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;




/*---------- Year 2 - pici----------*/
proc import out=work.data2pici
    datafile = "S:\SCC\Yu Wang\Spring 2022\Crystal Bishop\beetledata_year2_pici.xlsx"
                              DBMS=xlsx REPLACE;
            datarow = 2;
run;

proc sort data=data2pici;
 by treatment time replicate;
run;

proc sgscatter data = data2pici;
  plot pici_int * time;
```

```
  by treatment;
run;


proc genmod data = data2pici;
class treatment(ref="BNS") replicate(ref="1");
model pici_int = treatment|time treatment|time*time/ dist = poisson type3;
repeated subject=replicate*treatment  / type = ar ;
run;
```

CHAPTER 3

ASSOCIATIONS OF LONGLEAF PINE INFESTING BARK AND ROOT FEEDING BEETLES WITH OPHIOSTOMATOID FUNGI[2]

Bishop C[1, 2], Meinecke CD[1], Jimenez JA[3], Klepzig KD[2] and Villari C[1]. To be submitted to Persoonia.
[1]Warnell School of Forestry and Natural Resources, University of Georgia, [2]Jones Center at Ichauway, [3] Department of Cellular Biology, University of Georgia.

Abstract

The longleaf pine (*Pinus palustris*) ecosystem is a robust and resilient hot spot of diversity in North America yet is in endangered due to fire suppression and human encroachment. Other biotic factors can affect this ecosystem such as lower stem and root feeding beetles and their symbiotic Ophiostomatoid fungi. It is not well documented what fungal species are found in longleaf pine ecosystems, however we hypothesized that we would identify *Leptographium procerum, L. terebrantis*, and *Ophiostoma ips* on lower stem and root feeding beetles and find these same species in longleaf pine roots. We set up 15 plots to capture live *Hylobius pales* and *Pachylobius picivorus* in the Fall of 2020 with billet traps, and to capture live *Dendroctonus terebrans* with flight intercept traps in the summer of 2021 and collected root samples from mature longleaf pines (*Pinus palustris*) in January of 2021. We isolated the Ophiostomatoid fungi vectored by the collected beetles and Sanger sequenced the βT and TEF-1α gene regions of the obtained colonies. We created maximum likelihood phylogenetic trees using the sequenced regions and identified *O. ips* and *L. terebrantis*, but did not find any *L. procerum*. Instead, we identified *L. profanum*, a fungus previously only identified in Alabama, USA within hardwoods. We also found an unidentified *Leptographium* species that separated from other *Leptographium* species in both the βT (bootstrap value of 72%) and the TEF-1α (bootstrap value > 54%) maximum likelihood phylogenetic trees. The information we determined here shows that more research needs to be conducted in southeastern forests to identify what species of *Leptographium* are present.

3.1 Introduction

Longleaf pine (*Pinus palustris*) ecosystems have a robust history in the southeastern USA where they were well-established before European colonization of the North American continent and maintained by indigenous populations through fire (Van Lear et al. 2005). Historically, the longleaf pine system encompassed geographical regions including Piedmont, Blue Ridge, Ridge and Valley, and the Cumberland Plateau, dominating about 30 million ha, but as of today, approximately 97% of its previous range has been lost or converted to other uses (Van Lear et al. 2005). As recently as 1995, the longleaf pine system was listed as one of the top three most endangered ecosystems in the USA (Van Lear et al. 2005). In addition to the threat of habitat loss, longleaf pines are also affected by biotic agents that can impact their growth. Among them, are the lower stem and root-feeding black turpentine beetle (*Dendroctonus terebrans*), pales weevil (*Hylobius pales*), and pitch-eating weevil (*Pachylobius picivorus*), which are pests of coniferous trees in forests of the southeastern USA (Baker 1972). These beetles bore into nutrient rich phloem layers of trees to mate and lay eggs, disrupting critical vascular tissue. As they do so, they introduce fungal spores they have carried phoretically and inoculate the host trees (Munro et al. 2019).

Vectored fungi may include *Leptographium terebrantis* (S.J. Barras & T.J. Perry), *Grosmannia huntii* (R.C. Robinson-Jeffrey), *Grossmania alacris* , *Leptographium procerum* (M.J. Wingfield) and *Ophiostoma* spp (Sydow and Sydow) (Matusick et al. 2012, Zanzot et al. 2010). While not known as aggressive tree pathogens, these fungi can be weakly virulent and locally affect the vascular system of their host (Harrington 2005, Jankowiak 2013, Kirisits 2004, Six and Wingfield 2011, Wingfield 1986). For example, in New Zealand, *L. procerum* is thought to contribute to a root disease complex (Shaw and Dick 1980) and, as a fungal associate of the

invasive red turpentine beetle (*Dendroctonus valens*), may contribute to the high rate of mortality of trees in native pine forests in China (Yin et al. 2015). *Ophiostoma ips* has been found in many countries, including North America and is associated with *Dendroctonus* and *Ips* species (Kim et al. 2003).

Considered different species in the past, members of the *Leptographium* genus are now recognized as the asexual state of some *Grosmannia* species and according to the one fungus one name principle the older genus name, *Leptographium,* is preferred over the newer name, *Grosmannia* (Yin et al. 2015). For clarity purposes, the *Leptographium* name will now on be used throughout this chapter. These fungi are commonly associated with conifer-infesting bark beetles and weevils and while over 90 species of *Leptographium* have been identified, only 12 have been associated with hardwoods (Jacobs and Wingfield 2001, Jankowiak et al. 2017, Linnakoski et al. 2012). Many species of *Leptographium* regularly reproduce asexually, and rarely sexually. For instance, recent genetic analysis of *L. procerum* and *L. profanum,* for which no sexual states have ever been observed, revealed that both possess loci associated with mating types, suggesting that cryptic sexual reproduction occurs in these species (Duong et al. 2012). Generally, *Leptographium* species have mononematous, darkly pigmented conidiophores terminating in several series of branches and produce slimy conidia that easily adhere to the exoskeletons of passing by insects, where they are phoretically carried and disseminated (Jankowiak et al. 2018). Their sexual fruiting structures, when present, are characteristically long necked perithecia (Yin et al. 2015).

Leptographium fungi belong to the Ascomycota order Ophiostomatales, which are commonly known as blue-stain fungi due to the characteristic blue-staining they cause in the colonized tissues (Harrington 2005, Jankowiak and Bilański 2013, Kirisits 2004, Six and

Wingfield 2011). Within these fungi, also referred to as Ophiostomatoid fungi, *L. terebrantis*, *G. huntii*, *L. procerum*, and *G. alacris* have been associated to the so-called Southern Pine Decline (Eckhardt et al. 2004, Eckhardt et al. 2007); however, the extent to which these fungi impact the health of southern pine trees is not yet well established (Coyle et al. 2015, Otrosina et al. 1999).

There is an extensive body of literature documenting the association of fungi with bark beetles, but there is relatively little information concerning the impacts of these insect-fungal associations on longleaf pine (Seifert et al. 2013). Further clarification is needed to determine which species of fungi are vectored by lower stem and root feeding beetles within the longleaf pine ecosystem, and what impacts they may have on longleaf pine health in the southeastern USA. We hypothesized that lower stem and root feeding beetles in the longleaf pine system would yield positive identification of the Ophiostomatoid fungi *L. procerum, O.ips,* and *L. terebrantis*, similarly to what have been already observed in the system (Eckhardt et al. 2007, Otrosina et al. 1999) and other southern pine systems (Wingfield 1986, Zhou et al. 2007), and that the same species would be observed in longleaf pine root tissue. We thus established a project in which we identified blue-stain fungi either found within mature longleaf pine roots or on the bodies of *D. terebrans*, *H. pales,* and *P. picivorus* beetles collected within a longleaf pine ecosystem.

3.2 Material and Methods

3.2.1 Field site

Our study site is The Jones Center at Ichauway, an 11,400 ha restored longleaf pine forest located in Baker County, Georgia (31.22N, 84.48W). This area has a humid subtropical climate with a mean annual temperature of 19 °C that has long, hot summers and short, moderate, cool winters, and annual precipitation of 1,310 mm per year (Wagner et al. 2019). The site is

dominated by longleaf pine, which covers between 60-90% of the canopy basal area, averaging between 6 to 20 m^2 ha^{-1} basal area (Wagner et al. 2019). The area is divided into burn blocks that are comprised mostly of mature, 80 to 100-year-old second-growth trees that are prescription burned on a two-year interval (Wagner et al. 2019). The soil is comprised mostly of siliceous, loamy, thermic Arenic Paleudults and poorly separated horizons (Hendricks et al. 2006).

3.2.2 Live beetle collection

We established 15 plots to collect live beetle specimens of *D. terebrans*, *H. pales* and *P. picivorus* utilizing two different trap designs, billet traps and flight intercepts traps, to target the different niches for lower stem feeding (*D. terebrans*) and root feeding (*H. pales* and *P. picivorus*), respectively (Baker 1972). We only used beetles collected alive to reduce contaminants and insect decomposers. We placed one billet trap within each plot of planted longleaf pine at least 10 m away from the nearest tree and at least 50 m away from roads or fire breaks within the burn block. Billet traps consisted of three small logs, placed inside an approximately 100 cm deep hole, which we covered with soil and longleaf pine branches (Flechtmann et al. 1999). Logs were approximately 30 cm long and with a diameter of approximately 13 cm. We removed strips of bark along the sides of logs to promote the release of pine volatiles and increase the attractiveness of the logs to root feeding beetles (Flechtmann et al. 1999, Tilles et al. 1986). These traps were active between September and October 2020.

To target *D. terebrans* beetles, we installed flight intercept traps by attaching one trap to the base of a mature longleaf pine tree in each plot. Flight intercept traps were baited with 95% ethanol (Pharmco by Greenfield Global, Brookfield, Connecticut) and 100% turpentine (Sunnyside Corporation, Wheeling, Illinois) and consisted of a one-gallon milk jug with one side removed from it before we attached it upside down to the tree. The inverted milk jug functioned

as a funnel and had its flat side flush with the tree surface so that when beetles struck it, they would fall into a 50 mL Falcon tube collection container connected to the threaded section of the jug. To increase attraction to the trap we scraped three strips of bark, approximately 0.5 m long and no more than 5 cm wide, off of the pine tree above the trap (Klepzig et al. 1991). Flight intercept traps were active from May 2021 to July 2021.

We collected live beetles from all traps every three days until we had collected a total of five live beetles per species for each plot or until the end of the peak beetle flight period for each species, as identified by a previous study (see Chapter 2). We placed each live beetle individually into a 2 ml centrifuge tube labeled with date and plot and transported them on ice to the laboratory at the Jones Center at Ichauway, where they were stored at 4 °C for up to one week. We transferred the beetles on ice to the University of Georgia; during transportation, the centrifuge tubes containing live beetles were stored in plastic deli cups to prevent direct contact with ice. Once at destination, we stored the beetles at 4 °C until further processing, which occurred no later than 4 weeks after arrival.

3.2.3 Root collection

In January of 2021, we collected root samples from longleaf pine roots at the Jones Center at Ichauway within each of the 15 plots previously described. Within each plot, we selected 4 mature longleaf pines, two asymptomatic and two symptomatic (i.e., displaying yellowing or dying needles and reduced crown vigor) with a diameter at breast height (DBH) of no more than 152 cm and no less than 50 cm, which we recorded using a DBH tape before collecting the roots. We collected two root samples from opposite cardinal directions of each tree (i.e., West and East), which we randomly assigned with the use of a random number generator. We dug around the base of each tree until we located a root that was connected to the selected pine tree. We then used a shovel and knife to clear a 20 x 20 cm area from around each selected

root and debarked about 10 x 10 cm of the top root surface with a drawknife. Finally, we used a hammer, hatchet, and chisel to cut a 5 x 5 cm square of root tissue that included the phloem and approximately the first few rings of wood. We sterilized all cutting tools by spraying them with 70% EtOH and letting them air dry before use. We stored samples individually in plastic Ziplock bags labeled by date, plot, tree number, and cardinal direction and transported all samples on ice to the laboratory, where we stored them at 4 °C until further processing. We washed the samples under tap water until all visible dirt and debris were removed and dried them at room temperature. Within a week after collection, we transported all samples on ice to the University of Georgia for further processing.

3.2.4 Isolation of Blue-stain Fungi

For blue-stain fungi isolation from live beetles, we used a serial dilution plating technique modified from Harrington and Fraedrich (2010). To minimize contamination, we used only live or recently dead (within 3 days) beetles that have been stored at -4 °C for no more than three days. Following a modified method from Battisti et al (1999), we used sterile tweezers to place beetles into 2 ml centrifuge tubes containing 1 mL of sterile 1% tween 80 (Sigma-Aldrich, Burlington, Massachusetts, USA) solution. We vortexed the beetles at 30 HZ for 60 seconds and plated 100 µL of 10X and 100X dilutions of the washing solution onto 90 mm plates of 2% malt agar media (VWR, Radnor, Pennsylvania, USA), amended with 300 ppm of streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA) and 200 ppm of cycloheximide (Sigma-Aldrich) (CSMA) (Harrington and Fraedrich 2010). We used cycloheximide to inhibit non-blue-stain fungal growth since all *Ophiostomatales* tolerate high levels of this fungistatic antibiotic (Jacobs and Wingfield 2001, Zhou et al. 2007). Immediately after pipetting, we used a sterile spreader to evenly distribute the solution over the entire surface of the media. Lastly, we wrapped plates in

Parafilm® (Bemis Company, Inc., Neenah, Wisconsin, USA) and stacked them three high to be incubated at 19 °C in the dark for seven days. On the seventh day of incubation, we classified growing colonies into morphotypes based on color, shape, yeasty consistency, and mycelial patterns. For morphotypes displaying the characteristic aspect of blue-stain fungi (e.g., darkly pigmented hyphae, presence of mononematous or synnematous conidiophores) (Jacobs and Wingfield 2001), we removed one colony of each morphotype per beetle from the original plate, transferred it to a fresh 90 mm 2% malt agar plate, and incubated it at room temperature until we observed sufficient growth for further processing.

For the isolation of blue-stain fungi from plant tissues, we worked in a laminar flow hood and cut root samples into 2x2 mm segments which we surface sterilized by rinsing them in 10% commercial bleach (The Clorox Company, Oakland, California) for 10 s, 70% EtOH for 10 s, and then sterile water for 10 s. Once sterile, we air dried the samples and placed them onto CSMA media in 90mm plates (Hamilton et al. 2020). We checked the plates every two days for fungal growth and promptly transferred mycelia to new plates to obtain pure cultures as needed.

3.2.5 DNA extraction, PCR, and Sequencing

To obtain fresh, medium-free mycelium, we grew each isolated pure culture on 2% potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, Maryland, USA) cellophane-covered plates for an average of 5 days, until we were able to scrape approximately 60 mg of mycelial tissue into a 1.5 mL microcentrifuge tube. We then extracted DNA from the fresh mycelium using the E.Z.N.A.® Fungal DNA Kit (Omega Bio-tek, Inc. Norcross, Georgia, USA), following the manufacturer's recommended protocol. To identify the isolates to species, we sequenced the β-tubulin (βT), and elongation factor 1-α (TEF-1α) gene regions, which have been shown to better discriminate species in the *Ophiostomatales* group than the commonly used

Internal Transcribed Spacer (ITS) region (Duong et al. 2015, Duong et al. 2012, Glass and Donaldson 1995, Stielow et al. 2015). We amplified the βT gene region using the primers Bt2a and Bt2b (Glass and Donaldson 1995) and the TEF-1α gene region using the primers EF1F and EF2R (Jacobs et al. 2004). For amplification, we used 25 μL of a reaction mixture consisting of 12.8 μL molecular grade water, 5.0 μL 5x Green GoTaq® Reaction Buffer (Promega, Madison, Wisconsin, USA) 2.5 μL dNTPs (2mM, Promega), 2.5 μL MgCl2 (25mM, Promega), 1.0 μL each of forward and reverse primers (10 μM), 0.2 μL GoTaq DNA Polymerase (5u/μL, Promega), and 2 μL of template DNA. Each PCR reaction contained at least one negative control in which water replaced the template DNA. We performed PCR reactions using a Mastercycler thermal Cycler (Eppendorf, Hamburg, Germany) for 30 cycles for each reaction. Reactions to amplify the βT region ran at 95 °C for 5 minutes for the initial denaturation step, followed by 30 cycles of 94 °C for 60s, 61 °C for 60s, and 72 °C for 60s, and a final chain elongation step at 72 °C for 10 minutes. For the TEF-1α PCR reaction, we set the initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30s, 55 °C for 30s, and 72 °C for 60s, and a final chain elongation at 72 °C for 8 minutes. We separated PCR products using a 1.0 % agarose gel (VWR, Radnor, Pennsylvania, USA) using gel electrophoresis. The gel contained 1x SYBR Safe DNA binding fluorescent stain (Invitrogen, Waltham, Massachusetts, USA) and we viewed the PCR products under UV light. We determined the sizes of amplicons by comparing them against the GeneRuler DNA Ladder 100 bp DNA standard (Thermo Fisher, Waltham, Massachusetts, USA). We sent final products to Eton Bioscience, Inc. (Research Triangle, North Carolina USA) for purification and Sanger DNA sequencing.

3.2.6 Sequence analyses

We received forward and reverse sequence reads for each gene region of each isolate, formatted as .fastq files and utilized the program Geneious prime v2021.1 (Biomatters, Inc., San Diego, California, USA) to process and analyze sequence data. We aligned forward and reverse reads to form consensus sequences using MUSCLE 8 (Edgar 2004) and manually trimmed consensus sequences for length and quality. We identified the isolates on a preliminary basis using BLAST searches (Altschul 2001) against the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov).

To confirm the preliminary species identification of fungal isolates that were initially identified as *Leptographium*, we performed phylogenetic analyses using maximum likelihood and inferred the species by determining their phylogenetic relationship to known reference strains. We chose to reference *L. procerum*, *L. profanum*, and *L. terebrantis* based on previously published findings of these Ophiostomatoids in similar ecosystems (Buland et al. 2019, Eckhardt et al. 2004, Mensah et al. 2021, Zanzot et al. 2010). Maximum likelihood phylogenies were constructed for each βT and TEF-1α gene regions using RAxML v4.0 and 1000 bootstrap replicates (Stamatakis 2014) as shown in figures 3.1 and 3.2. We used a total of 9 corresponding reference sequences obtained from the Gene Bank to show the placement of our representative isolate sequences within the *Leptographium* genus and used *L. longiconidiophorum* (CMW2004) as our outgroup taxon.

3.3 Results

3.3.1 Fungal Identification

We obtained a total of 33 Ophiostomatoid fungal isolates from lower stem and root feeding beetles collected in the longleaf pine ecosystem, which we preliminary identified through Sanger DNA sequencing as belonging to the three species *O. ips*, *L. profanum*, and *L.*

terebrantis (Table 3.1). Twenty of the forty-two *H. pales* beetles collected during the peak flight season carried Ophiostomatoid fungi, which we identified as *O. ips* and *L. profanum*. Ten of the twenty-one collected *P. picivorus* beetles harbored *L. profanum*. Finally, out of the fourteen live captured *D. terebrans* included in this study, just one of the beetles carried an Ophiostomatoid fungus we identified as *L. terebrantis*.

Out of 45 mature longleaf pines from which we collected root samples, only a single root sample from an asymptomatic longleaf pine yielded an Ophiostomatoid fungus, which we identified as *L. profanum* (Table 3.1).

Of the 33 Ophiostomatoid isolates, preliminary BLAST searches identified 27 as *Leptographium* spp. and 6 as *O. ips*. Sequences that matched to *O. ips* were excluded from further phylogenetic analysis as the BLAST search results were deemed to be sufficiently specific for species-level identification.

3.3.2 Phylogenetic analysis

The βT and TEF-1α sequences of 11 isolates (Table 3.2) were selected for phylogenetic analysis to confirm the preliminary identification achieved through the BLAST comparison. These sequences of the isolates represented all genetic variants per each beetle.

We examined the relatedness of collected isolates shown in Figure 3.1 for the βT gene region and identified three distinctive taxa. The sequence of the isolate CV_2020_032 grouped most closely with those of *L. terebrantis* (CBS337.70) and our sequence of the isolate CV_2021_030, which grouped together, but the isolate remains unidentified. The sequences of both reference and putative *L. profanum* isolates grouped together with a bootstrap value of 68.5%, but then formed three distinct groups. Our TEF-1α tree (Figure 3.2) showed the sequence of the isolate CV_2020_032 to from an unidentified taxon by itself, thus confirming the results

obtained with the βT gene region. The sequence of the isolate CV_2021_030 was identical to that of the CBS337.70 *L. terebrantis* strain, with which it grouped. Both sequences derived from *D. terebrans* specimens, but came from different pine systems, longleaf and loblolly, respectively. All our *L. profanum* isolate sequences grouped together at a bootstrap value of 65 with all available strains of *L. profanum,* which were isolated from hardwood hosts. In the TEF-1α tree we also identified several genotypes of *L. profanum* that differed by one or two base pairs (Figure 3.2 and Table 3.3).

3.4 Discussion

We sought to characterized Ophiostomatoid fungi carried by lower stem and root feeding beetles within the longleaf pine ecosystem. We sought to document which Ophiostomatoid species were found with the roots of asymptomatic and symptomatic longleaf pine roots, to determine if there was any overlap between the species carried by the beetles and those found in the trees. We hypothesized that lower stem and root feeding beetles would commonly harbor Ophiostomatoid genera such as *Leptographium* and *Ophiostoma*, which are known to be associated with these beetles (Eckhardt et al. 2004, Eckhardt et al. 2007, Kim et al. 2003, Otrosina et al. 1999). We also hypothesized that these same genera would be found within the roots of longleaf pine trees.

The analysis of the βT gene region identified three species, *L. terebrantis*, *O. Ips*, and three different genotypes for *L. profanum*, while the TEF-1α region was more discriminant, identifying *L. terebrantis*, *O. Ips*, and six different genotypes for *L. profanum*. As expected and previously reported (Eckhardt et al. 2007, Zanzot et al. 2010), we isolated *O. ips* from *H. pales* and *P. picivours*, and L. *terebrantis* from *D. terebrans* within longleaf pine woodlands. Surprisingly, we did not isolate any *L. procerum*. Instead, we isolated *L. profanum* from this

same group of beetles. This stands in stark contrast to previous work describing *L. profanum* as only colonizing hardwoods such as blackgum (*Nyssa sylvatica* Marshall), hickory (*Carya sp*. Nutt.), and flowering dogwood (*Cornus florida* L.) and not in association with any insect vector (Jacobs et al. 2006). In this study and in a previous recent thesis (Buland et al. 2019), however, we have isolated *L. profanum* from root feeding beetles associated with two different pine systems, loblolly and longleaf. While we did document *L. profanum* within longleaf pine roots, we were only able to recover one isolate from a single root sample. Interestingly, *L. profanum* exhibited eight different genotypes, suggesting a high genetic diversity of this fungal species in comparison to Jacobs et al (2006), who found only three genetically different sequences of isolates. We also found an unidentified *Leptographium* spp (isolate CV_2020_032) that most closely resembled *L. profanum* but differed from it by 6 base pairs in the βT gene region and 16-21 base pairs in the TEF-1α gene region. In comparison, other sequences of isolates identified as *L. profanum* differed by only one base pair or 10 base pairs in the βT and TEF-1α gene regions, respectively. Future studies could include other taxonomically useful regions, such as the internal transcribed spacer 2 and the 28S large subunit regions of the nuclear ribosomal DNA gene, the actin gene, and the calmodulin gene, which would help to better identify the CV_2020_032 isolate to the species level.

We expected to identify *L. procerum* as found by Eckhart (2007) and Otrosina (1999). *Leptographium procerum* and *L. profanum* are closely related, morphologically similar, and native to the southeastern USA (Jacobs et al. 2006). However, prior to our recent work, only *L. procerum* was reported as associated with root lower stem and root feeding beetles within loblolly pines (Eckhardt et al. 2004, Eckhardt et al. 2007, Jankowiak et al. 2017). The isolation of *L. profanum* within loblolly (Buland et al. 2019) and longleaf pines (current study) in the

Southeast and the lack of isolation of *L. procerum* suggests that previous reports of *L. procerum* in southern pines from work performed without the use of molecular tools for species identification (Otrosina et al. 1999, Otrosina et al. 2001, Zanzot et al. 2010) might have been due to a misidentification of the species, and that *L. profanum* is in truth the species more prevalent among pine stands in the Southeast.

In conclusion, we found *L. profanum* to be highly associated with southern pines, with an apparent absence of *L. procerum*. Further study is needed to better define the prevalence and occurrence of this species, as well as the taxonomy of *L. procerum* vs. *L. profanum.* We also confirmed that *L. terebrantis* is found within the longleaf pine ecosystem along with *O. ips*. In future work, we will identify the isolate CV_2020_032, survey a larger sample size of *D. terebrans,* and add other root feeding beetles, such as *Hylastes* species, which are commonly found in the south and associated with Ophiostomatoid fungi. This would provide information for a more comprehensive picture of the composition of Ophiostomatoid fungi in longleaf pine ecosystems.

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Table 3.1. Ophiostomatoid fungi isolated from live captured lower stem and root-feeding beetles collected in a longleaf pine (*Pinus palustris*) ecosystem and from longleaf pine root tissues at The Jones Center at Ichauway, Georgia. Preliminarily identification was achieved through Sanger DNA sequencing of the β-tubulin (βT) and elongation factor (TEF-1α) gene regions, and BLAST searches against the GenBank nucleotide database.

Fungal species	Collection number	Host/Source	βT	TEF-1α
			Percent pairwise ID	Percent pairwise ID
Leptographium profanum	CV_2020_009	*Pachylobius picivorus*	99.7	99.6
Leptographium profanum	CV_2020_010	*Hylobius pales*	99.7	N/A
Leptographium profanum	CV_2020_011	*N/A*	97.2	99.8
Leptographium profanum	CV_2020_012	*Pachylobius picivorus*	100	99.8
Leptographium profanum	CV_2020_013	*N/A*	100	99.6
Leptographium profanum	CV_2020_014	*Pachylobius picivorus*	99.7	99.4
Leptographium profanum	CV_2020_015	*Pachylobius picivorus*	99.7	99.8
Leptographium profanum	CV_2020_016	*Pachylobius picivorus*	100	99.4
Leptographium profanum	CV_2020_017	*Hylobius pales*	100	99.6
Leptographium profanum	CV_2020_019	*Hylobius pales*	100	99.8
Leptographium profanum	CV_2020_022	*Hylobius pales*	100	99.8
Leptographium profanum	CV_2020_023	*Hylobius pales*	100	99.1
Leptographium profanum	CV_2020_024	*Hylobius pales*	100	99.8

Leptographium profanum	CV_2020_025	*Hylobius pales*	100	N/A
Leptographium profanum	CV_2020_026	*Hylobius pales*	100	100
Leptographium profanum	CV_2020_029	*Pachylobius picivorus*	100	N/A
Leptographium profanum	CV_2020_030	*Pachylobius picivorus*	100	99.7
Leptographium profanum	CV_2020_031	*Hylobius pales*	99.8	99.6
Leptographium profanum	CV_2020_032	*Hylobius pales*	97.8	97.8
Leptographium profanum	CV_2020_033	*Hylobius pales*	100	N/A
Leptographium profanum	CV_2020_034	*Hylobius pales*	100	N/A
Leptographium profanum	CV_2021_028	*Pinus palustris*	100	N/A
Leptographium terebrantis	CV_2021_030	*Dendroctonus terebrans*	100	100
Ophiostoma ips	CV_2020_018	*Hylobius pales*	100	99.8
Ophiostoma ips	CV_2020_020	*Hylobius pales*	100	99.6
Ophiostoma ips	CV_2020_021	*Hylobius pales*	100	99.6
Ophiostoma ips	CV_2020_027	*Pachylobius picivorus*	100	N/A
Ophiostoma ips	CV_2020_028	*Hylobius pales*	100	100
Ophiostoma ips	CV_2020_039	*Hylobius pales*	100	N/A

Table 3.2. List of isolates used to build the phylogenetic trees of the β-tubulin (βT) and elongation factor (TEF-1α) gene regions.

Culture ID	**Fungal species**	**Host/Source**	**Location**	**Beta tubulin**	**Elongation factor**	**Publication**
CMW 10550	*Leptographium profanum*	*Carya sp.*	Alabama, United States	DQ354935	DQ354940	Jacobs *et al.*, 2006
CMW 10552	*Leptographium profanum*	*Nyssa sylvatica*	Alabama, United States	DQ354936	DQ354941	Jacobs *et al.*, 2006
CMW 10554	*Leptographium profanum*	*Cornus florida*	Alabama, United States	DQ354934	DQ354939	Jacobs *et al.*, 2006
CMW 661	*Leptographium procerum*	*Pinus resinosa*	New York, United States	KM491422	KM491478	Yin *et al.*, 2015
CMW 10217	*Leptographium procerum*	*Pinus strobus, Dendroctonus valens*	Vermont, United States	KM491370	KM491479	Yin *et al.*, 2015
CMW 34542	*Leptographium procerum*	*Pinus strobus, Dendroctonus valens*	Maine, United States	KM491374	KM491483	Yin *et al.*, 2015
CBS 337.70	*Leptographium terebrantis*	*Pinus taeda, Dendroctonus terebrans*	Louisiana, United States	JF798459	JF798470	Lu *et al.*, 2009
CMW 9	*Leptographium terebrantis*	*Pinus sylvestris*	Minnesota, United States	AY534932	EU652700	Lu *et al.*, 2009
CMW 2004	*Leptographium longiconidiophorum*	*Pinus densiflora*	Japan	KM491362	KM491471	Yin *et al.*, 2015
CV_2020_009	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_011	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_012	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_013	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study

CV_2020_014	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_015	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_016	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_017	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2020_019	*Leptographium profanum*	*Hylobius pales*	Georgia, United States	N/A	N/A	Current Study
CV_2021_028	*Leptographium terebrantis*	*Pinus palustris*	Georgia, United States	N/A	N/A	Current Study
CV_2021_032	*Leptographium terebrantis*	*Dendroctonus terebrans*	Georgia, United States	N/A	N/A	Current Study

Table 3.3. Genotypes of *Lepotgraphium profanum* produced from the elongation factor (TEF-1α) phylogenetic tree (shown in figure 2). Isolates were obtained from lower stem and root feeding beetles and a longleaf pine (*Pinus palustris*) roots collected at The Jones Center at Ichauway, Georgia.

Source	**Isolate**	**Genotype**
Hylobius pales	CV_2020_012	D
Hylobius pales	CV_2020_019	D
Hylobius pales	CV_2020_017	B
Hylobius pales	CV_2020_024	A
Hylobius pales	CV_2020_025	F
Hylobius pales	CV_2020_035	E
Pachylobius picivorus	CV_2020_009	F
Pachylobius picivorus	CV_2020_030	C
Pinus palustris	CV_2021_028	C

Figure 3.1. Phylogram created with maximum likelihood from a subset of selected collected Ophiostomatoid fungal species, for the β-tubulin (βT) gene region. This tree shows the placement of collected species along with sequences obtained from BLAST. The isolates collected in this study are from lower stem and root feeding beetles and a longleaf pine (*Pinus palustris*) root, are highlighted in yellow.

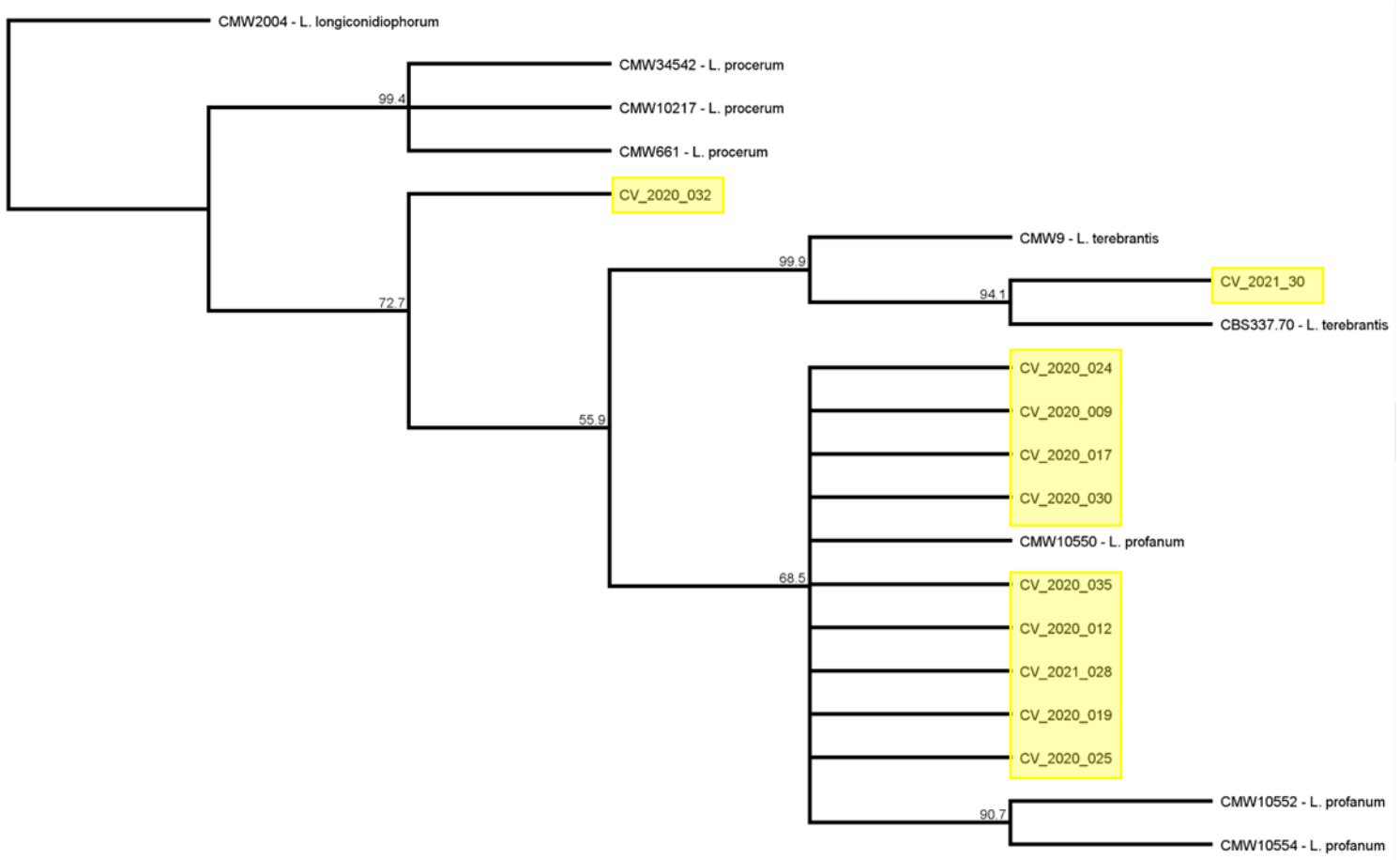
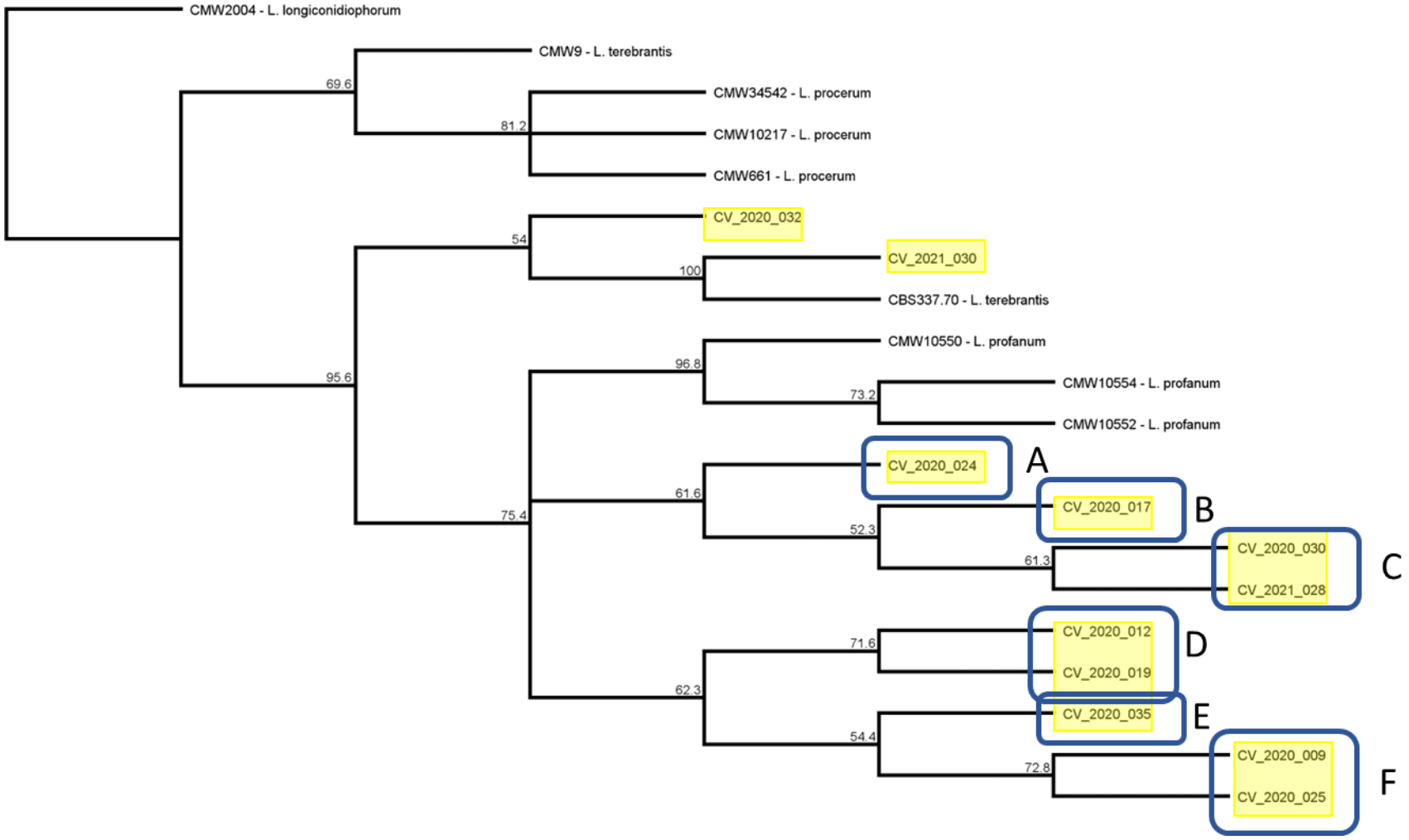


Figure 3.2. Phylogram created with maximum likelihood from a subset of selected collected Ophiostomatoid fungal species, using the elongation factor (TEF-1α) gene region. This tree shows the placement of collected species along with sequences obtained from BLAST. The isolates collected in this study from lower stem and root feeding beetles and longleaf pine (*Pinus palustris*) roots are highlighted in yellow. Blue squares indicate different genotypes of *Leptographium profanum*.



CHAPTER 4

THESIS CONCLUSIONS

4.1 Conclusions

Longleaf pine (*Pinus palustris*) is an important forest resource in the southeastern United States. Longleaf pine ecosystems are home to high amounts of biodiversity in North America and the system has high resilience to natural disturbances (Susaeta and Gong 2019). This ecosystem historically covered the majority of the Southeast, encompassing the Blue Ridge, the Cumberland Plateau, Ridge and Valley, and Piedmont regions and dominating about 37 million ha (Van Lear et al. 2005). This ecosystem was maintained by indigenous populations, before European colonization, through cultural burning - intentionally set, fires to enhance wildlife habitats and improve plant resources (Van Lear et al. 2005). Because of the suppression of wildfires, discouragement of intentionally set management fires, and land conversion for agricultural uses and human developments, longleaf pine has lost approximately 97% of its historical range and in 1995 was labeled as the third most endangered ecosystem in the United States (Van Lear et al. 2005). Because of its shade intolerance, longleaf pine is dependent upon disturbances like lightning strikes and even the occasional hurricane to create gaps in canopy cover for regeneration. (Ashton and Kelty 2018, Landers et al. 1995). However, hurricane damage is a concern due to anthropogenic climate change that is projected to increase the intensity of hurricanes (Cole et al. 2021). For example, Hurricane Michael, which struck the southeastern United States in October 2018, made landfall as a category five storm and plunged into the southwestern corner of Georgia as a category four (EPA 2019). This hurricane had wind speeds of up to 240 kph (150 mph), immense rainfall, and left massive amounts of coarse woody debris and snapped trees in its wake, resulting in nearly $3 billion of damage, including $1

billion in timber alone (Brett 2018, Georgia Forestry Commission 2018). However, this was only the immediate damage done. Hurricane damage, in fact, brings additional long-lasting effects, such as delayed tree mortality which may alter patterns of forest regeneration and succession while at the same time strengthening other disturbances such as fire and herbivory (Dale et al. 2001, Rutledge et al. 2021).

Forest pests, such as bark beetles and weevils, can take advantage of this large-scale disturbance, feeding and reproducing within the phloem of stressed trees and vectoring blue-stain fungi (Baker 1972, Zanzot et al. 2010). Specifically, *Hylastes*, *Hylobius*, *Pachylobius*, and *Dendroctonus* species (Coleeoptera: Curculionidae) are well known as destructive forest pests in the southeast United States (Baker 1972). The vectored blue-stain fungi, mostly mildly virulent, are in the Ascomycota order *Ophiostomatales*, in the genera *Ophiostoma*, *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia*, and related asexual morphs such as *Pesotum* and *Leptographium* (Harrington 2005, Jankowiak 2013, Kirisits 2004, Six and Wingfield 2011). These fungi and bark beetles form a symbiotic relationship in which the fungi create a better feeding environment by detoxifying tree defenses, while the beetles inoculate the fungi into new host trees (Biedermann and Vega 2020). Additionally, blue-stain fungi have been associated with the decline of southern pines (Eckhardt and Menard 2008); however, it is unknown if they are causing disease or merely secondary colonizers that are taking advantage of an already compromised tree (Coyle et al. 2015).

To explore lower stem and root feeding beetles and their symbiotic blue-stain fungi and how they interact with the longleaf pine ecosystem after a hurricane, we set up two projects: one where we studied lower stem and root feeding beetle abundance in various management treatments over two years after the occurring of a hurricane, and a second where we documented

the fungal community composition of selected beetle species trapped within treatments. For the first project, we set up three management treatments at the Jones Center at Ichauway, an 11,400 ha, restored longleaf pine ecosystem that had been impacted by Hurricane Michael in October 2018. These treatments were (i) salvage logging following the hurricane in winter of 2018 to spring of 2019 and prescribed burning in the spring of 2019; (ii) no salvage logging but prescribed burning in spring of 2019; and (iii) no salvage logging and but delayed prescribed burning in spring of 2020. We set out (i) to characterize the seasonal flight patterns for the lower stem and root feeding beetles *D. terebrans, H. porculus, H. salebrosus, H. tenuis, H. pales,* and *P. picivorus*, (ii) to determine if peak beetle abundance of these species are significantly different across treatments, and (iii) to determine if numbers of blue-stain propagules for *H. pales* and *P. picivorus* are different between these treatments. We hypothesized that beetle abundances would differ between treatments, being higher in treatments that have more coarse woody debris such as in year one in the no prescribed burning and no salvage logging plots, and in year two in the prescribed burning and no salvage logging. We also hypothesized that blue-stain propagules would be found in higher amounts in treatments with higher coarse woody debris.

We found that beetles in both years were significantly affected by time, but only three species were significantly affected by treatment and time in the second year. Our results indicate that beetle abundance varies by seasonality through both years of collection. In the second year , *D. terebrans, H. porculus*, and *H. tenuis* had a significantly higher abundance within the no prescribed burning and no salvage logging plots as time progressed.

When only looking at the peak flight of each beetle species for each year following the hurricane, we found that in the first year *H. salebrosus* abundance was significantly higher within the no prescribed burning no salvage logging plots than in the prescribed burning no

salvage logging. In the second year, *H. porculus* abundance was significantly higher in the prescribed burning and no salvage logging than in the other two treatments. The number of blue-stain propagules were not statistically different among our treatments.

For our second project, we identified to the species level the blue-stain fungi vectored by live specimens of *D. terebrans*, *H. pales,* and *P. picivorus*, as well as those present within mature symptomatic and asymptomatic longleaf pine roots. We hypothesized that that we would find species of Ophiostomatoid fungi including *L. procerum, O. ips,* and *L. terebrantis* and that the same species would be found within the roots of longleaf pine. As expected, we identified *L. terebrantis* and *O. ips.* However, we surprisingly never detected *L. procerum* in the system, but we isolated several isolates of *L. profanum*, a blue-stain fungus that was previously identified only from hardwoods (Jacobs et al. 2006). This suggests a possible case of species misidentification in the past literature.

While we did find that there is a delayed effect of no management activities directly after Hurricane Michael, as shown by our results on beetle abundance for *D. terebrans*, *H. porculus* and *H. tenuis* in the second year of sampling after the disturbance event,, it can come as a relief to longleaf pine forest managers that *H. salebrosus*, *H. pales*, and *P. picivorus* abundance and that of their symbiotic blue-stain fungi are unaffected by management practices that aim to reduce the amount of downed timber. We did positively identify *L. terebrantis* and *O. ips* in the longleaf pine ecosystem and found that *L. profanum* is associated with root feeding beetles and longleaf pine roots. These research studies combined show a more comprehensive picture of lower stem and root feeding beetles and their symbiotic blue-stain fungi within the longleaf pine ecosystem.

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