Developing novel molecular detection techniques for hemlock woolly adelgid (*Adelges tsugae*)

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DEDICATION

I dedicate this thesis to “She Don’t Use Jelly” by The Flaming Lips as I deemed this a theme song throughout my research. I also dedicate this thesis’s completion to Lil Nas X’s album “Montero.”

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ABSTRACT

Hemlock forests in eastern North America face a deadly threat: the invasive insect, hemlock woolly adelgid (HWA), *Adelges tsugae*. Early detection of this pest remains a key focus for management groups to ensure rapid response to control and stop the spread of HWA. In Chapter II, our goals were to develop an affordable, easy-to-use trap that is compatible with airborne eDNA sampling techniques and assess its efficiency as a monitoring tool for HWA. We tested three potential trap designs (i.e., passive trap, funnel trap, and motorized trap) against a standard sticky trap. Our passive, funnel, and motorized traps estimated adelgid capture success probabilities compared to sticky traps were 0.87, 0.8, and 0.4, respectively. We then further assessed the motorized trap after modifying the original design. In the secondary study, the motorized trap increased in estimated success probability to 0.67. We also evaluated how many traps would be needed in a set area size to maintain high probability of detecting HWA and measured how environmental variables affected trap performance in capturing adelgids. We found that number of traps placed within a 3-acre area did not impact trap capture success over a 16-week collecting period, but trap elevation and distance to an infested hemlock did affect adelgid numbers. In Chapter III, we continued to assess the motorized trap's performance across varied height and distance to an infested hemlock stand. We also determined how well a rapid molecular assay worked to detect HWA from the environmental samples caught by the trap. Again, trap distance to an infestation impacted trap capture success, but the height of a trap did not. The molecular assay reached a 0.9 probability of detecting HWA when a trap sample had approximately 14 adelgids present. This technology showed to be very promising as a monitoring tool for HWA and could help preserve valuable personnel and financial resources for HWA eradication efforts across its invasive range.

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ABBREVIATIONS

AIC = Akaike information criteria
ANOVA = Analysis of variance
bp = Base pairs
cm = centimeter
CO1 = Cytochrome oxidase 1 gene
DNA = Deoxyribonucleic acid
eDNA = Environmental DNA
GLM = Generalized linear model
GPS = Global Positioning System
GVSU = Grand Valley State University
HWA = Hemlock woolly adelgid
IDW = Inverse distance weighting
m = meter
m^2 = meters squared
mCO1 = mitochondrial CO1
MEGA = Molecular Evolutionary Genetics Analysis
mg = milligram
min = minutes
mL = milliliter
NODU = North Ottawa Dunes
PCR = Polymerase chain reaction
PIPK = Pioneer Park
PVC = Polyvinyl chloride
qPCR = Quantitative polymerase chain reaction
RBS = Random branch sampling
rtPCR = Real-time polymerase chain reaction
s = seconds
SLBR = Sleeping Bear Dunes National Lakeshore
USA = United States of America
U.S. = United States
USDA = United States Department of Agriculture
°C = Degrees Celsius
uL = microliter
uM = micromolar

CHAPTER I

INTRODUCTION

Hemlock trees are shade-tolerant species that commonly occur within mesic hardwood forests. They can be found in riparian areas along slopes where they form thick canopies with foliage that extends to the ground. This provides critical thermal cover, habitat diversity, and quality ecosystems for a variety of flora and fauna (Yamasaki et al., 2000; Toenies et al., 2018). Hemlocks can have important effects on aquatic ecosystems by shading streams to moderate temperatures, regulating streamflow, and decreasing runoff into their surrounding water bodies (Rogers, 1978; Snyder et al., 2002; Ford and Vose, 2007; Havill et al., 2014). They even influence soil temperatures, nitrogen cycling, and decomposition rates in their ecosystems (Havill et al., 2011).

Loss of hemlock trees can lead to many immediate, short-, and long-term alterations to their ecosystem structure and function (Orwig and Foster, 1998; Ellison et al., 2005; Ellison et al., 2018). In North America, hemlocks' economic value in terms of ecosystem services has been valued at $969 per hectare each year (Havill et al., 2014). A common cause of hemlock mortality in North America is the invasive insect, hemlock woolly adelgid, *Adelges tsugae* (HWA). Hemlocks infested with HWA typically do not survive without some involvement of pest management, and there is often a lack of natural re-establishment after hemlock death from this invasive species (Ford et al., 2012; Havill et al., 2014; McCarty and Addesso, 2019).

Hemlock woolly adelgid is an aphid-like insect native to Japan, China, Taiwan, and western North America (Havill et al., 2014; Limbu et al., 2018). HWA is not a threat to Asian and western North American hemlock species, and this has been attributed to a combination of

host tolerance, host resistance, and the presence of predators in those regions (Oten et al., 2014). As a member of Adelgidae, HWA has a complex life cycle, which slightly varies between its ranges (Havill et al., 2014). In its native ranges, HWA can alternate between hemlock and spruce species. Sexual reproduction occurs on the spruce trees by individuals known as sexuparae, while hemlocks are considered a secondary host only supporting asexual generations. In its native range of western North America, some areas of Japan, and its invasive range, a suitable host spruce is not present, so HWA will complete a shortened life cycle solely on hemlocks that consists of two asexual generations, each going through four nymph stages, repeating annually (Havill et al., 2014; Limbu et al., 2018). The first stage to hatch from each generation are referred to as crawlers since they are the only mobile life stage and disperse to feed on old or new growth needles, depending on the generation.

The earliest records of invasive HWA in eastern North America occurred in Richmond, Virginia, United States, in 1951 (Havill et al., 2011), and these populations are thought to have originated from Japan (Havill and Foottit, 2007). HWA quickly became a problem in this region as it most notably threatens eastern and Carolina hemlocks (*Tsuga canadensis*; *Tsuga caroliniana*). This insect feeds on hemlock nutrients by inserting a feeding stylet into the hemlocks' plant tissue and can kill healthy trees in four to ten years (Havill et al., 2014). The adelgids produce a white wax to cover themselves while feeding at the base of hemlock needles. This waxy or 'woolly' material is known as an ovisac and is what helped give HWA its name. HWA population growth rates are often highest during the initial stages of infestation on healthy hemlocks, and it is capable of explosive population increases in its invasive range since few natural predators have been identified (Havill et al., 2014). HWA can spread to new areas through birds, mammals, wind, and humans (McClure, 1990), and it can now be found from

Georgia to Canada. This invasive insect eventually expanded westward to Michigan with the most recent infestation believed to have been introduced in 2015 through nursery stock planted on private property (Michigan Department of Natural Resources, 2021), and HWA has since continued to spread within the state.

Management efforts have been ongoing across eastern North America to control and stop the spread of HWA since its introduction. Detecting new infestations are key for employing a rapid management response and having any hope of eradication (Lodge et al., 2006). Genetic approaches have been advancing as an affordable, efficient option to detect invasive species through methods such as environmental DNA (eDNA) techniques (Morisette et al. 2021). Any genetic material shed from organisms into surrounding water, soil, or air (Lodge et al., 2012) can be classified as eDNA. Invasive species managers can collect and analyze eDNA for the presence of targeted species. These eDNA approaches have been utilized for the invasive Asian carp (*Hypophthalmichthys* spp.) and sea lamprey (*Petromyzon marinus*) in the Great Lakes (Turner et al., 2014; Gingera et al. 2016) as well as invasive forest insects such as spotted lanternfly (*Lycorma delicatula*) in the northeastern United States (Valentin et al., 2020). Early detection of HWA is vital and exploring additional monitoring approaches such as eDNA techniques may improve those efforts.

PURPOSE

The purpose of this research is to identify an affordable, efficient trap to capture airborne HWA particles in a forest setting, assess long-term trap performance in capturing adelgids, and determine how well a rapid molecular assay can detect HWA from environmental samples.

SCOPE

The scope of this research focused on developing and testing HWA-specific eDNA technology in western Michigan. However, the traps and molecular assay analyzed in this study potentially can be used to monitor HWA across its invasive range in eastern North America.

ASSUMPTIONS

For adelgid counts in both chapters, we assumed crawlers that were captured on a petroleum-jelly-coated microscope slide (for motorized and passive traps) remained on a slide through the time of sample collection and analysis. For all traps used in this research, we assumed adelgids counted from each trap were collected from corresponding trap locations and were not the result of contamination.

In Chapter II, we assumed for the generalized linear model: our adelgid count data better fit a negative binomial distribution due to overdispersion; independence of our data values; and the model with the lowest Akaike information criteria (AIC) was optimal. Also, the inverse distance weighting (IDW) spatial interpolation method used in this chapter predicts values for unsampled locations by assuming those values are related more to closer data points than to those that are farther away.

In Chapter III, we assumed for the logistic regression: there was a linear relationship between the logit of the response and our explanatory variable; there were no outliers in our continuous predictor (adelgid counts); there was no multicollinearity among the predictors because we only used one explanatory variable; and our response variable was binary (positive vs negative rtPCR result).

For Chapter III, we assumed sistens counts to estimate site infestation level were representative of HWA density at each site and remained a consistent infestation level throughout the year-long study. We also assumed the negative controls used in this chapter to test our environmental samples with the molecular assay (field-blank, Vaseline-separation-blank, and rtPCR-blank) producing negative rtPCR results indicated there was no HWA contamination, and we assumed there was no HWA contamination for samples associated with those negative controls.

OBJECTIVES

Our main objectives of this project were to (1) develop an affordable, easy-to-use airborne HWA trap that is also compatible with genetic analysis and assess their efficiency (Chapter II), (2) further evaluate trap performance across varied height and distance to infestation (Chapter III), and (3) determine how well a rapid molecular assay works to detect HWA from environmental trap samples (Chapter III).

SIGNIFICANCE

This research was the first assessment to use molecular assays to detect HWA from airborne environmental samples. Our research also adds to the minimal scientific studies that have developed detection techniques for HWA monitoring and early detection. While some studies have evaluated the use of sticky traps in catching individuals in the mobile crawler stage, HWA identification in this life stage requires taxonomic expertise in areas where other adelgid species are present (Fidgen et al., 2019). The use of our DNA-compatible trap and this rapid molecular assay could allow for faster and easier confirmation of HWA presence within a

specific area. Many states, like Michigan, largely use on-the-ground visual surveys to find new HWA infestations, but these assessments are time and labor intensive and may miss early invasions (Evans and Gregoire, 2007). This technology could be used as a better early monitoring approach for natural resource managers to more easily detect early infestations or low populations of this invasive insect, and it can even be used in tandem with other monitoring efforts. For example, our methods could be used for initial surveillance, and a detection can initiate a more thorough site assessment through other means such as visual surveys. These techniques can help preserve valuable personnel and funds for HWA control and eradication efforts throughout eastern North America.

DEFINITIONS

Crawler: mobile, first-instar nymph stage of HWA.

eDNA: environmental DNA referring to genetic material collected from environmental sources like soil, water, or air.

Ovisac: white, waxy or woolly material produced by HWA to cover and protect itself as it feeds on the hemlock tree.

PCR: polymerase chain reaction is a molecular biology technique that makes numerous copies of DNA fragments.

Primers: short segments of DNA that allow for targeted copying of a piece of DNA.

Probe: a short segment of DNA that can be used with primers in some rtPCR techniques to allow for even more specificity binding to target DNA.

Progredientes: first of two annual asexual generations produced by HWA (progrediens = singular; progredientes = plural).

Quantitative PCR: another name for real-time PCR specifically referencing the method's ability to quantify the number of DNA copies present in the PCR reaction.

Real-time PCR: real-time PCR copies DNA fragments, and it also monitors amplification of targeted DNA as the reactions take place in real time.

Sexuparae: winged adults of HWA that disperse to sexually reproduce on spruce trees in HWA's native ranges (sexupara = singular; sexuparae = plural).

Sistentes: second annual asexual generation of HWA (sistens = singular; sistentes = plural).

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CHAPTER II

Development of novel early detection technology for hemlock woolly adelgid (*Adelges tsugae*)

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ABSTRACT

Hemlock woolly adelgid (HWA), *Adelges tsugae,* is an invasive pest that is a threat to hemlock forests throughout the eastern United States. Management efforts are underway to control and stop the spread of this pest, and in Michigan the primary focus is on early detection. The goal of this study was to identify an affordable, efficient trap that can aid with airborne eDNA sampling approaches as an early monitoring tool for HWA. Sticky traps are currently used as a monitoring tool for HWA but are not fully compatible with rapid DNA analysis. We initially assessed HWA detection success between three alternative trap designs potentially compatible with eDNA protocols (i.e., passive trap, funnel trap, and motorized trap) compared to a standard sticky trap. Our passive, funnel, and motorized traps estimated capture success probabilities compared to sticky traps were 0.87, 0.8, and 0.4, respectively. We also considered cost, ease of use, and sturdiness of each trap to determine which design may be best for land managers to use in the future. We then conducted a secondary evaluation of a modified version of the motorized trap to determine the number of traps needed in a set area size to maintain high probability of detecting HWA and further assess trap performance. By modifying the original motorized trap design, we increased its estimated success probability from 0.4 to 0.67, when compared to a sticky trap. We found that number of traps placed within a 3-acre area did not impact trap capture success over a 16-week collection period, but trap elevation and distance to an infested hemlock did affect the number of adelgids captured. This technology could help preserve valuable personnel and financial resources for HWA eradication efforts in Michigan as well as aid land managers in other states continuously monitoring HWA populations.

INTRODUCTION

Hemlock trees are critical to both terrestrial and aquatic systems as they provide thermal cover, habitat diversity, and quality ecosystems for a variety of flora and fauna (Yamasaki et al., 2000; Snyder et al., 2002; Ford and Vose, 2007; Toenies et al., 2018). Losing hemlocks can drastically alter their ecosystems (Orwig and Foster, 1998; Ellison et al., 2005; Ellison et al. 2018). One of the leading causes of hemlock death and decline in eastern North America is hemlock woolly adelgid (HWA), *Adelges tsugae*, an invasive insect. Economic impacts of HWA in the United States have been estimated to be over $250 million per year, primarily from decreased property values and the cost of treating and restoring infested hemlocks (Aukema et al., 2011). Hemlock forests require immediate attention to prevent the persistence of HWA.

Hemlock woolly adelgids feed on hemlock nutrients and can kill trees in as little as four years (Havill et al., 2014). The adelgids cover themselves with a white, 'woolly' wax while feeding on the hemlocks, and these white masses, also known as ovisacs, are the visible part of an infestation on a tree. HWA completes two asexual generations repeating annually in its invasive range (Havill and Foottit, 2007). Newly hatched adelgids are referred to as crawlers as they are the only mobile life stage and spread to find needles for feeding (Havill and Foottit, 2007). Birds, mammals, wind, and a variety of human activities (e.g., logging, planting nursery stock, and recreating) can all aid the dispersal of HWA (McClure, 1990). This pest has spread throughout much of the northeastern United States with expansion westward to Michigan, where it was found in 2015 (Michigan Department of Natural Resources, 2021).

Management efforts in Michigan are underway to control and stop the continued spread of HWA, and the main focus of management groups is on early detection with the goal of eradication. The primary method used in Michigan for detecting HWA is a visual assessment of

hemlock branches, typically those within reach from the ground, for the presence of ovisac material. This is a considerable task for land managers given the estimated 170 million hemlock trees in the state. Visual assessments alone may not allow for the earliest detection of this insect if initial HWA infestations begin in the top part of the canopy (Evans and Gregoire, 2007). These early infestations, as well as adelgid populations with low densities, may not be clearly visible on branches within reach of the ground and could give the false impression that HWA is not present in these areas (McClure, 1990; Evans and Gregoire, 2007). This could severely hinder early detection of this invasive insect, which is important for a rapid management response (Lodge et al., 2006).

Few scientific studies have assessed detection techniques for HWA. McClure (1990) and Fidgen et al. (2015, 2019) found sticky traps to be effective at catching individuals in the crawler stage but identifying HWA individuals in nymph life stages may require taxonomic expertise in areas where HWA is sympatric with other adelgids due to similar size and morphology (Limbu et al. 2018). Another method that may assist current monitoring efforts is the use of a combination of trap collection coupled with genetic analysis. Similar type work has been done through environmental DNA (eDNA) approaches, where DNA collected from the environment (i.e., soil, water, or air) is then genetically analyzed to determine if target species are present (Lodge et al., 2012). While sticky traps are effective at capturing adelgids, these traps are not easily compatible with genetic analysis to further confirm the species of adelgid present, due to difficulty in effectively removing material from the sticky glue (Fidgen et al., 2015, 2019). Several studies have successfully applied eDNA compatible traps in terrestrial settings to collect airborne samples to monitor species presence or absence for plants, fungi, and invertebrates (Folloni et al., 2012; Treguier et al., 2014; Johnson, 2017; Quesada et al., 2018; Valentin et al.,

2018; Thomsen and Sigsgaard, 2018). Given that wind can help facilitate the natural dispersion of HWA individuals and may also displace ovisac material within a forest canopy (McClure, 1990), the use of airborne traps for capturing individuals coupled with genetic analysis for species confirmation may be an effective method to monitor for the presence of HWA.

Our goal for this study was to identify an affordable, easy-to-use trap to capture airborne HWA material in a forest setting that would be compatible with further genetic testing. We conducted a preliminary study to assess three trap designs that potentially could be compatible with genetic analysis and evaluate their effectiveness in capturing HWA compared to sticky traps. We then conducted a secondary study in a low infested area to identify the minimum number of traps that would be needed within a given area to maintain a high potential of detecting an HWA infestation. We also evaluated how capture success was influenced by a trap's distance to an infested hemlock tree and landscape features including elevation, slope, and aspect. Implementing this technology could help maintain effective management of HWA without land managers having to perform time- and labor-intensive surveys to visually identify every infestation.

METHODOLOGY

1. Trap Design Testing

Trap Designs

The traps used in this study were: (1) motorized trap (Fig. 1A), (2) passive trap (Fig. 1B), (3) 8-funnel Lindgren funnel trap (Lindgren, 1983; Fig. 1C), and (4) standard sticky trap (Fidgen et al., 2019; Fig. 1D). (1) The motorized trap we used is a modification of a trap originally designed by Quesada et al. (2018) as a successful method for capturing airborne fungal spores in a forest setting. Our design included four petroleum jelly-coated (Vaseline) microscope slides affixed to the trap with two parallel (petroleum jelly facing upwards) and two perpendicular (petroleum jelly facing outward) to the ground to collect any airborne material. These slides were affixed to a battery-powered motor that rotated the slides in a clockwise direction at approximately 30 RPM (In the Breeze, Bend, OR). An aluminum pie pan and plastic bag covered the motor to protect it from the elements. (2) The passive traps were designed from a standing wind vane with all four petroleum-jelly-coated microscope slides affixed to the wind cups with jelly-coating facing upwards and slides parallel to the ground to capture airborne material; the slides rotated solely by the wind. Each microscope slide used in passive and motorized traps was 7.5 cm x 2.5 cm. (3) The 8-funnel Lindgren funnel traps consisted of eight 20 cm diameter openings of each funnel for material to fall into with a collection cup at the bottom. We kept 45 mL of propylene glycol in the attached cup of the funnel trap for preservation of material. (4) Due to not being able to affordably obtain the same materials used for Fidgen et al. (2019) sticky trap design, we slightly modified our sticky traps by assembling five sticky card insect traps on a 20 cm x 20 cm corrugated plastic board for each sticky trap.

Although sticky traps are useful in collecting adelgid count data, they are not compatible with genetic analysis due to difficulty in effectively removing material from the sticky glue (Fidgen et al., 2015, 2019). The traps using petroleum-jelly coated microscope slides (i.e., the motorized and passive traps) can be used for further genetic analysis, as Quesada et al. (2018) developed a method to successfully isolate captured airborne material from the petroleum jelly for DNA processing. Funnel traps are commonly used to capture insects (Lindgren, 1983; Klimaszewski et al., 2018) and have the potential to be compatible with DNA analysis. However, their use for specifically capturing HWA has not been evaluated previously.

Study Site

The trap design study took place at Pioneer Park, Muskegon, Michigan, USA (Fig. 2), a site with confirmed HWA infestation. Pioneer Park is 145 acres of county park and campground property along Lake Michigan. The public recreational areas are surrounded by eastern hemlock (*Tsuga canadensis*) dominated forests with some mixed hardwood and other conifers. All traps were deployed in areas with known infested hemlock trees to test our trap designs.

Trap Deployment

All four trap designs (motorized, passive, funnel, and sticky traps) were deployed for four weeks in the month of July 2020, which coincided with the second peak HWA crawler stage of the year. We organized our experiment in a randomized block design with five blocks (Fig. 3). Each block comprised 36 cells for a total area of 625 m^2. One of each trap type was randomly assigned a location within every block using a random number generator. The number randomly selected for each trap represents a cell within the block. We placed each trap at the latitude and

longitude of the central point of their randomly chosen cell. All traps were attached to standing poles 1.5 m from the ground. Trap contents were collected on a weekly basis for a total of 8 collecting periods. Slides from the passive and motorized traps and the funnel trap contents were collected in sterile 50 mL vials and stored in a refrigerator (4°C). The sticky trap panels were collected in clear, plastic storage bags due to their large size and stored in a freezer (-20°C).

Adelgid Capture Assessment for Each Trap

Motorized and Passive Traps

To assess the number of adelgids captured, we examined the petroleum jelly-coated microscope slides from the motorized and passive traps under a Nikon SMZ645 dissecting microscope and counted the total number of HWA crawlers from the four slides of each trap. Trap contents were then stored in a freezer (-20°C).

Funnel Traps

To assess adelgid capture success for the funnel traps, we counted crawlers in each funnel trap by placing each trap's contents into an individual petri dish and examining the contents underneath a dissecting microscope. The contents were placed back into their respective 50 mL vials when the counts were completed and stored in a freezer (-20°C).

Sticky Traps

To obtain adelgid counts for the sticky traps, we counted adelgids on each sticky trap using methods previously described by Dreistadt et al. (1998). Adelgids were counted on a one-inch-wide vertical column down the center of each sticky insect card using a dissecting microscope. We used this technique on each of the five cards that made up every sticky trap. When counting was completed, these sticky traps were stored in a freezer (-20°C).

HWA Estimates Within Each Block

To determine if variation in HWA prevalence across our sampling site might impact our capture results, we evaluated HWA presence within each designated block at Pioneer Park (See Figure 3) by counting the number of ovisacs on hemlock branches using a method from the Pennsylvania Department of Conservation and Natural Resources (Johnson, 2020). This was quantified at the block level since differing amounts of HWA between blocks could impact trap success in catching HWA. We randomly selected 10 trees within every block and numbered the lower crown branches within 7.5 m of the ground starting on the north side and moving clockwise around the tree. We used a random number generator to select five branches around each tree and counted the number of ovisacs within a 25 cm length of the distal part of each branch.

Statistical Analysis

All analyses were conducted using the program R v 4.0.3 (R Core Team, 2020). HWA estimates within each block and adelgid capture assessment data were non-normal despite transformations, thus we chose non-parametric analyses. To determine whether there were significant differences in HWA prevalence between blocks, we assessed differences between the average number of ovisacs counted from each block with a Kruskal-Wallis test using the package stats v 3.6.2. We evaluated HWA capture successes between non-sticky traps and sticky traps by estimating the probability that a non-sticky trap would capture HWA when a corresponding sticky trap (same block and same collection date) also captures HWA with a Wilson score interval (Wilson, 1927) using the package binom v 1.1-1. All statistical analyses used an alpha value of 0.05 to determine statistical differences.

2. Trap Efficiency Assessment

Once we determined the trap design that would be best suited for long-term monitoring (in this case, the motorized trap), we conducted further analysis to evaluate the number of traps that should be deployed in a given area to achieve a high potential of HWA detection. We also examined whether we could detect a relationship between the number of adelgids collected on a trap and the distance to an HWA-infested hemlock tree and general landscape features such as elevation, slope, and aspect.

Study Site

The second part of our study took place at North Ottawa Dunes (Fig. 2), a 593-acre Ottawa County Parks property of wooded sand dunes bordering Lake Michigan. The site consists of northern hardwood forest including many eastern hemlock trees and other conifers. This is a site with a known HWA infestation, and we designated the infestation level as low (see Chapter III). We obtained Ottawa County Parks survey data (January – October 2020) with GPS locations of all hemlock trees within the park, as well as the locations of hemlock trees where visual surveys previously detected the presence of HWA ovisacs. We conducted our study in the southern part of the park where the largest clusters of HWA-infested hemlocks were located, and our entire survey range included areas both with and without hemlock trees.

Trap Deployment

For the trap efficiency assessment, we deployed a modified version of the previous motorized trap (Fig. 4A) and sticky traps (Fig. 4B). While the motorized trap from the trap design study resulted in the lowest capture rate (see results), we made significant modifications

to this design that we felt corrected the flaws limiting its capture success. This included modifying the aluminum pan size to prevent the slides from being covered and arranging all petroleum jelly-coated slides so that they were parallel to the ground (i.e., facing upwards). The base of the trap was changed by putting a circle (cut from corrugated plastic board) over the top of the perpendicular metal piece the slides were previously attached to. We then clipped the slides directly to the plastic circle, which gave each glass slide a more secure and even surface to lay flat when attached to the base. This helped prevent slide breakage, and it made collection and redeployment easier and faster for the user. We also slightly extended the distance that the slides hung from the motor to better prevent petroleum jelly from being wiped away when the wind blew the slides upward and they contacted the motor. The same 20 cm x 20 cm sticky trap design applied in our previous study was used in this experiment.

Within North Ottawa Dunes, we established a 90-acre circle over our study area and sectioned it into 30 equal parts (Fig. 5). The 30 equal sections (3 acres each) were divided into five replicate groups (A-E), with six sections per group. Each of these six sections hosted a different number of paired motorized and sticky traps. Section one contained one pair of motorized and sticky traps, section two contained two pairs of traps, so on and so forth up to the sixth section containing six trap pairs. This resulted in a total of 105 motorized and 105 sticky traps for the entire 90-acre area, and the density of the traps within each section ranged from 1 trap per 0.5 acres to 1 trap per 3 acres. In every replicate group, the number of trap pairs and trap placement within each section was randomly assigned. Traps were attached to a 1.5 m pole, and the motorized and sticky traps were placed 2 m apart at each trap location. Traps were deployed for 16 weeks from April through July 2021 during both annual HWA egg hatching events. Petroleum jelly-coated slides from the motorized traps were collected biweekly and placed in 50

mL vials and sticky traps were collected in clear, plastic storage bags. Trap samples were stored at room temperature until processing.

Adelgid Capture Assessment

After each biweekly collection, we counted the number of adelgids observed on each trap. For the motorized traps, the number adelgids present on the four petroleum-jelly coated slides were observed using a Nikon SMZ645 dissecting microscope, counted, and recorded. We assessed the number of adelgids collected on each sticky trap using the same method previously described for our trap design assessment (Dreistadt et al. 1998). For both the motorized and sticky traps, 20% of traps per collection period were recounted for quality assurance ($R^2 = 0.99$). When counting was completed for the motorized trap samples, we used dish soap to clean all microscope slides and 50 mL vials used for sample collection. These slides and vials were reused for other trap deployment and sample collection events throughout the trap assessment study. Sticky traps were stored at either room temperature or in a freezer (-20°C) until the study was completed.

Inverse Distance Weighted Spatial Interpolation Mapping

We created maps predicting distribution of HWA with the count data for each motorized trap by means of the inverse distance weighted (IDW) spatial interpolation method using ArcMap v 10.4.1 (ESRI, 2016) to visualize how adelgid counts varied in our study area throughout the summer. The IDW method predicts likely HWA numbers based on a linear-weighted combination of count data for sample locations. This method is appropriate for clustered data. IDW predicts values for unsampled locations by assuming those values are related

more to closer data points than to those that are farther away. We used a power of 2 and a nearest neighborhood search of 8 points in the analysis, so more localized trap counts influenced predictions of the nearby unsampled locations and to account for all cardinal directions surrounding a location.

Statistical Analysis

Statistical analyses were conducted using the program R v 4.0.3 (R Core Team, 2020). To determine if the number of traps deployed within a 3-acre area significantly impacted whether an adelgid was captured within a section, we assessed adelgid capture success and failure throughout the 16-week study when one trap was used per section compared to when more than one trap was used per section with a Barnard's unconditional test (Barnard, 1945) using the package Barnard v 1.6. We did this to compare the following groups of trap numbers: one and two, one and three, one and four, one and five, and one and six traps. We again estimated the probability that a motorized trap would detect HWA when the corresponding sticky trap detected HWA for the entire 16-week study period with a Wilson score interval (Wilson, 1927) using the package binom v 1.1-1 to evaluate how our modifications to the motorized trap improved capture success compared to our initial trap design. We also assessed if trap elevation, slope, aspect, and Euclidean distance to the nearest HWA-infested hemlock impacted the number of adelgids caught in a motorized trap. The adelgid count data were non-normal, and they were heavily over-dispersed. Because of this, we used a GLM with a negative binomial distribution using the package MASS v 7.3-53.1. The full model consisted of adelgid counts as the dependent variable and Euclidean distance, elevation, slope, and aspect as the independent variables. A reduced GLM model was also run after removing the non-significant terms, and the optimal model was

selected using the lowest Akaike's Information Criterion (AIC). Analyses used an alpha value of 0.05 to determine statistical differences.

RESULTS

1. Trap Design Testing

For HWA estimates within each block, we accepted the null hypothesis that median values in ovisac counts were similar between blocks (Kruskal-Wallis test = 1.625, df = 4, P = 0.804). With the Wilson score interval, if a non-sticky trap detected HWA every time a corresponding sticky trap did, then the estimated success probability would be 1. The passive trap had the most success with its estimated success probability averaging around 0.87 (95% CI = 0.62, 0.96). The funnel trap had an average success probability of 0.8 (95% CI = 0.55, 0.93), and the motorized trap averaged a 0.4 success probability (95% CI = 0.2, 0.64).

2. Trap Efficiency Assessment

Out of the 105 traps placed in the 90-acre area, 100 traps captured at least one adelgid over the course of the 16-week study period. The Barnard's unconditional tests showed that number of motorized traps used did not have an effect on adelgid capture success (1 vs 2: P = 0.44; 1 vs 3: P = 0.58; 1 vs 4: P = 0.26; 1 vs 5: P = 0.97; and 1 vs 6: P = 0.92) when analyzed over the 16 weeks. For the Wilson score interval, the motorized traps had an estimated success probability of 0.67 (95% CI = 0.62, 0.71) for capturing adelgids when corresponding sticky traps also caught adelgids throughout the study.

To visualize and evaluate how adelgid capture success changed throughout the HWA crawler period, when HWA is most mobile, we created spatial interpolation maps. Figure 6 (A-G) shows the number of adelgids captured for each trap placed within the total 90-acre area, as well as the interpolated values. These interpolated values predict the potential number of adelgids captured if traps were placed in areas between our trap locations. We found that as the

HWA progredientes crawler stage progressed, we captured an increasing number of crawlers, and these numbers peaked on June 2nd. The number captured began to decrease on June 16, and only a few traps captured crawlers through the sistentes stage by the end of the study period on July 28th. At the peak HWA crawler stage, the interpolated values show that traps could be placed anywhere in the study area and have the potential to capture 1-5 adelgids. When crawler numbers were low (Figure 6A, F, G), the geographical area that is likely to not catch crawlers (interpolated values = 0) increased, but the majority of the study area still had interpolated values of 1-5 or higher. These maps also showed a close association between the number of adelgids captured and where hemlocks previously identified as containing ovisac material (red stars) were clustered. Traps near clusters of infested hemlock trees tended to have higher adelgid numbers and this pattern was most obvious during the May 19th – June 16th sampling period (Figure 6C, D).

We also assessed if trap elevation, slope, aspect, and Euclidean distance (EucDist) to the nearest HWA-infested hemlocks impacted adelgid capture for the motorized traps using a GLM with a negative binomial distribution. We first ran a full model with all explanatory variables, but slope and aspect were not significant. We then ran a reduced model with slope and aspect removed to see if this would improve the model. The reduced model with only elevation and EucDist was slightly better than the full model based on the AIC value (Table 1), thus we designated the reduced model as the optimal model. While the reduced model was slightly improved based on AIC values compared to the full model, it was not significantly better (ANOVA, $P = 0.08$).

DISCUSSION

Our first goal of this study was to identify an affordable, easy-to-use trap that could effectively capture airborne HWA material, be compatible with DNA analysis, and persist in a natural forest setting during use. Our results from the initial trap design testing show overall we accomplished this. The passive trap design was most similar to the sticky trap in catch rates (0.87 success probability), followed by the funnel trap (0.8 success probability), and then the motorized traps (0.4 success probability). We considered all the tested trap designs easy to use as they required about the same amount of time to deploy and collect from; each took 5-10 minutes to set up once in the field. Counting crawlers from the funnel and sticky traps took longer since these had much larger surface areas to inspect compared to the passive and motorized traps and much more bycatch of non-target species (i.e., $\geq$ 30 minutes compared to 5-10 minutes, respectively). These results show an estimated probability of HWA capture success for our tested non-sticky traps compared to sticky traps; however, these numbers alone do not paint the entire picture of how well each design worked through this experiment. Each trap had their pros and cons in use for our purpose (Table 2).

The passive and funnel traps, while most successful on average, did not meet all other criteria we were looking for in a trap that could be helpful to land managers. The passive traps were the cheapest and most successful design, but they were the least sturdy trap of this study with broken traps at every collection in each block. Continual replacement of these traps would lead to increased time and effort by management teams if they were to be used for long-term monitoring. We knew the sticky traps were not compatible with DNA analysis due to not being able to effectively extract material from the sticky glue (Fidgen et al., 2015, 2019), but we also found that the funnel trap contents may not be effective for DNA analysis, specifically when

testing for HWA. The funnel traps caught a large amount of non-target bycatch, and we believe this amount of bycatch would likely limit rapid DNA extraction and processing for HWA. The Lindgren funnel traps were also the most expensive traps in our study (See Table 2), and a lot of their success can likely be attributed to the eight 20 cm diameter funnel openings for material to fall in or be blown into. We suggest the motorized traps have the potential to be most helpful for land managers in long-term use to detect HWA because they met more of the criteria that we were looking for both long-term monitoring and potential for rapid DNA assessment, and we could easily make significant modifications to increase their capture efficiency.

While the motorized traps did not have the most success capturing HWA at first, we were able to modify the trap to be more similar to the more successful passive trap, which brought the motorized trap's estimated success probability up to 0.67 when compared to sticky traps. In our first experiment, our motorized trap had a 20 cm diameter aluminum pan covering the top of the trap to help protect the motor from the elements, and this allowed the pan to cover the width of the microscope slides hanging below the motor. We also initially had two slides facing up (parallel to the ground) and two slides on their side (perpendicular to the ground). We thought having two slides perpendicular with the petroleum-jelly coated side facing the direction the slide rotated in would help increase the chance of collecting airborne material with a motorized trap. However, our results showed this might not be the case for our target species since the parallel slides often had more crawlers on them compared to the perpendicular slides. In our second trap efficiency experiment with the motorized trap, we put all four slides parallel to the ground and face-up. We believe these modifications attributed the most to the motorized trap's much higher success in 2021. We were also able to collect data with a larger number of traps and for a much longer time period in 2021. Overall, the motorized trap is very cost effective, easy to

use, and extremely sturdy for long-term use in a forest setting while also being compatible with DNA analysis.

Our assessment of the number of motorized traps that are needed within a set area to have a high potential of detecting adelgids over the 16-week collecting period indicated that placing 2-6 traps within a 3-acre section did not have a significantly greater success than just using one trap within the same size section. We think this lack of difference could be because we analyzed capture success for the entire 16-week period. If one trap is placed in a hemlock stand for 16 weeks, then it has a lot of opportunity to capture at least one adelgid and adding more traps to that same area would not necessarily be more beneficial. However, if managers would like to shorten the collection period, it should be noted that using only one trap may not result in the same capture efficiency. In this case, having more than one trap could be more useful in providing additional opportunities to detect at least one adelgid. We do plan on re-analyzing this part of our data to look at adelgid capture success and failure in 4-week intervals of the study period to see if a higher number of traps is significantly different from when one trap is used.

In our assessment of how trap elevation, slope, aspect, and Euclidean distance to the nearest HWA-infested hemlocks impact adelgids captured for the motorized traps, we found that trap elevation and distance to infested hemlocks had more of an effect on the number of adelgids captured than slope or aspect. This makes sense as the data generally showed that traps closest to infested hemlocks caught the most throughout the study (See Figure 6), and traps at lower elevations typically caught more than those at the top of a dune. It is important to state that there could also be other variables outside of what our study evaluated that could explain variation in the number adelgid captures across our study site. Figure 6C and D show a cluster of traps in the northeast part of our survey area that captured many adelgids but are not as close to infested

hemlocks as most of the other highly successful traps. This could be due to wind pushing adelgids to those traps, as a lot of northeasterly winds prevail from Lake Michigan in this area. Those northeast traps are also downhill from the nearest infested trees, so this could help facilitate adelgid movement to them. There could even be a closer infested hemlock tree that we could not consider since Ottawa County Park's HWA survey data for this park ended October 2020, and our study took place summer 2021. Also, infestation level of each individual hemlock tree could play a role as a really infested tree would produce more adelgids than a tree with just a few individuals.

Among the few studies to assess the use of traps in detecting HWA is McClure (1990) and Fidgen et al. (2015, 2019), both of which used sticky traps to catch adelgids in the mobile crawler stage. Like McClure (1990), our study suggests that trap distance to HWA-infested hemlock trees does impact trap capture. Unlike Fidgen et al. (2019), we did not find the number of traps to significantly increase capture success (when assessed over a 16-week period). Many states, such as Michigan, primarily use visual assessments to find new HWA infestations, but these on-the-ground surveys can miss early invasions that may only be present in the top part of the trees' canopy (Evans and Gregoire, 2007). Our results showed the motorized trap can catch adelgids almost as often as sticky traps, even in a low-infestation area, and this makes them another viable option for detecting HWA. Use of sticky traps or any of our tested trap designs alone to detect HWA can require taxonomic expertise to confidently identify HWA in areas where other adelgids may be present but using a DNA-compatible trap like our motorized trap in tandem with DNA analysis of trap samples could alleviate this issue and allow for easier and more rapid confirmation of HWA presence in trap contents. This monitoring tool can be used to inspect larger geographic areas more easily and possibly detect early infestations of HWA before

visual assessments. These traps can also be paired with visual surveys, e.g., a trap's detection can initiate a more thorough site assessment.

This study opens the door for future research to continue assessing the use of eDNA compatible traps in HWA detection. We conducted research further testing our motorized trap's long-term performance in successfully capturing HWA after design modifications similar to those described in this study (Chapter III) where we evaluated how infestation level, HWA life stage, trap height, and trap distance to an infested hemlock stand impact our motorized trap's success in HWA detection. Dr. Mark Whitmore's lab at Cornell University also developed a rapid molecular technique (Kirtane, 2021) that we adapted to detect the presence of HWA from the DNA caught by our traps. We tested this molecular method in conjunction with our additional trap testing (Chapter III) to assess how well the molecular assay detects HWA from environmental samples caught with our motorized traps. Certainly, there is potential for future research to design traps outside of what we have tested or improve upon any of these designs. It could also be beneficial for further study of other environmental variables that may affect the success of a trap capturing HWA, such as wind direction and hemlock density.

Hemlock forests in the eastern United States continue to be threatened by HWA, and this pest is spreading in Michigan with the known leading-edge creeping further north each year. eDNA compatible trap use could be an efficient method for land managers to detect early infestations as well as low density HWA populations that can be difficult to identify visually. The sooner management efforts can be implemented, the better chance there is at eradicating this invasive species (Lodge et al., 2006). This technology can help protect our hemlocks by preserving valuable personnel and funds for HWA eradication efforts in Michigan.

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TABLES

Table 1. Results of the full and reduced generalized linear models. The full model used trap elevation, slope, aspect, and Euclidean distance (EucDist) to the nearest HWA-infested hemlock tree as explanatory variables for adelgid number caught by a motorized trap. The reduced model used only trap elevation and EucDist as explanatory variables for the number of adelgids caught.

Full Model				
Variables	Estimate	Standard Error	z-value	P
(Intercept)	18.284	5.489	3.330	8.67e-4
Aspect2	1.152	0.514	2.24	0.025
Aspect3	-0.66	0.565	-1.169	0.242
Aspect4	0.395	0.538	0.735	0.462
Aspect5	0.045	0.584	0.077	0.938
Aspect6	0.597	0.492	1.213	0.225
Aspect7	0.169	0.478	0.354	0.723
Aspect8	0.672	0.466	1.442	0.149
Slope	0.006	0.046	0.134	0.893
Elevation	-0.077	0.029	-2.597	0.009
EucDist	-0.004	8.21e-4	-5.263	1.42e-7
(AIC = 832.4)				
Reduced Model				
Variables	Estimate	Standard Error	z-value	P
(Intercept)	17.295	4.974	3.477	5.07e-4

Elevation	-0.069	0.027	-5.139	2.76e-7
EucDist	-0.004	8.04e-4	-2.624	0.009

(AIC = 830.31)

Table 2. Comparison of the different categories we assessed for each trap type (i.e., lab processing time, cost, sturdiness, DNA-analysis compatibility) in addition to HWA capture success for the initial trap design testing. Redeployment costs were averaged for a single trap from the total cost of redeployment over the four weeks of the study.

Trap Type	Lab Processing Time	Trap Cost	Redeployment Cost per Week	Sturdy	Compatible with DNA
Motorized	5-10 min	$30	$3.50	Yes	Yes
Passive	5-10 min	$20	$3	No	Yes
Funnel	≥ 30 min	$100	$0.60	Yes	Maybe
Sticky	≥ 30 min	$10	$7	Yes	No

FIGURE LEGENDS

Figure 1. Photos of each trap design used in this study: (A) motorized trap, (B) passive trap, (C) funnel trap, and (D) sticky trap.

Figure 2. Map of study sites: Pioneer Park (PIPK), Muskegon, Michigan, USA, and North Ottawa Dunes (NODU), Spring Lake, Michigan, USA, each denoted with a black star.

Figure 3. Map of Pioneer Park, Muskegon, MI, USA, showing our randomized block design with numbered cells. A dot indicates a trap location within the block.

Figure 4. Photos of the traps used in this study: (A) motorized trap and (B) sticky trap.

Figure 5. Map showing our trap efficiency assessment experimental design with 30 equal sections of a 90-acre survey area divided into five replicate groups (A-E) with six sections per group numbered to represent the amount of paired motorized and sticky traps in each section (e.g., section A1 contained one pair of motorized and sticky traps and A6 contained six trap pairs).

Figure 6. Inverse distance weighted spatial interpolation maps created for every 2021 collection period with the count data of each motorized trap. Collection dates: (A) May 5, (B) May 19, (C) June 2, (D) June 16, (E) June 30, (F) July 14, and (G) July 28.

FIGURES

Figure 1.

Figure 2.

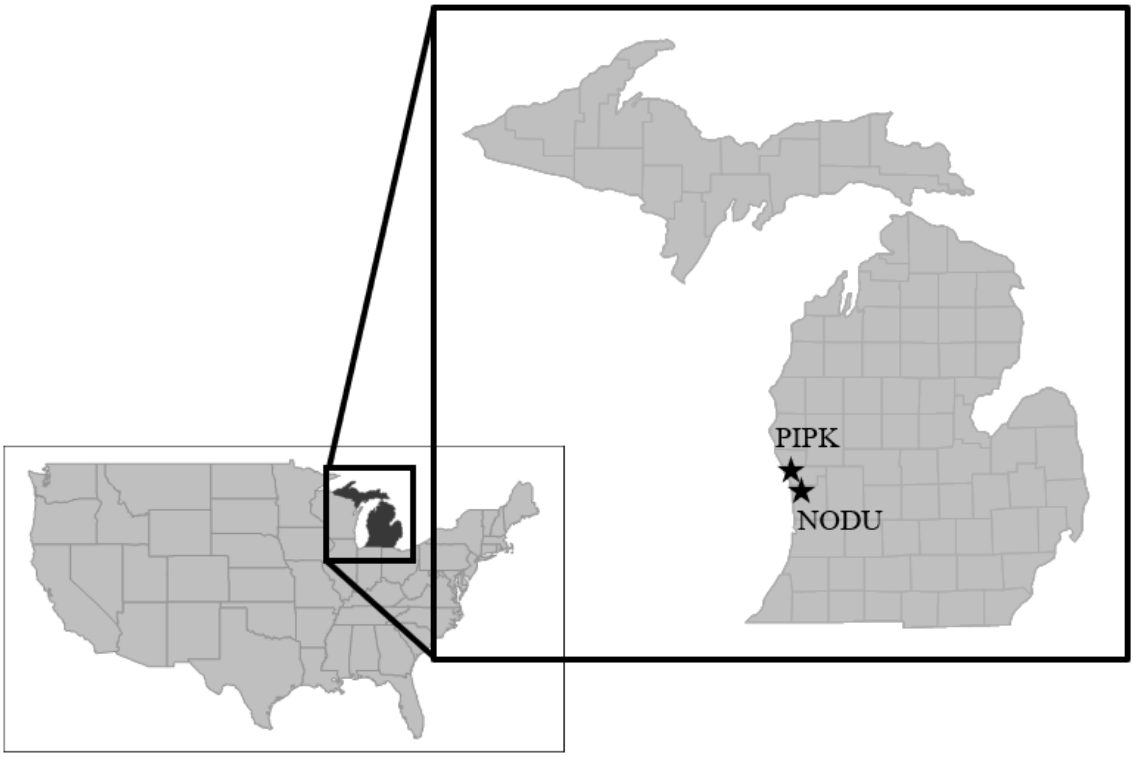


Figure 3.

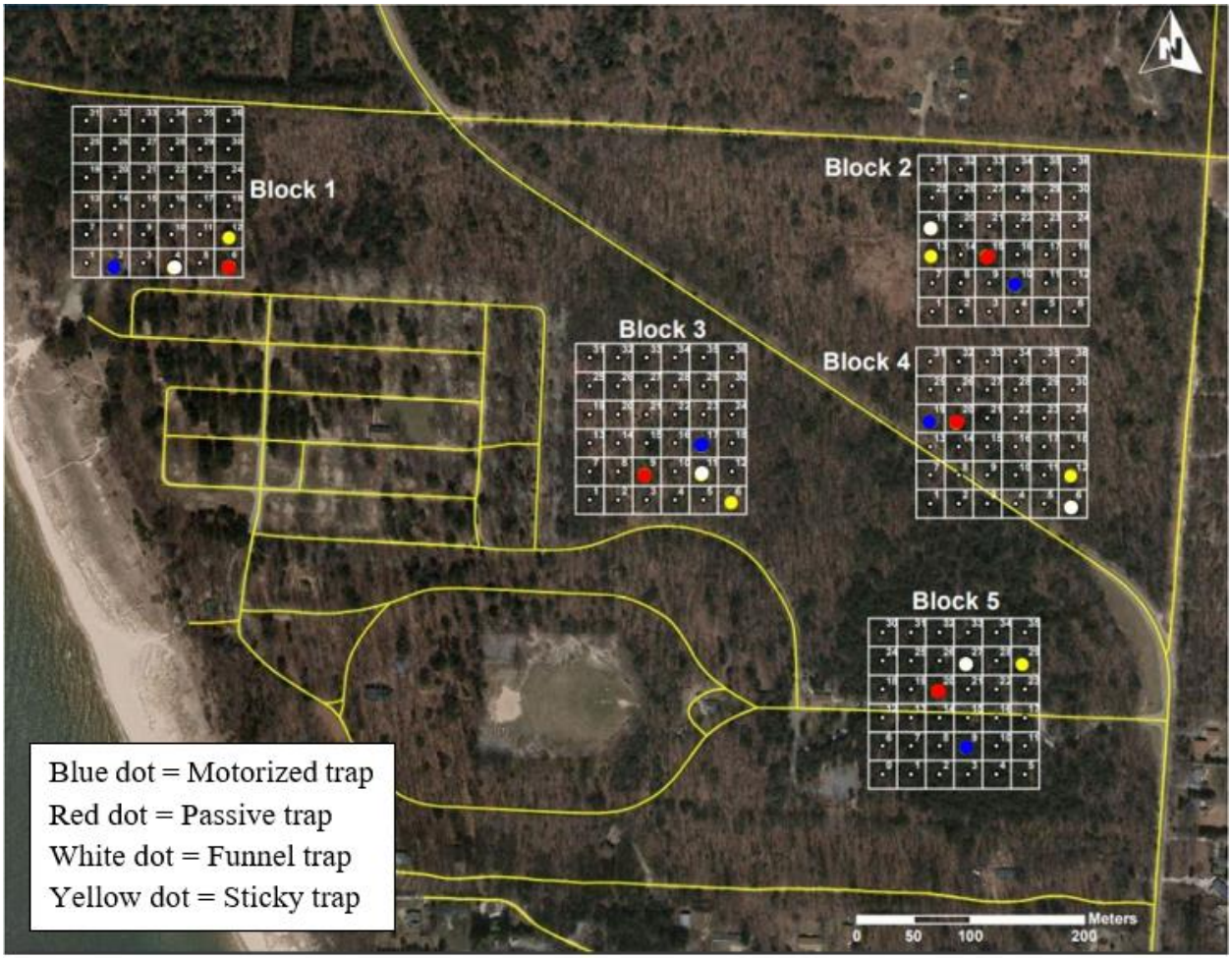

Block 1
Block 2
Block 3
Block 4
Block 5
Blue dot = Motorized trap
Red dot = Passive trap
White dot = Funnel trap
Yellow dot = Sticky trap
Meters
0
50
100
200

Figure 4.

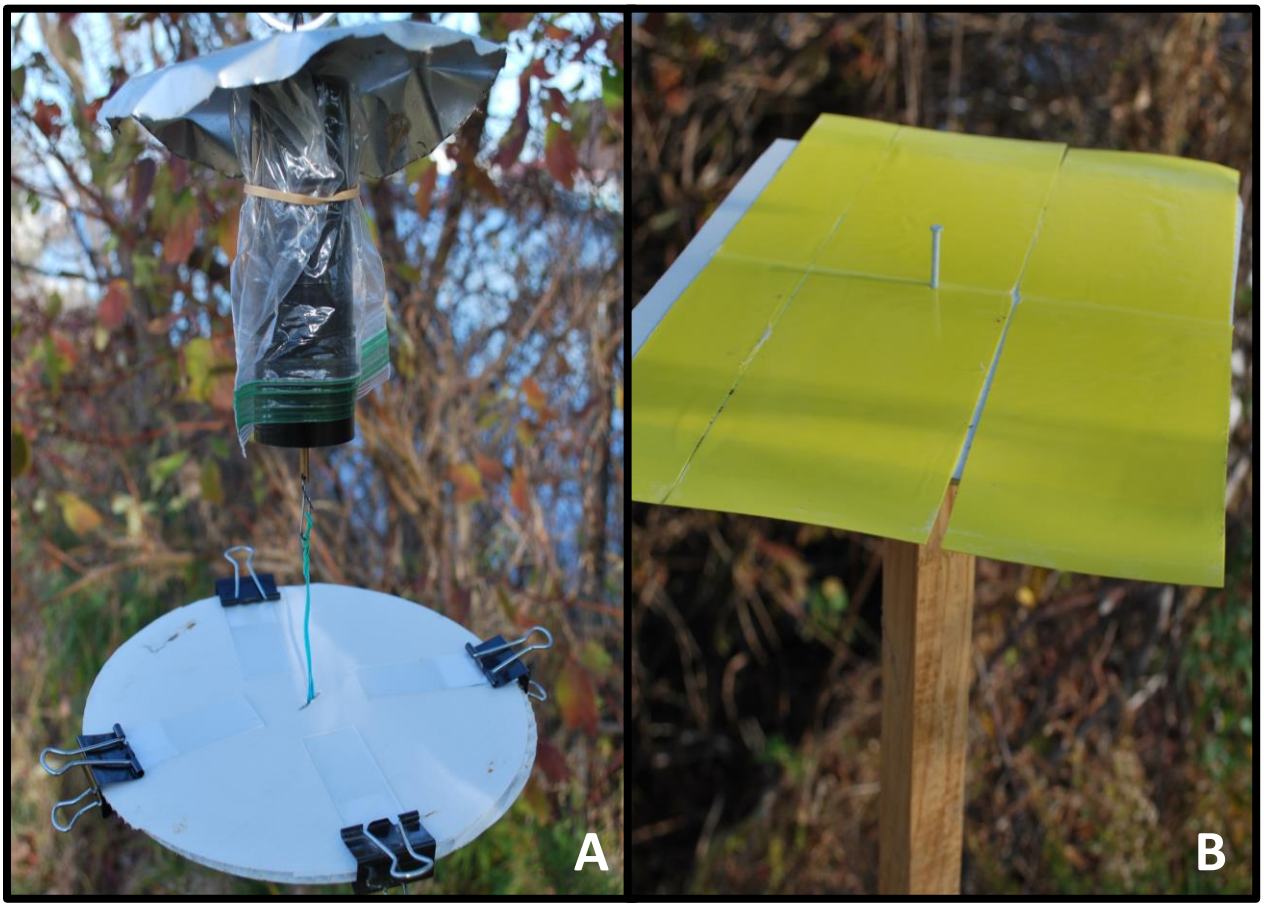


Figure 5.

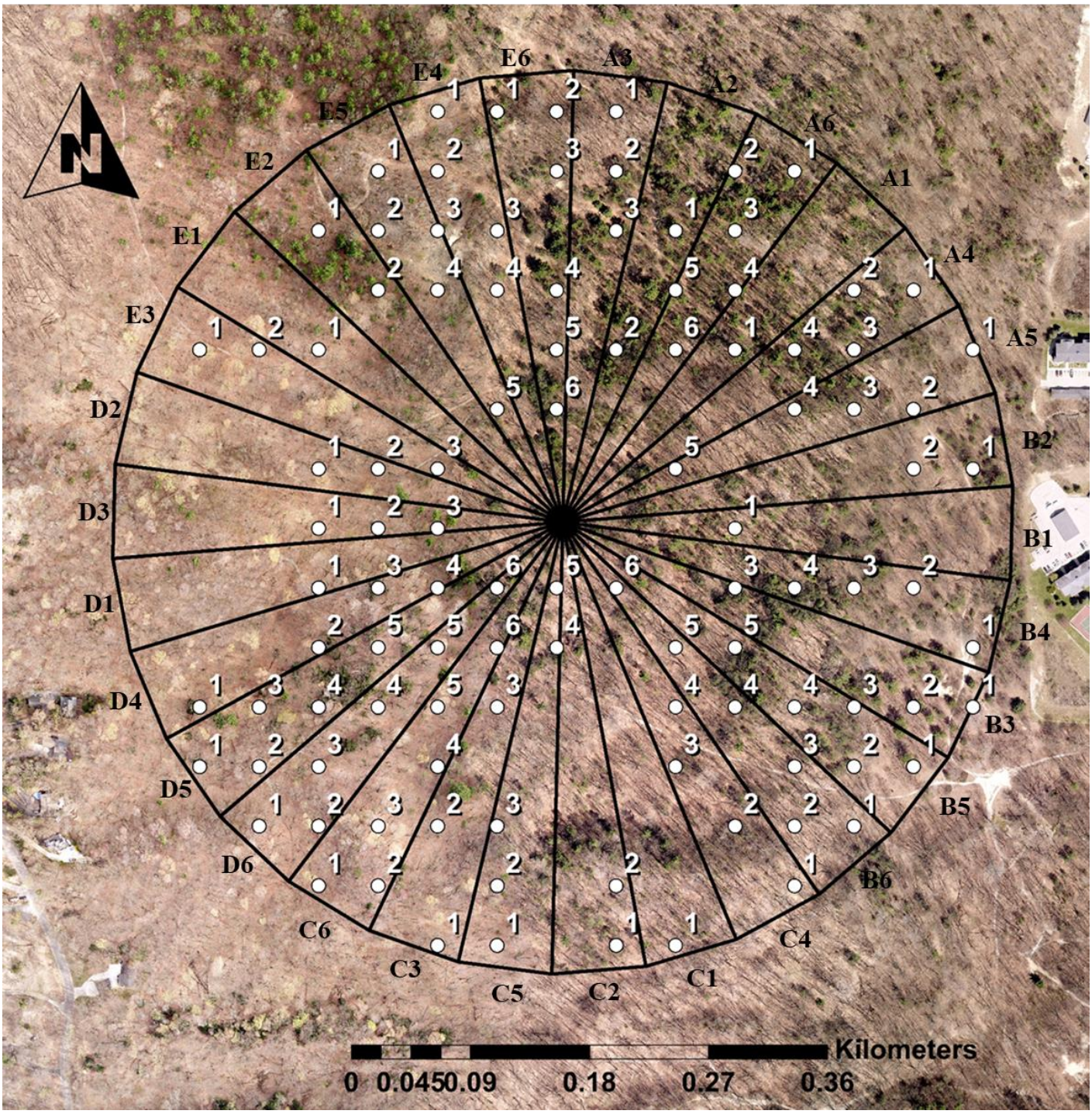
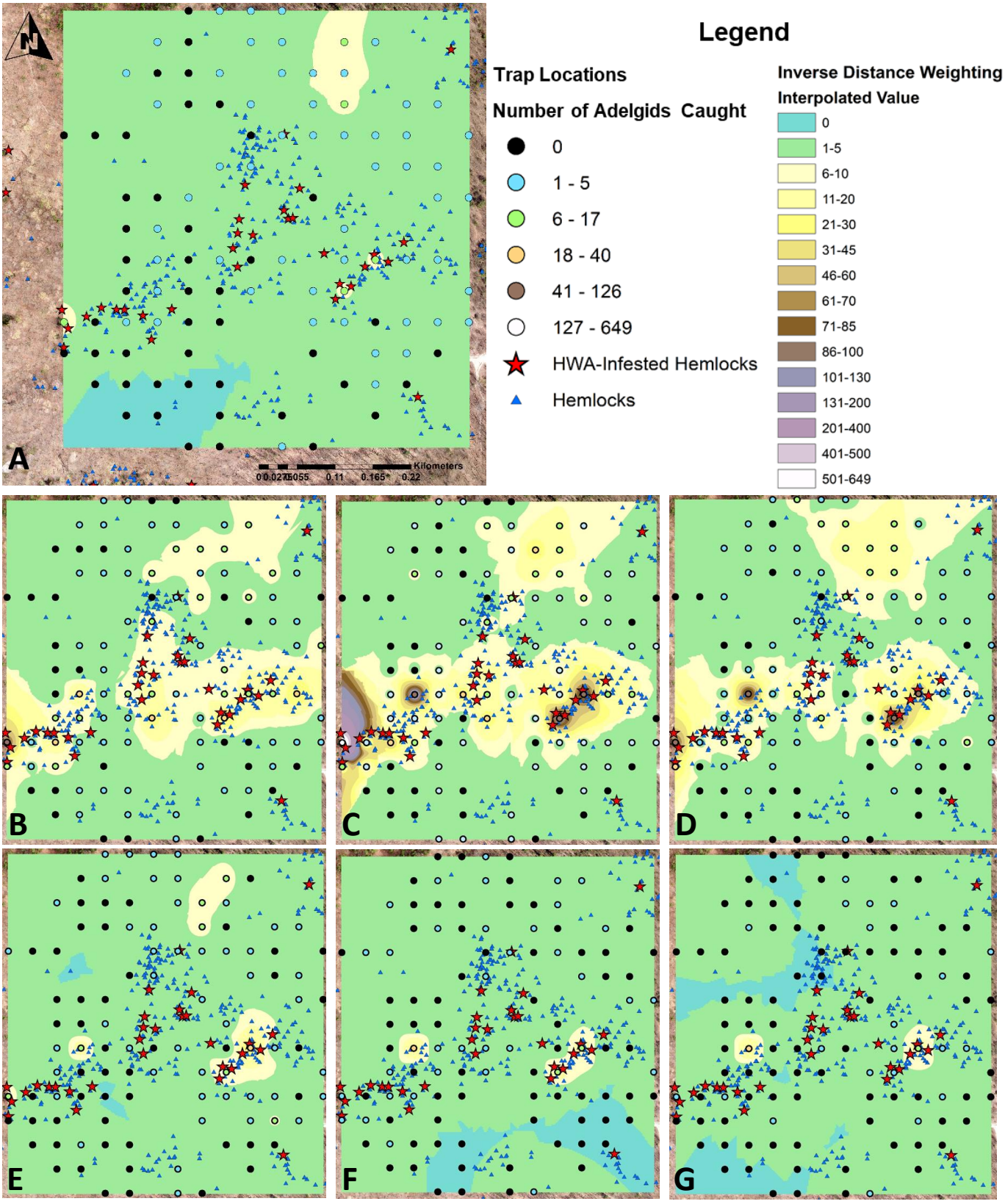

E6
A3
E4
A2
E5
A6
E2
A1
E1
A4
E3
A5
D2
B2
D3
B1
D1
B4
D4
B3
D5
B5
D6
B6
C6
C4
C3
C1
C5
C2
Kilometers
0 0.045 0.09 0.18 0.27 0.36

Figure 6.


Legend
Trap Locations
Number of Adelgids Caught
0
1 - 5
6 - 17
18 - 40
41 - 126
127 - 649
HWA-Infested Hemlocks
Hemlocks
Inverse Distance Weighting
Interpolated Value
0
1-5
6-10
11-20
21-30
31-45
46-60
61-70
71-85
86-100
101-130
131-200
201-400
401-500
501-649
Kilometers
0 0.0275 0.055 0.11 0.165 0.22
A
B
C
D
E
F
G

CHAPTER III

Developing airborne eDNA techniques for the early detection of hemlock woolly adelgid (*Adelges tsugae*)

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ABSTRACT

Hemlock woolly adelgid (HWA), *Adelges tsugae*, threatens hemlock forests throughout eastern North America with management efforts underway to control and stop the spread of this invasive pest. Natural resource managers' primary focus is on early detection, and we believe incorporating DNA-detection techniques can be a helpful tool for land managers. The goal of this study was to further assess our previously designed motorized trap in capturing HWA in a natural forest setting and determine how well a rapid, HWA-specific molecular assay could identify HWA presence in our traps' samples. We found that trap distance to infested hemlocks, infestation level, and timing of trap deployment can all impact HWA detection. The molecular assay proved to reach 90% detection effectiveness when approximately 14 adelgids are present in a trap sample. This technology could help preserve valuable resources for HWA control and eradication efforts along the leading edge of the invasion in the United States and Canada.

INTRODUCTION

Hemlock trees provide vital services and control ecosystem dynamics within both terrestrial and aquatic ecosystems (Yamasaki et al., 2000; Snyder et al., 2002; Ford and Vose, 2007; Toenies et al., 2018). Hemlock loss can lead to many negative impacts to their ecosystem structure and function (Orwig and Foster 1998; Ellison et al. 2005; Ellison et al. 2018). Hemlock mortality in eastern North America is largely due to the invasive insect, hemlock woolly adelgid (HWA), *Adelges tsugae*. The presence of this pest has significant economic impacts, primarily from decreased property values and the cost of treatment and restoration efforts (Aukema et al. 2011).

In its invasive range, HWA cycles through two asexual generations annually on hemlock trees (Havill et al. 2014). The first generation is comprised of progredientes and the second generation consists of sistentes. Each group goes through four instar nymph stages before they become adults. The first-instar nymphs, referred to as crawlers, are the only mobile life stage that spread to find old or new growth needles (this depends on the generation) for feeding (Havill and Foottit, 2007). Progredientes emerge in spring and early summer as the largest crawler hatch of the year, and they have a short lifespan ending later in the summer after they lay their eggs. Sistentes hatch in the late summer and feed for a short time before entering a period of dormancy known as aestivation. In the late fall, sistentes come out of dormancy to feed and develop through the winter months until laying their eggs in the spring to continue the cycle. Humans, wind, birds, and mammals can all disperse HWA to new areas (McClure 1990).

Along the leading edge of the HWA invasion in the United States, management efforts have been implemented to control and stop the spread of this invasive pest with a primary focus on early detection. A commonly used method for detecting new HWA infestations is a survey of

hemlock branches for the presence of ovisacs, a white woolly wax that individuals secrete to protect themselves. This is challenging for land managers given the expanse of hemlocks in eastern North America and the fact that ovisac material may be hard to visually identify if infestation density is low or infestations begin at the upper portions of a hemlock tree (McClure 1990; Evans and Gregoire 2007). Lack of detection during early infestation could impact rapid response of management to treat infested trees and limit the spread to new areas (Lodge et al. 2006).

The use of environmental DNA as an HWA monitoring tool could be another approach in in the effort for HWA early detection. Environmental DNA (eDNA) refers to genetic material collected from the environment such as soil, water, or air (Lodge et al. 2012). Organisms shed tissues and whole cells that eventually break down and release DNA into their environment, which can then be captured and used to monitor the presence or absence of a specific species (Barnes and Turner 2016). While eDNA detection techniques have often been implemented in aquatic systems (Goldberg et al. 2011; Turner et al. 2014; Barnes et al. 2014; Evans et al. 2016), many have successfully implemented these techniques in terrestrial settings with taxa such as plants, fungus, and invertebrates (Folloni et al. 2012; Treguier et al. 2014; Johnson 2017; Quesada et al. 2018; Valentin et al. 2018; Thomsen and Sigsgaard 2018). Airborne eDNA approaches have been used to detect fungal spores (Quesada et al. 2018) and pollen (Johnson 2017), and it can be applied to a wide range of species with wind dispersion. Airborne eDNA techniques may be an effective method to monitor for HWA presence because wind can assist HWA dispersion and may also move ovisac material within a forest canopy (McClure 1990).

This study aimed to further assess our previously designed motorized trap (Chapter II) in capturing airborne HWA particles in a forest setting and determine how well a rapid molecular

assay can identify HWA presence in the environmental trap samples. Our more specific trap assessment goals were to evaluate our motorized trap in detecting HWA across varied height from the ground and distance to infestation. This technology could be a helpful early monitoring tool for natural resource managers in their detection and eradication efforts of this invasion in eastern North America.

METHODOLOGY

Trap Assessment

Site Selection

We selected three sites of varying infestation level (no detection, one high, one low) to use in our study. Sleeping Bear Dunes in Leelanau County, Michigan, was our 'no detection' site. Pioneer Park in Muskegon, Michigan, and North Ottawa Dunes in Ottawa County, Michigan, were our two infested sites. In September 2020, prior to trap deployment, we assessed infestation levels at both Pioneer Park and North Ottawa Dunes using methods outlined in Evans and Gregoire (2007). In this approach, they conducted a full crown assessment of hemlock trees infested with HWA to determine adelgid density within the different crown levels of hemlock trees. They found that hemlock trees with lower-level branches (within a 7.5 m height from the ground) with more than 20 sistentes counted per 100 new growth needles had high HWA populations and those with less than 20 sistentes counted per 100 new growth needles in the lower crown branches had low HWA populations. We randomly selected 25 hemlock trees from each site and numbered the lower-level branches, always starting on the North side and moving clockwise around the tree. Nine branches from each tree were randomly chosen and assessed for number of sistentes per 100 new growth needles. The counts were averaged per tree to alleviate bias in HWA numbers on a single branch. We designated Pioneer Park as our high infestation site (averaged 24.2 sistentes) and North Ottawa Dunes as the low infestation (averaged 0.2 sistentes). Counting sistentes is an appropriate measure of HWA for a particular year since sistentes are the first generation to emerge after new growth looking to settle on the new hemlock needles (Evans and Gregoire 2007). Because of this, we were confident that our infestation level designations were consistent through the year of sampling.

Study Sites

Our study took place at three sites in western Michigan from October 2020 through October 2021: Crystal River Trailhead of Sleeping Bear Dunes National Lakeshore, Leelanau County, Michigan; Pioneer Park, Muskegon, Michigan; and North Ottawa Dunes, Ottawa County, Michigan (Fig. 1).

Sleeping Bear Dunes

The Crystal River Trailhead area of Sleeping Bear Dunes (SLBR) hosts an eastern hemlock (*Tsuga canadensis*) stand between the trailhead and the Crystal River itself. This site is in a county with no known HWA infestations, and HWA has also not been found at this site in any annual visual surveys performed by Sleeping Bear Dunes' staff. For these reasons, we chose to use this area as a 'no detection' control site in our study.

Pioneer Park

Pioneer Park (PIPK) is 145 acres of Muskegon County campground and park property along Lake Michigan. PIPK is a site with confirmed HWA infestation, which we designated as a high infestation level for this study. This park is dominated by eastern hemlock trees with some mixed hardwoods and other conifers. We used the eastern part of the park where the most hemlock trees were located.

North Ottawa Dunes

North Ottawa Dunes (NODU) is an Ottawa County Parks' 593-acre wooded sand dune property bordering Lake Michigan. The site is made up of northern hardwood forest, which includes many clusters and some scattered eastern hemlock trees as well as other conifers. We designated the infestation level as low for our study and worked in the southern part of the park where a larger cluster of HWA-infested hemlocks was located.

Trap Deployment and Sample Collection

We continued using our motorized trap in this study with a few modifications (Fig. 2A) from the design used in the initial trap design testing (Chapter II). We included four 7.5 cm x 2.5 cm petroleum jelly-coated microscope slides affixed to the trap with all four slides parallel to the ground and jelly-coated sides facing upwards. The traps had a battery-powered motor that rotated the slides in a clockwise direction at approximately 30 RPM (In the Breeze, Bend, OR) as well as an aluminum pan and plastic bag to protect the motor from the elements. The tall traps were affixed to a hollow PVC pole that slid over a thin metal pole secured in the ground, so it was easily removable for sample collection. Six traps were deployed at each site in October 2020 for 52 weeks. Within each site, two traps were centrally located in a hemlock stand (0 m), two traps were near the edge of the stand at 150 m from the central point, and two traps were 300 m from the central point continuing to move away from the hemlock stand. To assess how trap height could impact adelgid capture success, each set of two traps had one trap 1.5 m above the ground (Fig. 2A) and one trap 3 m above the ground (Fig. 2B), with the short and tall traps set 3 m apart from one another (Fig. 2C). Samples from each trap were collected in sterile 50 mL vials on a biweekly basis and stored in a freezer (-20°C) until further sample processing could occur. To employ contamination prevention techniques throughout sampling, we used latex gloves when collecting samples and sanitized them with 70% ethanol between touching samples and equipment; we used new latex gloves when going to a new site. To test for sample contamination during sample collection, we collected a field blank from each site for each collection week. The field blank was a petroleum-jelly coated microscope slide prepared in the lab at the same time as the microscope slides for each site. It was transported in its own 50 mL vial in the same container with the other microscope slides to be re-set at a site. At the time of sample collection

for each site, the field blank was temporarily removed from its 50 mL vial to be handled in the same manor we handled the environmental samples before being placed back into its container.

Vegetation Assessment

We assessed vegetation density between each set of traps at the 0 m, 150 m, and 300 m locations to determine if dense vegetation may impact how trap distance influenced capture success. At each site, we measured horizontal vegetation cover density using a 1 m x 1 m vertical profile cloth sheet with a 10 cm grid (Doggett and Locher, 2018). Measurements were taken in winter and summer at three locations per site: photos for the 0 m trap locations were taken 14 m away from the trap toward the direction of the 150 m traps; photos for the 150 m traps were taken 14 m away toward the direction of the 300 m traps, photos for the 300 m traps were taken 14 m away but back in the direction toward the 150 m traps. For all photos the top of the cloth was 1.5 meter above the ground to best capture the midstory vegetation that could possibly hinder HWA movement within the forest. We collected measurements in winter and summer to get a representation of leaf-off and leaf-on vegetation because our traps were deployed for a full year, and we chose 14 m as our distance since Turner et al. (2011) found 12-14 m as a mean dispersal distance in their simulation of HWA dispersal in forest understory. To calculate the vegetation percent cover, we recorded the percentage of cells in each grid that contained vegetation.

DNA extraction

We examined each petroleum-jelly coated slide under a Nikon SMZ645 dissecting scope to make note of any visible HWA material (e.g., ovisac, nymphs, adults, etc.) and hemlock

needles, and we counted the number of adelgids present. After slide inspection, each slide's petroleum jelly environmental sample was placed into individual sterile 1.5 mL microcentrifuge tubes. We then separated any environmental material from the petroleum jelly using methods from Quesada et al. (2018), which consisted of a series of heating and centrifugation steps. Once samples were separated from the petroleum jelly, they were preserved at -20°C until DNA extraction could occur. We extracted DNA using a Quick-DNA Tissue/Insect Microprep Kit (Zymo Research). To test for contamination during sample assessment, we prepared lab blanks for the petroleum-jelly separation and DNA extraction steps.

Presence/Absence Assessment for HWA

We assessed presence/absence of HWA in an environmental sample (e.g., trap sample representing a two-week period) using real-time polymerase-chain reaction (rtPCR) with primers and a probe specific to HWA previously designed and tested by Kirtane (2021) that amplify an approximately 250 base pair (bp) region of the cytochrome oxidase 1 (CO1) gene. We also tested the primers and probe for amplification with six other adelgid species commonly found in the northeastern U.S.: *Adelges abietis*, *A. piceae*, *A. cooleyi*, *A. laricis*, *Pineus pini*, and *P. strobi*. The primer and probe sequences are as follows: Forward – 5`-ACAGGATGAACAATTTACCCAC-3` and Reverse – 5`-AGCACCTGCTAGAACAGGTAAGG-3` and probe– 5`-CCA TTA TTC CCA TGA TCA ATT TTA ATT ACT GC-3`. The probe was fluorescently labeled with Fam and used a Zen/Iowa Black double quencher. Presence/absence analyses were performed on a Step-One Real Time PCR System (Applied Biosystems™). Each rtPCR reaction volume was 20 µl consisting of 2X TaqMan Environmental Master Mix 2.0 (Thermo Scientific Inc.), 2 mg/mL bovine serum

albumin (New England BioLabs Inc.), 0.6 μM of primers, 0.3 μM of probe, and 2 μl of DNA template. Cycling conditions were as follows: hold at 60°C for 30 s, 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a final hold at 60°C for 30 s. Samples were run in triplicate with each 96-well plate including a positive control (known HWA DNA) and rtPCR negative control (nuclease-free water used instead of DNA template). To minimize the chance of designating a false HWA positive, only samples where all three triplicates indicated a 'presence' rtPCR result were considered positive.

Statistical Analysis

All analyses were conducted using the program R v 4.0.3 (R Core Team, 2020). To evaluate if height impacted trap success in capturing HWA, we calculated the short traps' successes in catching HWA when corresponding tall traps (i.e., same trap location within a site) also caught HWA for each infested site (PIPK and NODU) throughout the year of sampling. We used these successes to estimate the probability that a short trap would detect HWA when a tall trap also detected HWA with a Wilson score interval (Wilson, 1927) using the package binom v 1.1-1. To assess if distance to an infested hemlock stand impacts trap capture success, we determined HWA capture successes and failures throughout the year for traps at the 0 m location compared to the 150 m and 300 m locations for each infested site with a Barnard's unconditional test (Barnard, 1945) using the package Barnard v 1.6. To determine how accurate of a predictor the rtPCR technique is for HWA presence in a trap sample, we assessed adelgid counts from each trap and rtPCR presence/absence results of HWA using a simple logistic regression with the function glm() in the package stats v 4.2.0. The explanatory variable was the adelgid counts, and the response variable was the presence/absence of HWA detected by rtPCR.

RESULTS

Vegetation Assessment

SLBR winter horizontal vegetation density percentages are listed as follows in order of trap pair locations (e.g., 0 m, 150 m, 300 m): 17%, 0%, and 14%. SLBR summer percentages were: 82%, 17%, and 67%. PIPK winter percentages of horizontal vegetation density were 4%, 98%, and 65%, while the summer percentages were 3%, 93%, and 79%. NODU winter percentages: 80%, 90%, and 14%; NODU summer 60%, 87%, and 34%.

Trap Assessment

Wilson score intervals were used to evaluate if trap height impacted trap success in capturing HWA. We calculated Wilson score intervals for our two infested sites, PIPK and NODU, since we were assessing success in capturing HWA. If a short trap caught HWA every time a corresponding tall trap also caught HWA, then the estimated success probability would be 1. At our PIPK site, the short traps had an estimated success probability of 0.83 (95% CI = 0.69, 0.92). Short traps at NODU had an estimated success probability of 0.94 (95% CI = 0.74, 0.99).

Barnard's unconditional tests were used to assess a difference in adelgid capture success of traps at the 0 m location compared to the 150 m and 300 m locations for each infested site (PIPK and NODU). The Barnard's tests showed that distance of traps to the infested hemlock stand can influence adelgid capture success. For PIPK, there was a significant difference in trap capture success between the traps at 0 m and 150 m ($P = 0.003$), while there was no difference between 0 m and 300 m ($P = 0.12$). For NODU, there was no difference between the traps at 0 m and 150 m ($P = 0.91$), but there was significance between 0 m and 300 m ($P = 2.7 \times 10^{-6}$).

Presence/Absence Assessment for HWA

Over the course of this 52-week study, we collected a total of 468 samples from all traps (156 trap samples from each site) with each sample representing a two-week sampling period. A simple logistic regression was used to determine how well the rtPCR method can accurately detect HWA in an environmental sample based on adelgid counts from each sample. Due to logistical constraints, we were only able to test 108 environmental samples (plus negative and positive controls associated with each sample) on the rtPCR assay. We chose an equal number of samples from each site (36 samples from each site) from six different collection periods to have a good representation of the year of sampling: December 30, 2020 – January 13, 2021; February 24 – March 10, 2021; April 7 – April 21, 2021; April 21 – May 5, 2021; May 19 – June 2, 2021; and June 30 – July 14, 2021. The rtPCR analyses resulted in 27 positive environmental trap samples out of the 108 tested. These 27 positive samples all had adelgids present, and we did not have any false positive results. Out of the 81 negative samples, 69 samples did not have adelgids and are considered true negative results. There were 12 samples that did have adelgids present but produced negative rtPCR results, and the majority of these samples had four or less adelgids present with the exception of two negative rtPCR samples having 8 and 11 adelgids present. This analysis indicated that rtPCR is a sensitive indicator of HWA presence: it showed that if a trap collected 14 or more adelgids during our two-week sampling periods, then the probability that rtPCR will detect HWA presence is greater than 90%. (Fig. 3). It is important to note that the associated negative controls for this set of 108 environmental samples were negative in the rtPCR analysis, and we assumed this indicated no HWA contamination for these samples.

DISCUSSION

The main goals of this study were to further assess our previously designed motorized trap (Chapter II) in capturing HWA in the environment and determine how well an HWA-specific rtPCR molecular assay could identify if HWA was present in our traps' samples. For further trap assessment, we evaluated the motorized trap's HWA capture success at different heights from the ground and distance to infestation and noted the trap's ability to capture HWA in varying infestation levels. We found that our short traps (1.5 m height) had success capturing HWA almost all of the time that our tall traps (3 m height) caught HWA in both our high and low infested sites. Distance to infested hemlock trees did impact our traps' success in capturing HWA, and we learned more about our traps' ability to capture HWA between high and low infested sites. We also discovered that number of adelgids caught in a trap is a good predictor of the rtPCR assay presence/absence outcome.

Our results show that using traps at greater heights (1.5 m vs 3 m) did not improve our ability to detect HWA, but trap distance to an infested hemlock stand probably does. At our highly infested site, PIPK, there was a significant difference in HWA capture between traps at the edge of a hemlock stand (150 m location) and traps more in the center of the hemlocks (0 m location), but there was no significant capture difference between traps within the hemlocks and traps moving furthest away from the hemlocks (300 m location). Our highly infested site had a high density of HWA present on all its hemlock trees; at the time of year when ovisacs were most visible, the trees appeared to be covered in snow just from ovisac presence. There were some individual hemlock trees scattered throughout the forest moving away from the main stand, and our farthest traps happened to end up near some of those individual highly infested trees. This could account for the 300 m location catching HWA almost as often as the 0 m traps. A

roadway was between the 0 m and 150 m locations, so this may have limited trap capture success compared to other locations. At our low infestation site, NODU, our results showed what we would expect with there being a significant difference in HWA capture between the traps furthest from the infested hemlock stand and the traps within the stand and no difference between the edge of the stand and within the hemlocks. Our traps at the 300 m NODU location did not catch HWA material throughout our yearlong sampling, and those samples also did not amplify on our rtPCR assay. This site's farthest trap location did not have any individual hemlock trees nearby the traps, and there was also a sand dune located between the 300 m location and the main hemlock stand. There were some small trails located in and around the hemlock stand, but these did not appear to impact the trap's capture success at the 0 m and 150 m locations. We believe infestation level and trap distance to hemlocks will have the biggest impact on trap success in capturing HWA.

Based on our data, the rtPCR approach we used to test for the presence of HWA had a 0.9 probability of detecting HWA when a trap had approximately 14 adelgids (See Figure 3A). If there were less than 14 adelgids, then the chance of obtaining a positive rtPCR result decreased, but we still had some success detecting HWA in samples with fewer adelgids present. One thing to note is that, as a conservative estimate, for a sample to be considered positive, all three replicates needed a positive reaction. In the future, we suggest that if only one or two sample replicates are positive, then these samples should be re-run to confirm those findings. There was one trap with a single adelgid that resulted in a positive rtPCR sample, but that sample also had ovisac material present. The ovisac likely provided more DNA for that sample to produce a positive rtPCR result. There were three instances where we had one or two adelgids present in a trap and did not get a rtPCR positive that were from our SLBR site where there was not a

confirmed HWA infestation. Our SLBR site did, however, have confirmed pine bark adelgids (*P. strobi*). Therefore, it is likely that adelgids captured at SLBR were *P. strobi,* and those were very likely accurate negative results from the rtPCR method.

While much of our work examining trap efficiency focused on capturing adelgid crawlers, we also wanted to assess if our traps and rtPCR assay could detect HWA outside of the crawler seasons to potentially provide other times of year these methods could be used. Our traps were able to catch HWA adults, exoskeletons, ovisac material, and other nymph stages throughout the year. So, our traps could be used outside of spring and summer months if land managers wanted to trap around the mobile crawler windows. However, our rtPCR method was not successful in detecting HWA from minimum material present in our traps for the samples we were able to test; we needed several adelgids to get a consistent positive result (> 0.9 probability of detection). From our lab testing, we know we can extract and amplify HWA from a single ovisac, but we did not always have the same success when we were removing this type of material from the traps for DNA extraction and amplification. This difference could be due to loss of DNA during the extraction process or inefficient removal of all environmental material during the petroleum-jelly separation. For example, the waxy ovisac material is also slightly buoyant, making separation harder. We plan to test more samples with this rtPCR method to further assess its limitations in detecting HWA material aside from crawlers. Future research could potentially enhance our current methods used in separating and extracting DNA from trap samples to make this process much more efficient and further minimize DNA loss before the rtPCR takes place. Although, we do acknowledge that some loss of DNA through these processes will happen and cannot be avoided.

Our research provides a baseline for helpful information on how best to utilize our motorized trap design for detecting HWA in a natural forest setting. This study complements our previous trap assessment work (Chapter II) and is the first instance of using an HWA-specific rapid molecular technique to detect the presence of HWA using DNA from environmental trap samples in Michigan. Future research could build off the motorized trap we tested, or new traps could be developed for this same use. While we used our rtPCR method for presence/absence, this method can potentially be used for quantitative assessment. With rtPCR, the amount of HWA material present on each trap can be quantified as the number of DNA copies present in the PCR reaction, otherwise known as quantitative PCR (qPCR). Future work can investigate incorporating qPCR methods in these monitoring efforts to not only allow managers to assess presence/absence of HWA but to also provide information on the level of infestation. This study will hopefully bring more implementation of these molecular techniques into HWA-detection.

Molecular assays using rtPCR methods are orders of magnitude more sensitive than standard PCR-based assays, and as such, will likely increase the efficiency of using DNA methods for early detection of HWA. These approaches allow for more accurate qualitative assessments of HWA presence. Identifying the optimal rtPCR method that is both cost effective and can produce accurate results was a necessary step for this technology to become incorporated into management efforts in the future. Our developed methods can provide a low-cost monitoring tool for land managers to better detect early infestations and HWA populations with low densities. These techniques can help preserve valuable personnel and funds for HWA detection and eradication efforts along the leading edge of this invasion in the United States and Canada.

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FIGURE LEGENDS

Figure 1. Map of study site locations in Michigan, USA: Crystal River Trailhead of Sleeping Bear Dunes National Lakeshore (SLBR), Pioneer Park (PIPK), and North Ottawa Dunes (NODU).

Figure 2. Photos of the traps used in this study: (A) our modified motorized trap, (B) the motorized trap at the 3 m height, and (C) both 1.5-m and 3-m traps paired.

Figure 3. Logistic regression estimated probability for the rtPCR method detecting HWA based on number of adelgids in a sample. Logistic regression shown (A) for all samples used with the rtPCR method (up to 2,500 adelgids per sample) and (B) for samples with only up to 100 adelgids per sample to better show the lower levels of detection with the rtPCR method. The horizontal dashed gray line represents a 0.9 detection probability, and the vertical dashed gray line represents the estimated number of adelgids needed to reach the 0.9 rtPCR detection threshold.

FIGURES

Figure 1.

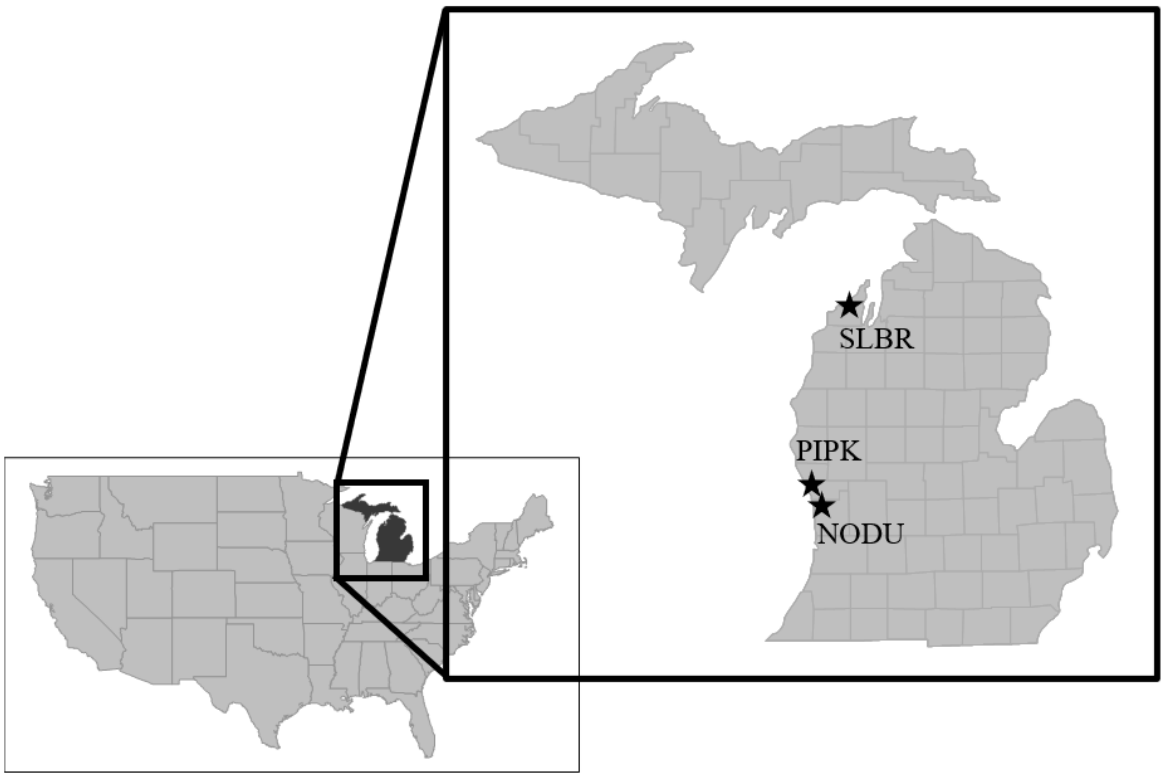


Figure 2.

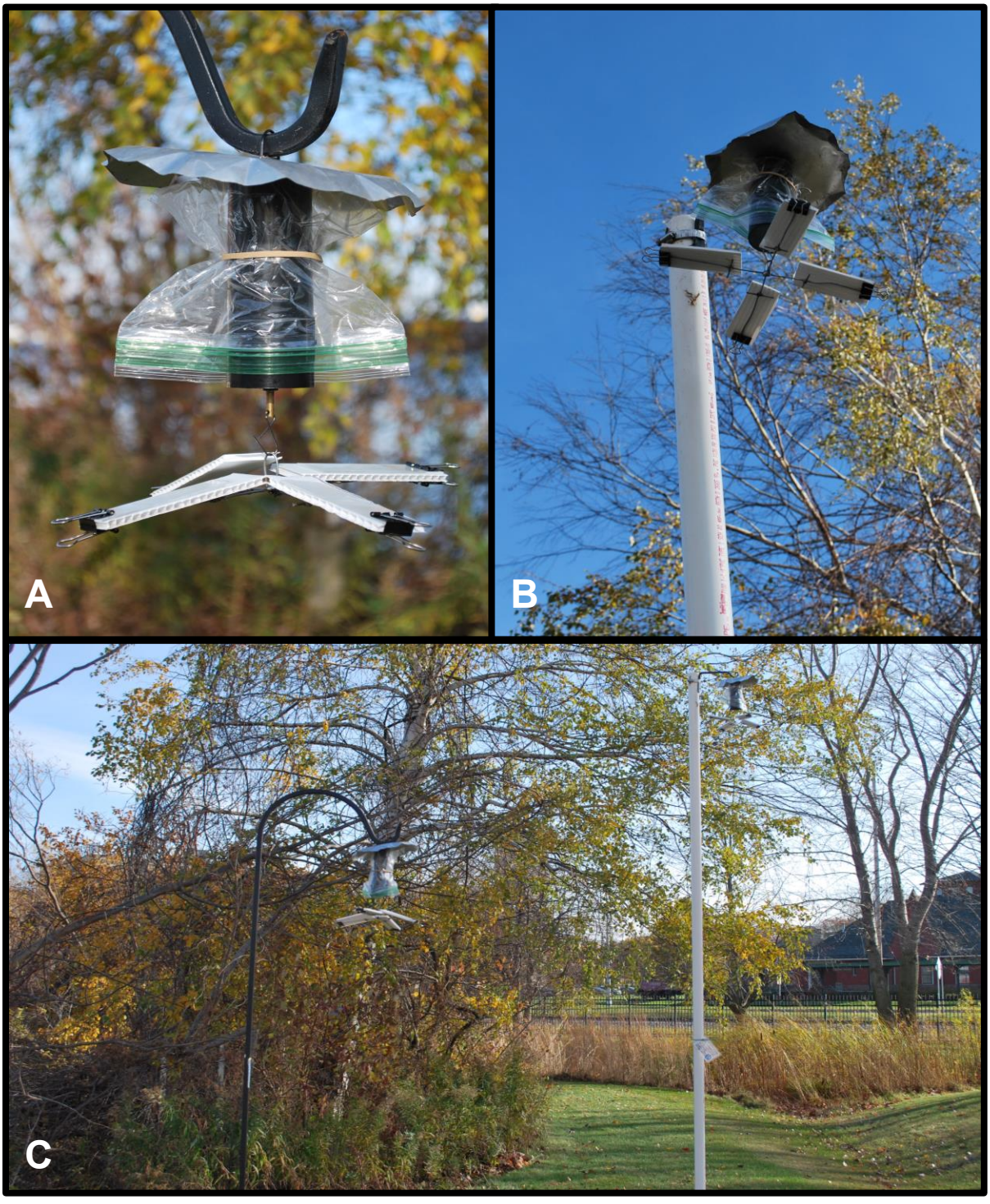
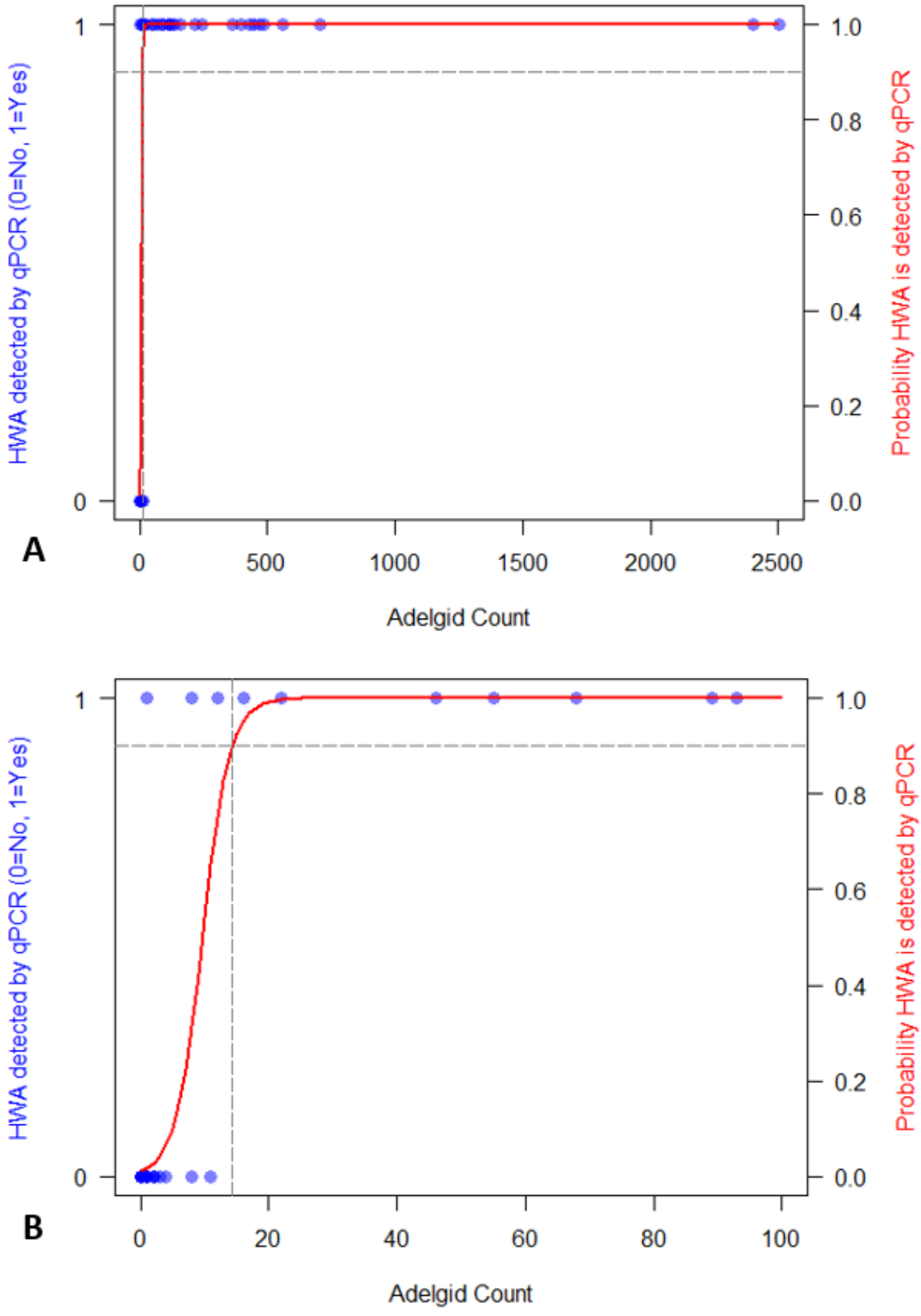


Figure 3.


HWA detected by qPCR (0=No, 1=Yes)
Probability HWA is detected by qPCR
1
0
1.0
0.8
0.6
0.4
0.2
0.0
A
0
500
1000
1500
2000
2500
Adelgid Count
HWA detected by qPCR (0=No, 1=Yes)
Probability HWA is detected by qPCR
1
0
1.0
0.8
0.6
0.4
0.2
0.0
B
0
20
40
60
80
100
Adelgid Count

CHAPTER IV

EXTENDED REVIEW OF LITERATURE

Introduction

Hemlock trees are commonly recognized as foundation tree species that define the forest structure and control ecosystem dynamics (Havill et al., 2014). Hemlocks are some of the most long-lived tree species in North America with one recorded to have lived more than 800 years (Ward et al., 2004). These trees can be found in riparian areas, typically within mesic hardwood forests, with thick, extensive canopies. Hemlocks stabilize soil, provide thermal cover for many birds and mammals, and are a preferred browse for white-tailed deer (Quimby, 1996; Yamasaki et al., 2000). Some bird species such as the black-throated green warblers (*Dendroica virens*) and the blue-headed vireo (*Vireo solitarius*) are only present in forests with hemlocks (Havill et al., 2014; Toenies et al., 2018). Hemlocks provide shade to streams, which helps moderate temperatures, regulate streamflow, and decrease runoff into surrounding aquatic systems. (Rogers, 1978; Snyder et al., 2002; Ford and Vose, 2007).

Losing hemlock trees can alter their ecosystems and potentially have damaging environmental effects (Orwig and Foster, 1998; Ellison et al., 2005; Ellison et al., 2018). Unfortunately, hemlock trees have been under attack in the eastern United States by the invasive insect, hemlock woolly adelgid (HWA), *Adelges tsugae*. Hemlocks infested with HWA have shown altered plant-water relations as well as overall reduced growth patterns (Miller-Pierce et al., 2010; Domec et al., 2013). HWA infestations negatively affect hemlock root composition and alter belowground interactions with ectomycorrhizal fungi and bacteria (Vendettuoili et al., 2015). Black birch tends to replace eastern hemlocks lost to HWA in riparian/wetland systems in

the eastern United States (Orwig and Foster, 1998). Daley et al. (2007) found that black birch trees use significantly more water than eastern hemlock, which could lead to unsustainable flow in streams that normally have light/moderate flows if black birch were to fully take over in a previously occupied hemlock stand.

Hemlock woolly adelgid is native to Japan, China, Taiwan, and western North America (Havill et al., 2014). HWA feeds on hemlock nutrients by inserting a feeding stylet into the hemlocks' plant tissue. This pest was accidentally introduced to eastern North America from Japan (Havill and Foottit, 2007), and it was first found in this region in 1951 in Virginia, United States (Havill et al., 2011). In its invasive range, HWA affects eastern (*Tsuga canadensis*) and Carolina (*Tsuga caroliniana*) hemlocks and can kill healthy trees in four to ten years (Havill et al., 2014). Asian and western North American hemlock species are not as negatively impacted by HWA, which may be due to host tolerance, host resistance, and the presence of predators that seem to regulate HWA populations (Oten et al., 2014). HWA got its name from the adelgids' ability to secrete a white, 'woolly' wax (also known as an ovisac) to cover and protect themselves while feeding and laying eggs on the hemlocks. Like most adelgids, this species has a complex life cycle. HWA rotates between sexual reproduction on spruce trees and asexual reproduction on hemlocks where these tree species coexist in its native ranges. In parts of HWA's native range as well as its invasive range in eastern North America, a suitable host spruce is not present and HWA will only reproduce asexually on hemlocks with two generations each year (Havill et al., 2014). The first generation is known as progredientes and the second generation is sistentes with each generation going through four instar nymph stages before adulthood. The first-instar nymphs of both generations are known as crawlers because they are

the only mobile life stage that disperses in search of hemlock needles to feed on (Havill et al., 2014).

Hemlocks infested with HWA in the eastern United States seldom survive invasion without some involvement of pest management, and there is often a lack of natural re-establishment after hemlock death from this invasive species (Preisser et al., 2011; Ford et al., 2012; Havill et al., 2014; McCarty and Addesso, 2019). This pest has accumulated over $250 million in damages per year for the United States, largely from the cost of treating infested hemlocks and trying to restore lost trees (Aukema et al., 2011). The United States Department of Agriculture (USDA) Forest Service currently runs a genetic resource conservation program for hemlock trees implemented with seed collections of Carolina and eastern hemlocks to preserve these species and their genetic variability as much as possible in the case that HWA were to wipe out most of these trees from their natural range (Jetton et al., 2013). Eastern and Carolina hemlock resistance to HWA is also being researched by the USDA Forest Service (Montgomery and Gottschalk, 2009), but it is recognized that this type of work will be a long process. This is another reason why early detection of HWA is most important for land managers to be able to enact control and treatment methods as quickly as possible.

Control and Treatment Techniques for Hemlock Woolly Adelgid

Biological control is a research priority because HWA does not have any natural predators in the eastern United States. One potential management strategy is to identify and release species that will specifically prey on HWA to gain a more long-term, cost-effective, and self-regulating control method. Fungal pathogens have been studied (Costa, 2011), although the species looked at thus far tend to be more generalists. Some states have brought in *Sasajiscymnus*

tsugae, a beetle known to prey on HWA in their native range of Japan, and did find some success (Cheah et al., 2005). However, these predators have shown that they can take up to seven years to establish before they have a noticeable impact on HWA. There have been several successful controlled releases of *Laricobius nigrinus*, a beetle native to western North America that is a known specialist predator of HWA in the eastern United States (Lamb et al., 2006; Mausel et al., 2010; Davis et al., 2012). Two *Leucopis* species of fly that prey on HWA in western North America were genetically assessed because while those same species of fly are found on the east coast, they do not prey on the HWA in the eastern United States (Havill et al, 2018). The western variety of these species were found to be genetically different from their counterparts in the East, which could account for the lack of preying on HWA by the eastern variety. The western variety of the *Leucopis* species could be a good candidate for release in the East for biological control of HWA. Research is ongoing to continue assessing the viability of these potential enemies of HWA in natural forest settings across the eastern states.

Chemical treatment of HWA has been around since the 1990's and is commonly used in management, but there are always challenges with finding a treatment that works effectively in all situations. A class of systemic insecticides known as neonicotinoids are regularly utilized for treating HWA. Imidacloprid is a commonly studied insecticide from this group of neonicotinoids well praised for its more long-lasting control of HWA (Cowles et al., 2006; Eisenback et al., 2010; Benton et al., 2016). However, imidacloprid can take up to a year before it starts killing HWA, and there are also concerns with the potential broad-spectrum impacts on non-target species from this insecticide. Dinotefuran is another compound that can be more fast-acting at killing HWA, but it is more expensive and may not have the same long-term control compared to imidacloprid (Cowles and Lagalante, 2009; Joseph et al., 2011; Faulkenberry et al., 2012). There

is also a general concern with insecticide use as it can leach into surrounding water bodies of hemlock habitat and have negative environmental impacts (Benton et al., 2016). Managers hope to implement an integrative pest management plan for HWA where they can use both chemical and biological control to help save hemlocks that otherwise would not survive long-term by any one control method on its own (Havill et al., 2014; Sumpter et al., 2018).

Hemlock Woolly Adelgid Monitoring Methods

Treatments are not always affordable or viable for every hemlock stand, so many eastern states' monitoring efforts evolved to cooperative programs between state and federal agencies to (1) implement annual surveys for monitoring long-term health of hemlock trees infested with HWA, (2) identify new infestations to track its expansion, and (3) potentially characterize the level of infestation (Williams et al., 2002). Many of the hemlock stands that were the first to be infested or could not be treated are the primary areas with goals of monitoring forest health overtime as HWA runs its course. HWA spreading to new areas is always a concern, and many states do want to treat this invasive if the infestation can be caught early enough to keep treatments cost-efficient and manageable. Early detection of new HWA infestations is key for the most effective management, and this can be a challenge. Detecting HWA has typically occurred through visual assessments of hemlock branches for ovisac presence (Costa and Onkin, 2006). This can be a considerable task for land managers, and some research has shown that early infestations as well as low HWA populations may not be visible in the lower canopy of an affected hemlock tree (Evans and Gregoire, 2007). If even one ovisac is found, HWA can be assumed to be present in the area, but not finding HWA does not mean it is not present in the hemlock stand.

Identifying easier, affordable, and efficient HWA detection techniques is an ongoing goal for many researchers and land managers. If HWA is found, land managers also like to characterize the percentage of infested trees in a stand to help determine next best management strategies (Costa, 2005). Fidgen et al. (2015 and 2019) studied the use of sticky traps to detect HWA via first-instar nymphs (crawlers) during their peak active time in spring and summer months as well as assess if crawler amounts on the traps can be associated with infestation level in the affected hemlock stand. These traps have shown to be a very effective detection method by capturing HWA crawlers, however, they found that incidences of ovisacs in the canopy over the traps was not related to the count of crawlers on the trap or the number of positive traps. Evans and Gregoire (2007) tested a random branch sampling (RBS) technique of the entire height of mature hemlock trees in a known HWA infestation area to investigate crown distribution of HWA. They counted the number of sistentes per 100 new growth needles of various branches from many infested trees and found that infestation level could be associated with these counts. Sistentes are the first HWA life stage to emerge after hemlocks produce new growth and can be connected to a specific year (Evans and Gregoire, 2007). While this shows promise as a technique to estimate infestation level in a stand, this method may not always be feasible with the amount of time and effort needed to count adelgids on individual trees.

Genetic Studies of Hemlock Woolly Adelgid

Genetic approaches are becoming a promising method for identifying target species within an environment that may otherwise be hard to detect, such as the cryptic Burmese python now invasive in Florida (Hunter et al., 2015) or other forest pests like the spotted lanternfly (Valentin et al., 2020). It was discovered that some Adelgidae species like HWA can be

distinguished between other adelgids using the standard 658-bp DNA barcode fragment of the mitochondrial cytochrome oxidase 1 (mCO1) gene (Foottit et al., 2009). Being able to genetically differentiate between HWA and other adelgid species could be a useful tool for future early detection and management techniques. Havill et al. (2016) expanded on the previous HWA genetic work with the use of microsatellites and mCO1 sequences to assess HWA's worldwide genetic structure and reconstruct its colonization history. Through this work they identified the invasive HWA in the eastern United States as a different genetic lineage from the HWA populations native to western North America. They recommend monitoring non-native sentinel host trees and focusing on invasion pathways to be more effective at preventing invasion than just making predictions using species traits or evolutionary history. While there is not a large body of genetic studies on HWA, this research has laid the groundwork for future genetic work to build upon.

Terrestrial Environmental DNA Research

A potential method to further develop genetic work of HWA and enhance monitoring techniques is the use of environmental DNA in monitoring efforts. Environmental DNA (eDNA) refers to when organisms shed or excrete cells and tissues into the environment, whether it is soil, water, or air (Lodge et al., 2012), and it is this material that eventually breaks down to release DNA. This genetic material can be collected from the environment and used to monitor for the presence of specific species (Barnes and Turner, 2016). Detection with eDNA techniques have often been used in aquatic systems (Goldberg et al., 2011; Treguier et al., 2014; Turner et al., 2014; Barnes et al., 2014; Evans et al., 2016), but several studies have successfully applied this technology in terrestrial environments. Terrestrial eDNA detection has been used to identify

invasive insects on crops (Valentin et al., 2018), invasive fungus through airborne spores (Quesada et al., 2018), as well as other insects and plant species via pollen or plant material (Folloni et al., 2012; Johnson, 2017; Thomsen and Sigsgaard, 2018). Airborne eDNA approaches can be applied to a wide range of species that are dispersed by wind.

Wind can facilitate the natural dispersion of HWA individuals and may also move ovisac material within a forest canopy (McClure, 1990). McClure (1990) was one of the earliest studies to assess dispersal of HWA, identifying wind, birds, deer, and humans as potential avenues to move HWA to new areas in the northeastern United States. These different avenues of dispersal make it hard to assess how and at what rate HWA may spread, but some models have estimated HWA spread at 12.5 kilometers per year (Evans and Gregoire, 2006). Several models have accurately predicted trends that have been observed in HWA spread and rate of spread such as faster infestation spread in southern states compared to more northern areas and stands closer to corridors have an increased risk of infestation (Koch et al., 2006; Evans and Gregoire, 2006; Fitzpatrick et al., 2012). These findings have been useful for land managers to prioritize the most at-risk hemlock stands for detection surveys and can serve as starting points as to where eDNA monitoring approaches for HWA could be most useful.

Conclusions

Some of the major contributions to our collective knowledge of HWA in its invasive range in North America have been research identifying its ecosystem impacts as well as studies focused on the biology and control of this invasive pest. Land managers can’t make informed decisions on how to stop this invasive species or how to restore hemlocks that have been lost without learning these things first. The genetic work that distinguished between adelgid

populations has led to great insight for future research looking into more efficient detection methods using DNA. The use of eDNA could be a cheaper and more efficient method for land managers to detect early infestations as well as low HWA populations. New detection technology could help preserve valuable personnel resources for HWA eradication efforts in Michigan as well as aid land managers in other states continuously monitoring HWA populations.

EXTENDED METHODOLOGY

Trap Design Testing

Trap Designs

The traps used in this study were: (1) motorized trap, (2) passive trap, (3) 8-funnel Lindgren funnel trap (Lindgren, 1983), and (4) standard sticky trap (Fidgen et al., 2019). (1) The motorized trap we used is a modification of a trap originally designed by Quesada et al. (2018) as a successful method for capturing airborne fungal spores in a forest setting. Our design included four petroleum jelly-coated (Vaseline) microscope slides affixed to the trap with two parallel (petroleum jelly facing upwards) and two perpendicular (petroleum jelly facing outward) to the ground to collect any airborne material. These slides were affixed to a battery-powered motor that rotated the slides in a clockwise direction at approximately 30 RPM (In the Breeze, Bend, OR). An aluminum pie pan and plastic bag covered the motor to protect it from the elements. (2) The passive traps were designed from a standing wind vane with all four petroleum-jelly-coated microscope slides affixed to the wind cups with jelly-coating facing upwards and slides parallel to the ground to capture airborne material; the slides rotated solely by the wind. Each microscope slide used in passive and motorized traps was 7.5 cm x 2.5 cm. (3) The 8-funnel Lindgren funnel traps consisted of eight 20 cm diameter openings of each funnel for material to fall into with a collection cup at the bottom. We kept 45 mL of propylene glycol in the attached cup of the funnel trap for preservation of material. (4) Due to not being able to affordably obtain the same materials used for Fidgen et al. (2019) sticky trap design, we slightly modified our sticky traps by assembling five sticky card insect traps on a 20 cm x 20 cm corrugated plastic board for each sticky trap.

Although sticky traps are useful in collecting adelgid count data, they are not compatible with genetic analysis due to difficulty in effectively removing material from the sticky glue (Fidgen et al., 2015, 2019). The traps using petroleum-jelly coated microscope slides (i.e., the motorized and passive traps) can be used for further genetic analysis, as Quesada et al. (2018) developed a method to successfully isolate captured airborne material from the petroleum jelly for DNA processing. Funnel traps are commonly used to capture insects (Lindgren, 1983; Klimaszewski et al., 2018) and have the potential to be compatible with DNA analysis. However, their use for specifically capturing HWA has not been evaluated previously.

Study Site

The trap design study took place at Pioneer Park, Muskegon, Michigan, USA, a site with confirmed HWA infestation. Pioneer Park is 145 acres of county park and campground property along Lake Michigan. The public recreational areas are surrounded by eastern hemlock (*Tsuga canadensis*) dominated forests with some mixed hardwood and other conifers. All traps were deployed in areas with known infested hemlock trees to test our trap designs.

Trap Deployment

All four trap designs (motorized, passive, funnel, and sticky traps) were deployed for four weeks in the month of July 2020, which coincided with the second peak HWA crawler stage of the year. We organized our experiment in a randomized block design with five blocks. Each block comprised 36 cells for a total area of 625 m^2. One of each trap type was randomly assigned a location within every block using a random number generator. The number randomly selected for each trap represents a cell within the block. We placed each trap at the latitude and longitude

of the central point of their randomly chosen cell. All traps were attached to standing poles 1.5 m from the ground. Trap contents were collected on a weekly basis for a total of 8 collecting periods. Slides from the passive and motorized traps and the funnel trap contents were collected in sterile 50 mL vials and stored in a refrigerator (4°C). The sticky trap panels were collected in clear, plastic storage bags due to their large size and stored in a freezer (-20°C).

Adelgid Capture Assessment for Each Trap.

Motorized and Passive Traps

To assess the number of adelgids captured, we examined the petroleum jelly-coated microscope slides from the motorized and passive traps under a dissecting microscope and counted the total number of HWA crawlers from the four slides of each trap. Trap contents were then stored in a freezer (-20°C).

Funnel Traps

To assess adelgid capture success for the funnel traps, we counted crawlers in each funnel trap by placing each trap's contents into an individual petri dish and examining the contents underneath a dissecting microscope. The contents were placed back into their respective 50 mL vials when the counts were completed and stored in a freezer (-20°C).

Sticky Traps

To obtain adelgid counts for the sticky traps, we counted adelgids on each sticky trap using methods previously described by Dreistadt et al. (1998). Adelgids were counted on a one-inch-wide vertical column down the center of each sticky insect card using a dissecting microscope. We used this technique on each of the five cards that made up every sticky trap. When counting was completed, these sticky traps were stored in a freezer (-20°C).

HWA Estimates Within Each Block

To determine if variation in HWA prevalence across our sampling site might impact our capture results, we evaluated HWA presence within each designated block at Pioneer Park by counting the number of ovisacs on hemlock branches using a method from the Pennsylvania Department of Conservation and Natural Resources (Johnson, 2020). This was quantified at the block level since differing amounts of HWA between blocks could impact trap success in catching HWA. We randomly selected 10 trees within every block and numbered the lower crown branches within 7.5 m of the ground starting on the north side and moving clockwise around the tree. We used a random number generator to select five branches around each tree and counted the number of ovisacs within a 25 cm length of the distal part of each branch.

Statistical Analysis

All analyses were conducted using the program R v 4.0.3 (R Core Team, 2020). HWA estimates within each block and adelgid capture assessment data were non-normal despite transformations, thus we chose non-parametric analyses. To determine whether there were significant differences in HWA prevalence between blocks, we assessed differences between the average number of ovisacs counted from each block with a Kruskal-Wallis test using the package stats v 3.6.2. We evaluated HWA capture successes between non-sticky traps and sticky traps by estimating the probability that a non-sticky trap would capture HWA when a corresponding sticky trap (same block and same collection date) also captures HWA with a Wilson score interval (Wilson, 1927) using the package binom v 1.1-1. All statistical analyses used an alpha value of 0.05 to determine statistical differences.

Trap Efficiency Assessment

Study Site

The second part of our study took place at North Ottawa Dunes, a 593-acre Ottawa County Parks property of wooded sand dunes bordering Lake Michigan. The site consists of northern hardwood forest including many eastern hemlock trees and other conifers. This is a site with a known HWA infestation, and we designated the infestation level as low (see Chapter III). We obtained Ottawa County Parks survey data (January – October 2020) with GPS locations of all hemlock trees within the park, as well as the locations of hemlock trees where visual surveys previously detected the presence of HWA ovisacs. We conducted our study in the southern part of the park where the largest clusters of HWA-infested hemlocks were located, and our entire survey range included areas both with and without hemlock trees.

Trap Deployment

For the trap efficiency assessment, we deployed a modified version of the previous motorized trap and sticky traps. While the motorized trap from the trap design study resulted in the lowest capture rate (see Chapter II results), we made significant modifications to this design that we felt corrected the flaws limiting its capture success. This included modifying the aluminum pan size to prevent the slides from being covered and arranging all petroleum jelly-coated slides so that they were parallel to the ground (i.e., facing upwards). The base of the trap was changed by putting a circle (cut from corrugated plastic board) over the top of the perpendicular metal piece the slides were previously attached to. We then clipped the slides directly to the plastic circle, which gave each glass slide a more secure and even surface to lay flat when attached to the base. This helped prevent slide breakage, and it made collection and

redeployment easier and faster for the user. We also slightly extended the distance that the slides hung from the motor to better prevent petroleum jelly from being wiped away when the wind blew the slides upward and they contacted the motor. The same 20 cm x 20 cm sticky trap design applied in our previous study was used in this experiment.

Within North Ottawa Dunes, we established a 90-acre circle over our study area and sectioned it into 30 equal parts. The 30 equal sections (3 acres each) were divided into five replicate groups (A-E), with six sections per group. Each of these six sections hosted a different number of paired motorized and sticky traps. Section one contained one pair of motorized and sticky traps, section two contained two pairs of traps, so on and so forth up to the sixth section containing six trap pairs. This resulted in a total of 105 motorized and 105 sticky traps for the entire 90-acre area, and the density of the traps within each section ranged from 1 trap per 0.5 acres to 1 trap per 3 acres. In every replicate group, the number of trap pairs and trap placement within each section was randomly assigned. Traps were attached to a 1.5 m pole, and the motorized and sticky traps were placed 2 m apart at each trap location. Traps were deployed for 16 weeks from April through July 2021 during both annual HWA egg hatching events. Petroleum jelly-coated slides from the motorized traps were collected biweekly and placed in 50 mL vials and sticky traps were collected in clear, plastic storage bags. Trap samples were stored at room temperature until processing.

Adelgid Capture Assessment

After each biweekly collection, we counted the number of adelgids observed on each trap. For the motorized traps, the number adelgids present on the four petroleum-jelly coated slides were observed using a Nikon SMZ645 dissecting microscope, counted, and recorded. We

assessed the number of adelgids collected on each sticky trap using the same method previously described for our trap design assessment (Dreistadt et al. 1998). For both the motorized and sticky traps, 20% of traps per collection period were recounted for quality assurance ($R^2 = 0.99$). When counting was completed for the motorized trap samples, we used dish soap to clean all microscope slides and 50 mL vials used for sample collection. These slides and vials were reused for other trap deployment and sample collection events throughout the trap assessment study. Sticky traps were stored at either room temperature or in a freezer (-20°C) until the study was completed.

Inverse Distance Weighted Spatial Interpolation Mapping

We created maps predicting distribution of HWA with the count data for each motorized trap by means of the inverse distance weighted (IDW) spatial interpolation method using ArcMap v 10.4.1 (ESRI, 2016) to visualize how adelgid counts varied in our study area throughout the summer. The IDW method predicts likely HWA numbers based on a linear-weighted combination of count data for sample locations. This method is appropriate for clustered data. IDW predicts values for unsampled locations by assuming those values are related more to closer data points than to those that are farther away. We used a power of 2 and a nearest neighborhood search of 8 points in the analysis, so more localized trap counts influenced predictions of the nearby unsampled locations and to account for all cardinal directions surrounding a location.

Statistical Analysis

Statistical analyses were conducted using the program R v 4.0.3 (R Core Team, 2020). To determine if the number of traps deployed within a 3-acre area significantly impacted whether an adelgid was captured within a section, we assessed adelgid capture success and failure throughout the 16-week study when one trap was used per section compared to when more than one trap was used per section with a Barnard's unconditional test (Barnard, 1945) using the package Barnard v 1.6. We did this to compare the following groups of trap numbers: one and two, one and three, one and four, one and five, and one and six traps. We again estimated the probability that a motorized trap would detect HWA when the corresponding sticky trap detected HWA for the entire 16-week study period with a Wilson score interval (Wilson, 1927) using the package binom v 1.1-1 to evaluate how our modifications to the motorized trap improved capture success compared to our initial trap design. We also assessed if trap elevation, slope, aspect, and Euclidean distance to the nearest HWA-infested hemlock impacted the number of adelgids caught in a motorized trap. The adelgid count data were non-normal, and they were heavily over-dispersed. Because of this, we used a GLM with a negative binomial distribution using the package MASS v 7.3-53.1. The full model consisted of adelgid counts as the dependent variable and Euclidean distance, elevation, slope, and aspect as the independent variables. A reduced GLM model was also run after removing the non-significant terms, and the optimal model was selected using the lowest Akaike's Information Criterion (AIC). Analyses used an alpha value of 0.05 to determine statistical differences.

Molecular Assay Development and Testing

One of our initial goals was to develop a molecular assay targeting the cytochrome oxidase 1 (CO1) gene to detect HWA in our environmental samples. We continuously developed and tested primers for a PCR-based assay throughout the time of this thesis research. To identify a set of potential primers to test, we used the Molecular Evolutionary Genetics Analysis (MEGA) software v6 (Kumar et al. 1994; Tamura et al. 2013) to align the CO1 genes of HWA and six other adelgid species. We found a few different regions with a high proportion of base pair differences between HWA and the other species. The six adelgid species we compared to HWA were *Adelges abietis*, *A. piceae*, *A. cooleyi*, *A. laricis*, *Pineus pini*, and *P. strobi* since these are species commonly found in the northeastern United States. All adelgid sequences were downloaded from the Barcode of Life Data System (BOLD) (https://www.boldsystems.org/). We used Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012) to design three primer sets throughout the project that could be specific to HWA based on the gene region we identified. The primers were named CO1, SYBR, and EHAP, and the primer sequences are as follows: CO1 Forward – 5`-TTGGAGGATTYGGAAAYTGA-3` and Reverse – 5`-TGGTGGYTAAATTGTTCATCC-3`; SYBR Forward – 5`-TTTGACTTCTTCCTCCATCTCTAAT-3` and Reverse – 5`-GTGGGTAAATTGTTCATCCTGTTC-3`; and EHAP Forward – 5`-CAATTGTAATTGGAGGATTTGG-3` and Reverse – 5`-GAGATGGAGGAAGAAGTCA-3`. We used Primer-BLAST (Ye et al., 2013) to predict the potential for cross-amplification across the six additional adelgid species listed previously. SYBR had a small potential to cross amplify with *A. cooleyi*, while EHAPs showed no potential for cross-amplification. We performed a gradient PCR for each set of primers with annealing temperatures between 50℃ - 60℃ to

pinpoint the optimal annealing temperatures for the PCR reactions. With PCR conditions optimized, we tested the specificity of the primers by running a PCR of each primer set with a negative control (NFW used instead of DNA template), HWA DNA (positive control), and the other six adelgids' DNA. PCR gradients were performed on a 5341 Mastercycler epGradient Thermal Cycler (Eppendorf ®) and other standard PCR runs were performed on SimpliAmp Thermal Cyclers (Applied Biosystems™). PCR conditions were as follows: hold at 94°C for 2 min followed by 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with a hold at 72°C for 2 min and a final hold at 10°C. We ran PCR product on 2% agarose gels to check for DNA amplification. Some of the tested primers (CO1 and EHAP) amplified non-HWA adelgids in addition to amplifying HWA DNA, so we decided those primers would not work for our purposes. For the SYBR primers, we saw successful amplification of the HWA DNA only. We repeated this procedure for each primer set to confirm these results. We tested the SYBR primers further due to their HWA-specificity, however, we later learned that this set of primers was not efficient and did not produce reliable results with every PCR when tested on our environmental samples. For this reason, we could not use these primers for our standard PCR-assay moving forward, and unfortunately, we were not able to develop a successful PCR-based assay for this project. Future research could attempt to design and test other primers for this purpose or investigate gene regions other than the CO1 gene for designing HWA-specific primers that can be used with a standard PCR assay.

While working on our PCR-based assay, we developed and tested a SYBR Green rtPCR assay since rtPCR can be orders of magnitude more sensitive than standard PCR assays. SYBR Green is the most cost-efficient rtPCR method, so we wanted to test this assay before considering the TaqMan rtPCR method. Our SYBR primers mentioned previously were also designed to be

compatible with a SYBR Green-based rtPCR assay, and the primers initially showed to be HWA-specific in our standard PCR-based assay. We tested these primers against HWA and the six other adelgid species with SYBR Green rtPCR presence/absence analyses performed on a Step-One Real Time PCR System (Applied Biosystems™). Each SYBR Green reaction volume was 25 µl consisting of 2X PowerUp SYBR Green Master Mix (ThermoFisher Scientific Inc.), 0.5 µM of primers, and 2 µl of DNA template. Cycling conditions were as follows: hold at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min with a melt curve step and hold stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Samples were run in triplicate including a positive control and negative control. We found that our SYBR Green rtPCR reactions using these primers produced a moderate amount of primer dimer, and the SYBR Green dyes used in this chemistry will bind to anything that is double stranded, which includes primer dimer. We also experimented with a smaller concentration of primers (0.3 µM) to see if that would alleviate the primer dimer issue, but this was also unsuccessful. The primer dimer confounded our results with the SYBR Green method and would generate a false positive for HWA presence, so this method would not work for our intended purposes moving forward.

We quickly moved on to developing and testing a TaqMan-based rtPCR method, which uses a combination of primers and a DNA probe that are complementary to our target HWA DNA. In this case, the DNA probe contains a combination of a fluorescent dye (a fluorophore) and a quencher (which absorbs the excitation energy of the fluorophore). When the dye and quencher are near one another, the quencher masks the fluorescence of the dye. However, during rounds of rtPCR, the fluorescent dye is removed, and as more copies of the DNA target are produced, the higher the intensity of the fluorescent signal. Because of this, the TaqMan

approach can be more species-specific, and non-specific PCR product (such as primer dimer) is not an issue.

We designed a primer and probe set to use with the TaqMan-based rtPCR method using methods previously described, and those sequences (HWA1) are as follows: Forward – 5`-GATCATGGGAATAATGGAATTTGAG-3` and Reverse – 5`-TTGACTTCTTCCTCCATCTCT-3` and probe– 5`-AGGAACAGGATGAACAATTTACCCACCA-3`. The probe was fluorescently labeled with Fam and used a Tamra quencher. Our previously used EHAP primers in our standard PCR-based assay were also developed for use with the TaqMan-based rtPCR method, and we designed a complementary probe (probe sequence– 5`-AGA ACA CCT GAT ATA TCC TTT CCA CGA-3`). The probe was fluorescently labeled with Fam and used a Zen/Iowa Black double quencher. We tested these HWA1 and EHAP primers/probe against HWA and the six other adelgid species with the TaqMan quantitative analyses performed on a Step-One Real Time PCR System (Applied Biosystems™). Each rtPCR reaction volume was 20 µl consisting of 2X TaqMan Environmental Master Mix 2.0 (Thermo Scientific Inc.), 2 mg/mL bovine serum albumin (New England BioLabs Inc.), 0.6 µM of primers, 0.3 µM of probe, and 2 µl of DNA template. Cycling conditions were as follows: hold at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run in triplicate including a positive and negative control. Our reactions were not successful with either primer/probe set, so we decided to put a hold on developing our TaqMan method to test and hopefully move forward with another set of primers and probe that had recently been developed to be HWA-specific with a TaqMan-based method.

While we were working to develop our molecular assays, Dr. Mark Whitmore's lab at Cornell University also developed HWA-specific primers and probe that amplify an approximately 250 bp region of the CO1 gene to be used with a TaqMan-based rtPCR assay (Kirtane, 2021). The primer and probe sequences are as follows: Forward – 5`-ACAGGATGAACAATTTACCCAC-3` and Reverse – 5`-AGCACCTGCTAGAACAGGTAAGG-3` and probe– 5`-CCA TTA TTC CCA TGA TCA ATT TTA ATT ACT GC-3`. The probe was fluorescently labeled with Fam and used a Zen/Iowa Black double quencher. We also tested Cornell's primers and probe with our equipment to confirm their efficiency and HWA-specificity. We tested the primers and probe against HWA and the six other adelgid species with the TaqMan quantitative analyses performed on a Step-One Real Time PCR System (Applied Biosystems™). Each rtPCR reaction volume was 20 µl consisting of 2X TaqMan Environmental Master Mix 2.0 (Thermo Scientific Inc.), 2 mg/mL bovine serum albumin (New England BioLabs Inc.), 0.6 µM of primers, 0.3 µM of probe, and 2 µl of DNA template. Cycling conditions were as follows: hold at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run in triplicate with a positive control and negative control. Our results showed that the primers and probe were HWA-specific, and we decided to use this TaqMan assay with our environmental sample collection to test for HWA presence in the samples.

We also tested Cornell's primers with our standard PCR-based assay to learn if they could be used for both PCR and rtPCR methods. We performed a gradient PCR of Cornell's primers with HWA DNA and the six other adelgid species with annealing temperatures between 50℃ - 60℃ to pinpoint the optimal annealing temperatures for the PCR reactions and to see if annealing temperature impacted if the primers co-amplified any of the other adelgid species. We

ran the PCR product on a 2% agarose gel to check for amplification. There was some very slight amplification of another adelgid species (*P. strobi*) at the annealing temperature that showed to be most efficient for HWA, but the amplification for HWA showed a much brighter band when visualized on the gel. We repeated this process to confirm our results. We thought we could differentiate between a bright band representing HWA material and a light-colored band possibly showing up if there happened to be *P. strobi* in any environmental samples we collected as part of our other projects. We continued testing these primers with some of our environmental samples at the optimized PCR conditions we identified. PCRs were performed on both our 5341 Mastercycler epGradient Thermal Cycler (Eppendorf ®) and SimpliAmp Thermal Cyclers (Applied Biosystems™). PCR conditions were as follows: hold at 94°C for 2 min followed by 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with a hold at 72°C for 2 min and a final hold at 10°C. Unfortunately, when we used the primers with our environmental samples, we could not clearly identify HWA material with the bands visualized on the gel. There were multiple samples with HWA material that did not produce bright, distinguishable bands, and we also saw bands from samples that did not have HWA material in them. While the primers and probe showed to be very HWA-specific with rtPCR, we learned that the primers alone were not HWA-specific with a PCR-based assay; it was the DNA probe used with the primers in the TaqMan rtPCR approach that made this method more species-specific in amplifying HWA DNA only. We continued all environmental sample testing with the TaqMan-based rtPCR method developed by Kirtane (2021).

Trap Assessment for rtPCR

Site Selection

We selected three sites of varying infestation level (no detection, one high, one low) to use in our study. Sleeping Bear Dunes in Leelanau County, Michigan, was our 'no detection' site. Pioneer Park in Muskegon, Michigan, and North Ottawa Dunes in Ottawa County, Michigan, were our two infested sites. In September 2020, prior to trap deployment, we assessed infestation levels at both Pioneer Park and North Ottawa Dunes using methods outlined in Evans and Gregoire (2007). In this approach, they conducted a full crown assessment of hemlock trees infested with HWA to determine adelgid density within the different crown levels of hemlock trees. They found that hemlock trees with lower-level branches (within a 7.5 m height from the ground) with more than 20 sistentes counted per 100 new growth needles had high HWA populations and those with less than 20 sistentes counted per 100 new growth needles in the lower crown branches had low HWA populations. We randomly selected 25 hemlock trees from each site and numbered the lower-level branches, always starting on the North side and moving clockwise around the tree. Nine branches from each tree were randomly chosen and assessed for number of sistentes per 100 new growth needles. The counts were averaged per tree to alleviate bias in HWA numbers on a single branch. We designated Pioneer Park as our high infestation site (averaged 24.2 sistentes) and North Ottawa Dunes as the low infestation (averaged 0.2 sistentes). Counting sistentes is an appropriate measure of HWA for a particular year since sistentes are the first generation to emerge after new growth looking to settle on the new hemlock needles (Evans and Gregoire, 2007). Because of this, we were confident that our infestation level designations were consistent through the year of sampling.

Study Sites

Our study took place at three sites in western Michigan from October 2020 through October 2021: Crystal River Trailhead of Sleeping Bear Dunes National Lakeshore, Leelanau County, Michigan; Pioneer Park, Muskegon, Michigan; and North Ottawa Dunes, Ottawa County, Michigan.

Sleeping Bear Dunes

The Crystal River Trailhead area of Sleeping Bear Dunes (SLBR) hosts an eastern hemlock stand between the Crystal River Trailhead and the Crystal River itself. This site is in a county with no known HWA infestations, and HWA has also not been found at this site in any annual visual surveys performed by Sleeping Bear Dunes' staff. For these reasons, we chose to use this area as a 'no detection' control site in our study.

Pioneer Park

Pioneer Park (PIPK) is 145 acres of Muskegon County campground and park property along Lake Michigan. PIPK is a site with confirmed HWA infestation, which we designated as a high infestation level for this study. This park is dominated by eastern hemlock trees with some mixed hardwoods and other conifers. We used the eastern part of the park where the most hemlock trees were located.

North Ottawa Dunes

North Ottawa Dunes (NODU) is an Ottawa County Parks' 593-acre wooded sand dune property bordering Lake Michigan. The site is made up of northern hardwood forest, which includes many clusters and some scattered eastern hemlock trees as well as other conifers. We designated the infestation level as low for our study and worked in the southern part of the park where a larger cluster of HWA-infested hemlocks was located.

Trap Deployment and Sample Collection

We continued using our motorized trap in this study with a few modifications from the design used in the initial trap design testing (Chapter II). We included four 7.5 cm x 2.5 cm petroleum jelly-coated microscope slides affixed to the trap with all four slides parallel to the ground and jelly-coated sides facing upwards. The traps had a battery-powered motor that rotated the slides in a clockwise direction at approximately 30 RPM (In the Breeze, Bend, OR) as well as an aluminum pan and plastic bag to protect the motor from the elements. The tall traps were affixed to a hollow PVC pole that slid over a thin metal pole secured in the ground, so it was easily removable for sample collection. Six traps were deployed at each site in October 2020 for 52 weeks. Within each site, two traps were centrally located in a hemlock stand (0 m), two traps were near the edge of the stand at 150 m from the central point, and two traps were 300 m from the central point continuing to move away from the hemlock stand. To assess how trap height could impact adelgid capture success, each set of two traps had one trap 1.5 m above the ground and one trap 3 m above the ground, with the short and tall traps set 3 m apart from one another. Samples from each trap were collected in sterile 50 mL vials on a biweekly basis and stored in a freezer (-20℃) until further sample processing could occur. To employ contamination prevention techniques throughout sampling, we used latex gloves when collecting samples and sanitized them with 70% ethanol between touching samples and equipment; we used new latex gloves when going to a new site. To test for sample contamination during sample collection, we collected a field blank from each site for each collection week. The field blank was a petroleum-jelly coated microscope slide prepared in the lab at the same time as the microscope slides for each site. It was transported in its own 50 mL vial in the same container with the other microscope slides to be re-set at a site. At the time of sample collection for each site, the field

blank was temporarily removed from its 50 mL vial to be handled in the same manor we handled the environmental samples before being placed back into its container.

Vegetation Assessment

We assessed vegetation density between each set of traps at the 0 m, 150 m, and 300 m locations to determine if dense vegetation may impact how trap distance influenced capture success. At each site, we measured horizontal vegetation cover density using a 1 m x 1 m vertical profile cloth sheet with a 10 cm grid (Doggett and Locher, 2018). Measurements were taken in winter and summer at three locations per site: photos for the 0 m trap locations were taken 14 m away from the trap toward the direction of the 150 m traps; photos for the 150 m traps were taken 14 m away toward the direction of the 300 m traps, photos for the 300 m traps were taken 14 m away but back in the direction toward the 150 m traps. For all photos the top of the cloth was 1.5 meter above the ground to best capture the midstory vegetation that could possibly hinder HWA movement within the forest. We collected measurements in winter and summer to get a representation of leaf-off and leaf-on vegetation because our traps were deployed for a full year, and we chose 14 m as our distance since Turner et al. (2011) found 12-14 m as a mean dispersal distance in their simulation of HWA dispersal in forest understory. To calculate the vegetation percent cover, we recorded the percentage of cells in each grid that contained vegetation.

To quantify vertical canopy coverage of the hemlock stands at each site, we used the image classification tool in ArcMap v 10.4.1 (ESRI, 2016) to analyze aerial satellite imagery. For our SLBR site, we had access to 12-inch resolution imagery, we used 6-inch resolution imagery for PIPK, and NODU had 3-inch resolution imagery. First, we classified land cover

based on the following categories: water, forest, open, and impervious. These categories were sufficient to capture the variation in reflectance values across the imagery and minimize misclassifications between classes. We then reclassified forest as 1 and anything else that was not forest as 0. We used a focal mean with a window size of 1 acre to calculate the percentage of forest cover per acre. We did this using leaf-off imagery for each site, so the hemlock forests would be more identifiable from deciduous trees also found in our sites.

DNA extraction

We examined each slide under a Nikon SMZ645 dissecting scope to make note of any visible HWA material (e.g., ovisac, nymphs, adults, etc.) and hemlock needles, and we counted the number of adelgids present. After slide inspection, each slide's petroleum jelly environmental sample was placed into individual sterile 1.5 mL microcentrifuge tubes. We then separated any environmental material from the petroleum jelly using methods from Quesada et al. (2018), which consisted of a series of heating and centrifugation steps. Once samples were separated from the petroleum jelly, they were preserved at -20℃ until DNA extraction could occur. We extracted DNA using a Quick-DNA Tissue/Insect Microprep Kit (Zymo Research). Post-DNA extraction, the samples continued to be stored at -20℃ until they could be analyzed for HWA presence with the molecular assay. Contamination prevention procedures were put in place at all stages of sample assessment in the lab and included the following: sterilizing work surfaces and tools with DNA AWAY™, also sterilizing gloves with DNA AWAY™ between handling samples, changing gloves between each assessment step, and sterilizing tools used to place petroleum-jelly environmental samples into individual microcentrifuge tubes by dipping them in 70% ethanol and flaming them over a Bunsen burner. To test for contamination during sample

assessment, we prepared lab blanks for each step, e.g., petroleum-jelly separation and DNA extraction, in the same room where the samples were assessed and stored. All extractions were done in a lab space reserved for extracting DNA samples, where no PCR products or other HWA material were handled.

Presence/Absence Assessment for HWA

We assessed presence/absence of HWA in our environmental samples using real-time polymerase-chain reaction (rtPCR) with primers and a probe specific to HWA previously designed and tested by Kirtane (2021) that amplify an approximately 250 bp region of the CO1 gene. The primer and probe sequences are as follows: Forward – 5`-ACAGGATGAACAATTTACCCAC-3` and Reverse – 5`-AGCACCTGCTAGAACAGGTAAGG-3` and probe– 5`-CCA TTA TTC CCA TGA TCA ATT TTA ATT ACT GC-3`. The probe was fluorescently labeled with Fam and used a Zen/Iowa Black double quencher. Presence/absence analyses were performed on a Step-One Real Time PCR System (Applied Biosystems™). Each rtPCR reaction volume was 20 µl consisting of 2X TaqMan Environmental Master Mix 2.0 (Thermo Scientific Inc.), 2 mg/mL bovine serum albumin (New England BioLabs Inc.), 0.6 µM of primers, 0.3 µM of probe, and 2 µl of DNA template. Cycling conditions were as follows: hold at 60°C for 30 s, 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a final hold at 60°C for 30 s. Samples were run in triplicate with each 96-well plate including a positive control (known HWA DNA) and rtPCR negative control (NFW used instead of DNA template). To minimize the chance of designating a false HWA positive, only samples where all three triplicates indicated a

'presence' rtPCR result were considered positive. Contamination prevention included UV sterilization of tools and reagents and sterilizing work surfaces with DNA AWAY™.

Statistical Analysis

All analyses were conducted using the program R v 4.0.3 (R Core Team, 2020). To evaluate if height impacted trap success in capturing HWA, we calculated the short traps' successes in catching HWA when corresponding tall traps (i.e., same trap location within a site) also caught HWA for each infested site (PIPK and NODU) throughout the year of sampling. We used these successes to estimate the probability that a short trap would detect HWA when a tall trap also detected HWA with a Wilson score interval (Wilson, 1927) using the package binom v 1.1-1. To assess if distance to an infested hemlock stand impacts trap capture success, we determined HWA capture successes and failures throughout the year for traps at the 0 m location compared to the 150 m and 300 m locations for each infested site with a Barnard's unconditional test (Barnard, 1945) using the package Barnard v 1.6. To determine how accurate of a predictor the rtPCR technique is for HWA presence in a trap sample, we assessed adelgid counts from each trap and rtPCR presence/absence results of HWA using a simple logistic regression with the function glm() in the package stats v 4.2.0. The explanatory variable was the adelgid counts, and the response variable was the presence/absence of HWA detected by rtPCR.

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