PROTEIN SELF-MARKING BY EMERALD ASH BORER: AN EVALUATION OF EFFICACY AND PERSISTENCE

by

Scott Gula

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Entomology

West Lafayette, Indiana

August 2019

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Matthew Ginzel, Co-Chair

Departments of Entomology and Forestry and Natural Resources

Dr. Ann Ray, Co-Chair

Department of Biology

Dr. Michael Scharf

Department of Entomology

Approved by:

Dr. Stephen Cameron

Head of the Graduate Program

To John and Carol

ACKNOWLEDGMENTS

I would like to thank my committee members, Dr. Matthew Ginzel, Dr. Ann Ray, and Dr. Michael Scharf for their guidance, wisdom, and commitment to my development as a scientist. I am especially grateful to my co-advisers: Dr. Ray and Dr. Ginzel for their patience, support, and for constantly encouraging my curiosity. I also thank my lab mates Gabe Hughes, Matt Ethington, Bridget Blood, Sara Stack, and Tyler Stewart for their friendship and their assistance with various aspects of this project. I thank Brian Beheler for providing ash logs and Jay Young, Sue Cambron, Grzegorz Buczkowski, and Ameya Gondhalekar for the use of their facilities for the completion of this project. Finally, thanks to my family and friends for their love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Author: Gula, Scott, W. MS
Institution: Purdue University
Degree Received: August 2019
Title: Protein Self-Marking by Emerald Ash Borer: An Evaluation of Efficacy and Persistence
Committee Chair: Dr. Matthew Ginzel and Dr. Ann Ray

Understanding the dispersal ability of invasive insects provides useful insights for developing effective management strategies. Historically, methods for marking insects for dispersal studies have been expensive, time-consuming, and labor-intensive, especially for woodboring beetles. In addition, capturing or rearing insects requires human handling, which can alter behavior. Immunomarking is a well-established technique for studying the dispersal of insects, however, it has not been broadly applied to woodborers. This study evaluates the potential for using immunoproteins applied directly to woodborer-infested trees to mark emerging beetles. Specifically, in the first experiment I sprayed varying concentrations of ovalbumin (egg white) solution directly onto logs infested with emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) (Buprestidae: Agrilini) and ELISA was used to detect the presence of protein on emerged beetles. To test the persistence of the mark, I applied varying concentrations of albumin to freeze-killed beetles, mounted them on pins, and placed them in an exposed location outdoors. Adult EAB self-marked as they emerged from protein-treated trees, with higher protein concentrations persisting for longer on the cuticle when exposed to sun and rain. This technique offers a convenient, inexpensive and durable means of marking woodborers and circumvents the need for human handling, allowing for more natural behavior and more realistic estimates of dispersal. Protein self-marking may find application in studies of woodborer dispersal within natural forest environments.

CHAPTER 1: INVASIVE WOODBORING BEETLES AND THEIR MANAGEMENT

1.1 Value of Forest Resources

Products from the nearly 4 billion hectares of natural and managed forests support a $600 billion global industry comprised of timber, pulp, fiber, and fruit and nuts (FAO, 2015). Forests also provide ecosystem services including carbon sequestration, purifying water and air, mitigating flood risk, supporting biodiversity, enriching soils and reducing erosion, and providing recreation, entertainment, and esthetic value (Costanza et al., 1997; Boyd et al., 2013; Liebhold & Kean, 2019). Although quantifying the actual economic value of ecosystem services is difficult, their economic contribution is thought to far exceed those of forest products (Costanza et al., 1997). Nevertheless, the health and productivity of forests are threatened by human-mediated disturbances (e.g., climate change, pollution, and habitat fragmentation) and a litany of native and invasive insect pests, parasites, and pathogens (Dale et al., 2001; Levine, 2008; FAO, 2015; Laurance, 2015; Miller & Stephenson, 2015). Among native species that affect forest health, notable examples include the western and southern pine beetles (*Dendroctonus ponderosa* Hopkins and *D. frontalis* Zimmerman) that have devastated millions of hectares of *Pinus* spp. forests during outbreak years (Turchin et al., 1991; Kurz et al., 2008). Despite the episodic and widespread damage caused by outbreaks of native species, exotic invasive insects pose a far greater threat to forest sustainability (Liebhold et al., 2017).

Biological invasions progress along three conceptual stages: introduction, establishment, and spread (Liebhold & Tobin, 2008; Levine, 2008). Introduction is the first step in the invasion, where an exotic organism first arrives in a location outside of its native range. Generally, these organisms are introduced through accidental or intentional human transport. Establishment

occurs when a founder population grows and expands its distribution beyond the point of introduction. During this stage, populations are relatively small and localized making them highly vulnerable to extinction, especially due to stochastic abiotic influences and Allee effects (Liebhold & Tobin, 2008). Allee effects consists of a wide array of potential factors that drive sparse populations into decline and set a critical threshold on the minimum number of individuals required for a population to remain viable. Once established, local extinction of the founder population is highly unlikely. During the spread phase, populations of exotic organisms begin to expand or invade new territory. An exotic organism is considered invasive when it begins to displace native species (Liebhold & Tobin, 2008). Because incipient exotic populations are small and geographically limited, most are not detected until they have spread and become invasive.

Invasive arthropods cause more than $17 billion in damage in the United States every year (Pimentel et al., 2002; Follet & Neven, 2006) and introductions of forest pests have steadily increased concomitant with a rise in international trade and travel (Aukema et al., 2010; Roques, 2010). Insect introductions are expected to increase at an even faster rate with expanded economic globalization (Levine & D'Antonio, 2003; Roques, 2010; Early et al., 2016). Of these introductions, relatively few species will establish, and fewer still will become pests (Aukema et al., 2010). However, with thousands of introductions each year, several will undoubtedly become invasive and cause serious harm.

1.2 Damage Caused by Invasive Woodborers

Woodboring insects feed subcortically by boring through the stem or branches of a tree. This feeding behavior damages the vascular cambium, disrupting the flow of water and nutrients and ultimately girdling the tree. Members of this feeding guild often have close associations with

disease causing agents such as fungi that are introduced into trees as they bore under the bark (Fraedrich et al., 2008; Paap et al., 2018). As a result, woodborers are among the most destructive and most economically important forest pests compared to other feeding guilds such as defoliators (Aukema et al., 2011). Moreover, woodborers spend the majority of their lifecycle as larvae where they are concealed beneath the bark of trees. This cryptic lifestyle contributes to woodborer invasiveness because they are often transported unwittingly within nursery stock, solid wood packing material, and dunnage where they go undetected from inspections (Haack, 2006; Brockerhoff et al., 2006). In fact, the rate of woodborer introduction is increasing relative to other forest pests. When they emerge as adults in new territories without coevolved predators or pathogens to regulate populations, non-native woodborers can become established, spread, and have devastating impacts as invasive species on native trees within the introduced range (Aukema et al., 2010).

Among the most commonly intercepted woodborers are the longhorned beetles and jewel beetles in the families Cerambycidae and Buprestidae, respectively (Haack, 2006; Haack et al., 2014; Wu et al., 2017). The Asian longhorned beetle (ALB; *Anoplophora glabripennis* (Motschulsky)), was likely introduced to the United States in the 1980s but was not detected until the 1990s in New York City (Haack, 2006). Since that time, populations of ALB have been discovered in numerous cities in the United States, Canada, and Europe (Haack et al., 2010). Native to Asia, ALB preferentially feeds on *Populus* and *Acer* species, however it is highly polyphagous and can attack and kill dozens of hardwood species (Haack et al., 2010). Economic damage caused by ALB has the potential to exceed $900 billion dollars, but ALB population is currently contained in New York, Massachusetts, and Ohio, and several satellite infestations have even been eradicated (Nowak et al., 2001; Haack et al., 2010; Asian longhorned beetle,

2019). The emerald ash borer (EAB; *Agrilus planipennis* Fairmaire), a buprestid from Asia, is among the most destructive forest pests in the United States (Herms & McCullough, 2014). Originally introduced into Michigan in the 1990s, it has since spread to 35 US states and five Canadian provinces killing tens of millions of ash trees in its wake (Kovacs et al., 2010; Poland et al., 2015; Emerald ash borer, 2019). The cost of treating or removing and replacing urban ash trees within 25 US states over a ten-year period has been estimated to exceed $10.7 billion (Kovacs et al., 2010). All native ash species are susceptible to EAB, however EAB appears to prefer green and black ash (*Fraxinus pennsylvanica* Marshall and *F. nigra*, Marshall respectively) while blue ash (*F. quadrangulata* Michx.) is more resistant (Herms and McCullough, 2014; Poland et al., 2015). In some locations near the epicenter of infestations, EAB has killed 100% of ash trees and it is poised to functionally extirpate ash from North America (Herms & McCullough, 2014). Unlike ALB, the EAB population is continuing to grow rapidly and expand into new locations despite efforts to slow its spread (Herms & McCullough, 2014).

1.3 Strategies to Manage and Mitigate Losses to Invasive Woodborers

Management strategies to combat invasive woodborers fall into two broad categories: eradication and suppression, depending on the ultimate objective of the management program (Liebhold & Tobin, 2008; Liebhold & Kean, 2019). The choice of strategy is largely dictated by the size and distribution of the invasive population upon detection (Liebhold & Tobin, 2008; Brockerhoff et al., 2010; Liebhold & Kean, 2019). Eradication is the preferred option when attempting to protect a forest ecosystem because it involves eliminating a pest rather than just reducing the damage it causes (Liebhold et al., 2016). Another advantage to eradication is that it has the potential to eliminate a population while it is still small and before it causes widespread

damage (Liebhold et al., 2016). Successful eradication is predicated on early detection and a rapid response to an invasive species (Tobin et al., 2014). Eradication involves delimiting the area occupied by the population, containing all individuals using quarantines and exclusion zones, and eliminating all individuals of the invasive species within the designated area (Liebhold et al., 2016). One of the most commonly used and effective methods for eradicating woodborers is to remove and destroy all host trees – host trees surrounding the infestation are also generally removed to form an exclusion zone or "firebreak" to stop beetles from spreading (Brockerhoff et al., 2010; Tobin et al., 2014). Finally, the infested area is also quarantined to prevent humans from translocating infested material with extensive surveying and monitoring efforts to capture any escaping individuals (Liebhold & Kean, 2019). Unfortunately, eradication is not always achievable as evidenced by the EAB invasion (Herms & McCullough, 2014). Moreover, eradication is an expensive undertaking that can take several years to accomplish which requires considerable public education to overcome resistance to sometimes large-scale removal of trees (Liebhold et al., 2016; Liebhold & Kean, 2019). Eradication is often supplemented with insecticide applications, the release of biocontrol agents or use of pheromones for mating disruption within the infested area (Liebhold et al., 2016).

If a population cannot be eradicated, generally because the area occupied by the invader is too great for eradication to be attainable, then the goal shifts from elimination of the population to minimization of potential destruction (Liebhold & Kean, 2019). Suppression efforts are also expensive, though often less than eradication efforts on a per year basis (Liebhold & Kean, 2019). However, because they have no definite endpoint, suppression efforts can end up costing much more than a successful eradication (Liebhold & Kean, 2019). Like eradications, population suppression requires implementing quarantines and often utilizes exclusion zones, but

instead of destroying all suitable hosts, the population is managed through the use of biocontrol, trapping, sterile insect technique, planting resistant or non-host trees, or some combination of these tactics (Liebhold & Kean, 2019). Suppression also requires extensive surveying efforts both to monitor the pest population and to evaluate the efficacy of treatments and to ensure that the population does not spread beyond quarantined areas (Liebhold & Kean, 2019).

The most effective strategy to combat invasive woodborers is to prevent their entry into new environments (Leung et al., 2002; Wittenberg & Cock, 2005). However, because of the astronomical volumes of goods reaching ports-of-entry each day, it is impossible to inspect all packing material and dunnage for woodborers. In fact, only an estimated 2% of goods are inspected by the US Port Authority (Surkov et al., 2008). Because the exclusion of all exotic insects is unrealistic, the most efficient strategy to combat invasive species is to combine extensive monitoring efforts with timely management practices, referred to as "early detection, rapid response" (Wittenberg & Cock, 2005; Liebhold & Kean, 2019). In fact, the cost of management increases and the likelihood of eradication decreases as a population grows and the area it occupies expands (Wittenberg & Cock, 2005; Liebhold & Kean, 2019). Therefore, the probability of successful eradication and the overall costs of management can be optimized if a pest population is detected early (Wittenberg & Cock, 2005). To this end, the goal of early detection, rapid response is to quickly detect and delineate an exotic population of insects and enact management efforts while the population is still small and localized (Wittenberg & Cock, 2005).

Gaps in knowledge regarding the natural history of early forest invaders and a lack of tools to combat their introduction and spread resulted in a relatively large number of pests establishing in the United States (Aukema et al., 2010; Liebhold & Kean, 2019). For example,

when gypsy moth was introduced in the late 1800s, there were few options to combat this pest beyond manually removing insects and burning forests that were believed to be infested (Liebhold & Kean, 2019). There were no effective tools for monitoring or delimiting populations and effective pesticides and biocontrol were lacking (Liebhold & Kean, 2019). Consequently, gypsy moth spread for years without effective remediation and was therefore able to expand beyond the point where eradication was possible (Liebhold & Kean, 2019). Synthetic pheromone lures were first developed in the 1970s which enabled effective monitoring and delineation of pest insects in a cost-efficient manner (Bierl et al., 1970). In addition to semiochemical lures, numerous other advancements for combating invasive insects have become widely used including, aerial applications of pesticides, mating disruption, biocontrol, sterile insect technique, and government quarantines, survey teams, and educational campaigns to raise awareness of invasive insects (Cameron et al., 1974; Lee et al., 1996; Hajek & Tobin, 2010; Liebhold et al., 2016; Hajek & van Frankenhuyzen, 2017; Liebhold & Kean, 2019). However, despite these new advances, invasive insects still present a challenge to countries around the world.

1.4 Factors that Affect Success of Eradication Efforts

Eradication of woodborers often fails due to issues related to their natural history. Insecticides sprays are a common tool used to combat invasive folivores, however they are ineffective against woodborers because most of the lifecycle is spent as larvae protected under the bark (Poland et al., 2006). Rather, insecticides that target larvae must be delivered systemically through injection or root drenches, which is prohibitively expensive at the scale of an entire forest (Poland et al., 2006). Additionally, the cryptic lifecycle of woodborers, coupled with the fact that trees rarely show symptoms of damage until several years after the initial attack, provides incipient populations time to grow and spread before they are detected and

control efforts implemented (Liebhold et al., 2017; Liebhold & Kean, 2019). Moreover, exotic woodborers can be introduced into forests that are geographically removed from human populations and logistically difficult to access, further complicating detection efforts (Liebhold & Tobin, 2008). The monitoring requirement that accompanies eradication and containment efforts can be enormously expensive due to the labor needed to survey forests (Liebhold et al., 2016). Although effective traps paired with semiochemical lures can reduce the costs significantly, a workforce is required to service traps and process the captured insects (Liebhold et al., 2016). Quarantines can fail when infested material is transported beyond the boundaries of a quarantine zone (Liebhold & Kean, 2019). Moreover, management fails when the exclusion zone is not large enough to prevent the insects from naturally dispersing beyond this boundary and establishing satellite populations (Liebhold & Kean, 2019). Firebreaks are often employed but frequently fail if too narrow (Liebhold & Kean, 2019). Underestimation of the dispersal of an insect is generally due to poor knowledge of its ability to fly (Tobin et al., 2014). During the years soon after the initial EAB invasion, multiple firebreaks established to contain them proved ineffective (Herms & McCullough, 2014). In fact, 27 EAB eradications have been attempted, and 26 of them have failed (Liebhold & Kean, 2019).

1.5 Dispersal Ability of Invasive Woodborers

The most important factor in successfully eradicating an invasive insect is containment within the area under management and the most common reason eradications are unsuccessful is because quarantines and exclusion zones fail (Tobin et al. 2014; Liebhold et al., 2016; Liebhold & Kean, 2019). New technologies greatly assist our ability to control invasive species, however, eradication and monitoring efforts must be informed by knowledge of the dispersal ability of the insect. The primary reason the EAB eradication efforts have failed is because beetles have

routinely circumvented efforts to contain them (Herms & McCullough, 2014; Liebhold & Kean, 2019). The failure of containment can primarily be attributed to two factors: the lack of a highly effective semiochemical lure for many years (Silk et al., 2011) and misunderstanding of EAB dispersal ability (Lyttek et al., 2019). The lack of a lure made monitoring populations, especially small and cryptic satellite populations difficult, but of greater consequence for the eradication of EAB, was that its dispersal was routinely underestimated. Moreover, EAB was further spread through infested nursery stock and firewood, especially during the early stages of the infestation.

Human-mediated transportation of EAB undoubtedly increased the rate of spread across the country, but that is not the only way EAB disperses. Adult EAB are strong fliers and capable of dispersing over 800 linear meters, however the maximum distance and the proportion of individuals that disperse is unknown (Muirhead et al, 2006; Taylor et al., 2010; Herms & McCullough, 2014). EAB disperse in a pattern known as stratified dispersal (Muirhead et al, 2006; Liebhold & Tobin, 2008). In this model, a few EAB disperse long distances and establish satellite populations far beyond the boundaries of the initial population. These populations continue to expand outwards, eventually meeting and coalescing into one large population. These long-distance dispersal events include instances of transport by humans but can also include those that resulted from natural dispersal (Muirhead et al, 2006). The aggressive spread of EAB across the country has been driven by two factors: EAB has been highly successful in colonizing new locations after human transport and EAB are also strong fliers capable of naturally dispersing effectively (Muirhead et al, 2006).

Measuring the dispersal capability of woodborers has proven to be a challenge, in part due to a lack of tools that can be used to quantify their dispersal ability in the field (Mercader et al., 2016). In such studies, dispersal distance can be inferred from releasing a marked individual

and subsequently capturing it in a semiochemical baited traps some distance away (Fraser et al., 2007). A mark-release-recapture study to quantify EAB dispersal involved the release of several thousand EAB, but a lack of effective trapping methods prevented the researchers from being able to collect sufficient data (only 4% were recovered) to draw meaningful conclusions (Fraser et al., 2007). There are also logistical problems that hamper dispersal studies with woodborers. There are few established protocols for rearing them in a laboratory-based colony, therefore infested trees must be located, felled, cut into logs, and brought to the laboratory rearing facility for emergence. This process can be arduous depending on the size of the logs and distance they need to be transported. In addition, the costs of rearing substantial numbers of woodborers can dramatically increase due to the requirement for assistance from laboratory staff. Given the time, effort, and cost of rearing woodborers, limited budgets often mean funding is spent directly on management efforts rather than conducting mark-release-recapture experiments.

Flight mills have also been employed to estimate natural dispersal of EAB (Taylor et al., 2010), but unfortunately, they have limited biological relevance with regard to the natural dispersal ability of an insect (Herms & McCullough, 2014). Flight mills remove an insect from its natural environment and, thus, may not provide a realistic estimate of its dispersal ability in the field. Taylor et al. (2010), for example, found that gravid female EAB fly further than unmated females and male EAB generally fly further than females, but the linear distances these beetles flew cannot be extrapolated to dispersal in the field and provide limited information for delimiting population boundaries.

Multiple computer models of EAB spread have also been created to estimate and predict dispersal (BenDor et al., 2006; Muirhead et al., 2006, Siegert et al., 2015; Lyttek et al., 2019). These models were constructed from data related to various stages of the invasion and all have

differing levels of accuracy. A common source of data on insect spread used to inform models is county-level records of first detections. Models created at different times during the invasion can have different conclusions based on the size of the population and data available at that time. Another source of data for modeling is the dendrochronological record of the invasion (Siegert et al., 2014). These data were used to create an accurate model of EAB spread, however its creation required EAB to have been established for a long period of time and over a large geographic area for it to be informative. By the point in time the model was useful, EAB had had years to inflict damage and spread beyond the point of containment or eradication. Like the other models, its predictive power is weakest early in the invasion when that information would be most valuable.

Few approaches are capable of being used to quantify dispersal early in the stages of invasion soon after an insect is discovered, and several even require the insect to have already dispersed. It is critical to measure dispersal as early as possible because eradication of a small population is both a less expensive endeavor and more likely to succeed. The most effective method used to date to model EAB dispersal has utilized an extensive survey conducted in the field to inform the model (Mercader et al., 2016). This colossal effort involved cutting down and debarking infested trees over several years to count larval populations in order to measure EAB dispersal. This strategy provided a highly accurate estimate of EAB dispersal – a dispersal distance of approximately two km per year. However, measuring dispersal in this way is expensive, labor intensive, and time consuming, and it is unknown the extent to which the destructive sampling of ash trees affected the dispersal of the observed population. A far better method would involve directly measuring the dispersal of adults over the course of a single generation.

1.6 Integrating Estimates of Dispersal into Management Efforts

Understanding insect dispersal is critical for monitoring efforts when detecting cryptic populations, delineating infested areas, and estimating population densities (Liebhold & Tobin, 2008; Mercader et al., 2016; Liebhold et al., 2016). Knowledge of dispersal ability also informs trap placement when establishing quarantine and exclusion zone boundaries for invasive insects. This information also informs management efforts such as insecticide applications, mating disruption, natural enemy releases, and mass-trapping efforts to locations that optimize their efficacy (Brockerhoff et al., 2010; Tobin et al., 2014; Liebhold et al., 2016).

If EAB dispersal had been better understood many of the failures that occurred in the early years of the North American invasion may have been avoided. For instance, if the natural dispersal ability of EAB was accurately quantified, an adequate firebreak could have been implemented to contain the beetles inside the relatively small geographical area to which they were introduced. Even the satellite populations that spread through human transport could have been contained if discovered early enough to enforce quarantines and erect firebreaks. Once contained, the populations could then be effectively suppressed or eradicated. Now that a synthetic lure enables enhanced monitoring of EAB, knowledge of their dispersal can inform trap placement to effectively survey for incipient populations. With the combination of attractive lures and knowledge of EAB dispersal, a slow-the-spread campaign as well as the eradication of satellite populations is now possible.

1.7 Mark Capture Techniques for Measuring Dispersal

Traditionally, dispersal studies have been conducted through mark-release-recapture, a technique that requires insects to be reared or collected in mass, marked, released into the environment, and then recaptured (Hagler & Jackson, 2001; Hagler 2019). There are numerous

options for marks including fluorescent dusts, paints, tags, hydrocarbons, mutilation (e.g. elytra clipping), radioactive isotopes (Hagler & Jackson, 2001). Several of these options, such as tags, allow the identity of individual insects to be known after recapture, though they require more time for application and the development of unique numbering or code systems than a broadcast marker (Hagler & Jackson, 2001). Other marks can be applied rapidly but do not allow individual identification because all insects receive the same mark (Hagler & Jackson, 2001). However, the major drawback to these techniques is they all require insects to be reared or collected prior to marking. Rearing or collecting insects for marking can be expensive and time-consuming as well as requiring space and facilities for maintaining a colony. Rearing woodboring beetles can be especially labor intensive due to the need to collect large numbers of logs. Finally, human handling of insects is known to affect behavior and could bias studies of dispersal ability (Taylor et al., 2010).

An alternative to mark-release-recapture studies is to mark insects directly in the field, a technique described as mark-capture (Hagler, 2019). A marking technique called immunomarking makes this a feasible option (Hagler, 1997). Immunomarking involves applying a vertebrate protein onto an insect for later detection by ELISA (enzyme-linked immunosorbent assay). The first instance of immunomarking used chemical reagents like bovine albumen serum as the marking protein, however new ELISAs have been created that allow inexpensive proteins such as chicken ovalbumin (egg whites) to be used instead (Hagler, 2019). This more economic option allows researchers to broadly apply the protein in the environment where the insect of interest can encounter it and mark itself (Hagler et al., 2004). Mark-capture removes the need to collect or rear insects before the study and also preserves natural behaviors as there is no human contact until capture at the end of the experiment. This technique has been effective in

understanding dispersal of insects within traditional agricultural systems and could be used to study woodborer dispersal as well (Hagler & Naranjo, 1997; Jones et al., 2006; Boina et al., 2009; Sivakoff et al., 2012; Hagler, 2019). By applying the protein to the bark of a tree, an emerging insect will self-mark by acquiring the protein before dispersing. The insect can later be captured and the distance from the marked tree can be easily measured, which informs the researcher that the insect can disperse at least as far as the location of collection.

Protein self-marking would not only reduce the time, effort, and costs of quantifying woodborer dispersal as well as preserve natural dispersal habits of the insect, but also provide reliable data that can be immediately utilized to combat the invasion (Hagler, 2019). It can inform governments and municipalities about quarantines and firebreaks and will inform researchers of trap placement for monitoring efforts. This technique would allow governments to create effective quarantines rather than boundaries that are either not based on dispersal data or on potentially misleading data from flight mill studies. Moreover, protein self-marking would allow the quantification of dispersal immediately upon discovery rather than years after the insect has established as when using historical data to create models. In addition, most of what is known about on insect dispersal is from limited locations, so this technique can be rapidly applied to populations if discovered in a new environment to quantify the extent to which a novel landscape and climate impact dispersal.

The central objective of the present study is to evaluate the potential for using immunoproteins applied directly to woodborer-infested trees to mark emerging beetles. I tested the hypothesis that ovalbumin (egg whites) is an effective and durable means of self-marking woodboring beetles. Specifically, in the first experiment we sprayed varying concentrations of ovalbumin solution directly onto logs infested with EAB and an ELISA was used to detect the

presence of the protein on emerged beetles. To evaluate the persistence of the mark, we applied varying concentrations of ovalbumin to freeze-killed beetles, mounted them on pins, and placed them in an exposed location outdoors. This study provides a novel technique to quantify dispersal of woodborers and the results can be applied to enhance the management of invasive forest pests.

1.8 References

Asian longhorned beetle, July 2019, USDA APHIS, viewed 2 July 2019, <https://www.aphis.usda.gov/aphis/resources/pests-diseases/asian-longhorned-beetle>.

Aukema JE, McCullough DG, Holle BV, Liebhold AM, Britton K & Frankel SJ (2010) Historical accumulation of nonindigenous forest pests in the continental United States. BioScience 60:886-897.

Aukema, JE, Leung B, Kovacs K, Chivers C, Britton KO, Englin J, Frankel SJ, Haigh RG, Holmes TP, Liebhold AM & McCullough DG (2011) Economic impacts of non-native forest insects in the continental United States. PLOS One 6:e24587.

BenDor TK, Metcalf SS, Fontenot LE, Sangunett B & Hannon B (2006) Modeling the spread of the emerald ash borer. Ecological Modelling 197:221-236.

Bierl BA, Beroza1 M & Collier CW (1970) Potent sex attractant of the gypsy moth: Its isolation, identification, and synthesis. Science 170:87-89.

Boina DR, Meyer WL, Onagbola EO & Stelinski LL (2009) Quantifying dispersal of *Diaphorina citri* (Hemiptera: Psyllidae) by immunomarking and potential impact of unmanaged groves on commercial citrus management. Environmental Entomology 38:1250-1258.

Boyd IL, Freer-Smith PH, Gilligan CA & Godfray HCJ (2013) The consequence of tree pests and diseases for ecosystem services. Science 342:1235773.

Brockerhoff EG, Bain J, Kimberley M & Knížek M (2006) Interception frequency of exotic bark and ambrosia beetles (Coleoptera: Scolytinae) and relationship with establishment in New Zealand and worldwide. Canadian Journal of Forest Research 36:289-298.

Brockerhoff EG, Liebhold AM, Richardson B & Suckling DM (2010) Eradication of invasive forest insects: concepts, methods, costs and benefits. New Zealand Journal of Forestry Science 40:S117-S135.

Cameron EA, Schwalbe CP, Beroza M & Knipling EF (1974) Disruption of gypsy moth mating with microencapsulated disparlure. Science 183:972-973.

Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O'Neill RV, Paruelo J, Raskin RG, Sutton P & van den Belt M (1997) The value of the world's ecosystem services and natural capital. Nature 387:253-260.

Dale VH, Joyce LA, McNulty S, Neilson RP, Ayres MP, Flannigan MD, Hanson PJ, Irland LC, Lugo AE, Peterson CJ, Simberloff D, Swanson FJ, Stocks BJ & Wotton BM (2001) Climate change and forest disturbances: Climate change can affect forests by altering the frequency, intensity, duration, and timing of fire, drought, introduced species, insect and pathogen outbreaks, hurricanes, windstorms, ice storms, or landslides. BioScience 51:723-734.

Early R, Bradley BA, Dukes JS, Lawler JJ, Olden JD, Blumenthal DM, Gonzalez P, Grosholz ED, Ibañez I, Miller LP, Sorte CJB & Tatem AJ (2016) Global threats from invasive alien species in the twenty-first century and national response capacities. Nature Communications 7:e12485.

Emerald Ash Borer, July 2019, USDA APHIS, viewed 2 July 2019, <https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases/emerald-ash-borer>.

Follet PA & Neven LG (2006) Current trends in quarantine entomology. Annual Review of Entomology 51:359-385.

Food and Agriculture Organization of the United Nations (FAO), Global Forest Resource Assessment 2015 (Second Edition) (FAO, Rome, 2015).

Fraedrich SW, Harrington TC, Rabaglia RJ, Ulyshen MD, Mayfield AE III, Hanula JL, Eickwort JM & Miller DR (2008) A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. Plant Disease 92:215-224.

Fraser I, Mastro VC & Lance DR (2007) 'Emerald ash borer dispersal-a release and recapture study' In Gottschalk KW (ed.), Proceedings, 17th US Department of Agriculture interagency research forum on gypsy moth and other invasive species 2006; Gen. Tech. Rep. NRS-P-10. Newtown Square, PA: US Department of Agriculture, Forest Service, Northern Research Station: 42.

Haack RA (2006) Exotic bark- and wood-boring Coleoptera in the United States: recent establishments and interceptions. Canadian Journal of Forest Research 36:269-288.

Haack RA, Britton KO, Brockerhoff EG, Cavey JF, Garrett LJ, Kimberley M, Lowenstein F, Nuding A, Olson LJ, Turner J & Vasilaky KN (2014) Effectiveness of the international phytosanitary standard ISPM No. 15 on reducing wood borer infestation rates in wood packaging material entering the United States. PLOS One 9:e96611.

Haack RA, Hérard F, Sun J & Turgeon JJ (2010) Managing invasive populations of Asian longhorned beetle and citrus longhorned beetle: A worldwide perspective. Annual Review of Entomology 55:521-546.

Hagler JR & Jackson CG (2001) Methods for marking insects: current techniques and future prospects. Annual Review of Entomology 46511-543.

Hagler JR & Naranjo SE (1997) Measuring the sensitivity of an indirect predator gut content ELISA: detectability of prey remains in relation to predator species, temperature, time, and meal size. Biological Control 9:112-119.

Hagler JR (1997) Field retention of a novel mark–release–recapture method. Environmental Entomology 26:1079-1086.

Hagler JR (2019) Super mark it! A review of the protein immunomarking technique. Annals of the Entomological Society of America 112:200-210.

Hagler JR, Blackmer JL, Henneberry TJ, Daane K, Groves RL & Jones VP (2004) Quantifying landscape-scale movement patterns of glassy-winged sharpshooter and its natural enemies using a novel mark-capture technique. CDFA Pierce's Disease Control Program Research Symposium 256-259.

Hajek AE & Tobin PC (2010) Micro-managing arthropod invasions: eradication and control of invasive arthropods with microbes. Biological Invasions 12:2895-2912.

Hajek AE & van Frankenhuyzen K (2017) 'Use of entomopathogens against forest pests' In Lacey LA (ed.), Microbial Control of Insect and Mite Pests, Academic Press, San Diego, pp.313-330.

Herms DA & McCullough DG (2014) Emerald ash borer invasion of North America: History, biology, ecology, impacts, and management. Annual Review of Entomology 59:13-30.

Jones VP, Hagler JR, Brunner J, Baker C & Wilburn T (2006) An inexpensive immunomarking technique for studying movement patterns of naturally occurring insect populations. Environmental Entomology 35:827-836.

Kovacs KF, Haight RG, McCullough DG, Mercader RJ, Siegert NW & Liebhold AM (2010) Cost of potential emerald ash borer damage in US communities, 2009–2019. Ecological Economics 69:569-578.

Kurz WA, Dymond CC, Stinson G, Rampley GJ, Neilson ET, Carroll AL, Ebata T & Safranyik L (2008) Mountain pine beetle and forest carbon feedback to climate change. Nature 452:987-990.

Laurance WF (2015) Emerging threats to tropical forests. Annals of the Missouri Botanical Garden 100:159-169.

Lee MK, Curtiss A, Alcantara E & Dean DH (1996) Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar*. Applied and Environmental Microbiology 62:583-586.

Leung B, Lodge DM, Finnoff D, Shogren JF, Lewis MA & Lamberti G (2002) An ounce of prevention or a pound of cure: Bioeconomic risk analysis of invasive species. Proceedings of the Royal Society of London B - Biological Sciences. 269:2407-2413.

Levine JM & D'Antonio CM (2003) Forecasting biological invasions with increasing international trade. Conservation Biology 17:322-326.

Levine JM (2008) Biological invasions. Current Biology 18:57-60.

Liebhold AM & Kean JM (2019) Eradication and containment of non-native forest insects: Successes and failures. Journal of Pest Science 92:83-91.

Liebhold AM & Tobin PC (2008) Population ecology of insect invasions and their management. Annual Review of Entomology 53:387-408.

Liebhold AM, Berec L, Brockerhoff EG, Epanchin-Niell RS, Hastings A, Herms DA, Kean JM, McCullough DG, Suckling DM, Tobin PC & Yamanaka T (2016) Eradication of invading insect populations: from concepts to applications. Annual Review of Entomology 61:335-352.

Liebhold AM, Brockerhoff EG, Kalisz S, Nuñez MA, Wardle DA & Wingfield MJ (2017) Biological invasions in forest ecosystems. Biological Invasions 19:3437-3458.

Lyttek E, Lal P, Nieddu G, Forgoston E & Wieczerak T (2019) Modeling *Agrilus planipennis* F. (Coleoptera: Buprestidae) spread in New Jersey. Journal of Economic Entomology doi.org/10.1093/jee/toz122.

Mercader RJ, McCullough DG, Storer AJ, Bedford JM, Heyd R, Siegert NW, Katovich S & Poland TM (2016) Estimating local spread of recently established emerald ash borer, *Agrilus planipennis*, infestations and the potential to influence it with a systemic insecticide and girdled ash trees. Forest Ecology and Management 366:87-97.

Millar CI & Stephenson NL (2015) Temperate forest health in an era of emerging megadisturbance. Science 349:823-826.

Muirhead JR, Leung B, van Overdijk C, Kelly DW, Nandakumar K, Marchant KR & MacIsaac HJ (2006) Modelling local and long-distance dispersal of invasive emerald ash borer *Agrilus planipennis* (Coleoptera) in North America. Diversity and Distributions 12:71-79.

Nowak DJ, Pasek JE, Sequeira RA, Crane DE & Mastro VC (2001) Potential effect of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) on urban trees in the United States. Journal of Economic Entomology 94:116-122.

Paap T, de Beer ZW, Migliorini D, Nel W & Wingfield MJ (2018) The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. Australasian Plant Pathology 47:231-237.

Pimentel D, Lach L, Ziniga R & Morrison D (2002) 'Environmental and economic costs of alien arthropods and other organisms in the United States' In Hallman GJ & Schwalbe CP (eds.) Invasive arthropods in agriculture: Problems and solutions. Science Publishers, Enfield, NH, pp.107-117.

Poland TM, Chen Y, Koch J & Pureswaran D (2015) Review of the emerald ash borer (Coleoptera: Buprestidae), life history, mating behaviours, host plant selection, and host resistance. The Canadian Entomologist 147:252-262.

Poland TM, Haack RA, Petrice TR, Miller DL, Bauer LS & Gao R (2006) Field evaluations of systemic insecticides for control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) in China. Journal of Economic Entomology 99:383-392.

Roques A (2010) Alien forest insects in a warmer world and a globalised economy: Impacts of changes in trade, tourism and climate on forest biosecurity. New Zealand Journal of Forestry Science 40:S77-S94.

Siegert NW, McCullough DG, Liebhold AM & Telewski FW (2014) Dendrochronological reconstruction of the epicenter and early spread of emerald ash borer in North America. Diversity and Distributions 20:847-858.

Siegert NW, Mercader RJ & McCullough DG (2015) Spread and dispersal of emerald ash borer (Coleoptera: Buprestidae): estimating the spatial dynamics of a difficult-to-detect invasive forest pest. The Canadian Entomologist 147:338-348.

Silk PJ, Ryall K, Mayo P, Lemay M, Grant G, Crook D, Cossé A, Fraser I, Sweeney JD, Lyons DB, Pitt D, Scarr T, & Magee D (2011) Evidence for a volatile pheromone in *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) that increases attraction to a host foliar volatile. Environmental Entomology 40:904-916.

Sivakoff FS, Rosenheim JA & Hagler JR (2012) Relative dispersal ability of a key agricultural pest and its predators in an annual agroecosystem. Biological Control 63:296-303.

Surkov IV, Lansink A, Kooten O & der Werf W (2008) A model of optimal import phytosanitary inspection under capacity constraint. Agricultural Economics 38:363- 373.

Taylor RA, Bauer LS, Poland TM & Windell KN (2010) Flight performance of *Agrilus planipennis* (Coleoptera: Buprestidae) on a flight mill and in free flight. Journal of Insect Behavior 23:128-148.

Tobin PC, Kean JM, Suckling DM, McCullough DG, Herms D & Stringer LD (2014) Determinants of successful arthropod eradication programs. Biological Invasions 16: 401-414.

Turchin P, Lorio PL, Taylor AD & Billings RF (1991) Why do populations of southern pine beetles (Coleoptera: Scolytidae) fluctuate? Environmental Entomology. 20:401-409.

Wittenberg R & Cock MJW (2005) ‘Best practices for the prevention and management of invasive alien species’ In Mooney HA, Mack RN, McNeely JA, Neville LE, Schei PJ & Waage JK (eds.), Invasive Alien Species: A New Synthesis Island Press, Washington D.C., pp.209-232.

Wu Y, Trepanowski NF, Molongoski JJ, Reagel PF, Lingafelter SW, Nadel H, Myers SW & Ray AM (2017) Identification of wood-boring beetles (Cerambycidae and Buprestidae) intercepted in trade-associated solid wood packaging material using DNA barcoding and morphology. Scientific Reports 7:e40316.

CHAPTER 2: PROTEIN SELF-MARKING BY EMERALD ASH BORER: AN EVALUATION OF EFFICACY AND PERSISTENCE

2.1 Abstract

Understanding the dispersal ability of invasive insects provides useful insights for developing effective management strategies. Historically, methods for marking insects for dispersal studies have been expensive, time-consuming, and labor-intensive, especially for woodboring beetles. In addition, capturing or rearing insects requires human handling, which can alter behavior. Immunomarking is a well-established technique for studying the dispersal of insects, however, it has not been broadly applied to woodborers. This study evaluates the potential for using immunoproteins applied directly to woodborer-infested trees to mark emerging beetles. Specifically, in the first experiment I sprayed varying concentrations of ovalbumin (egg white) solution directly onto logs infested with emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) (Buprestidae: Agrilini) and ELISA was used to detect the presence of protein on emerged beetles. To test the persistence of the mark, I applied varying concentrations of albumin to freeze-killed beetles, mounted them on pins, and placed them in an exposed location outdoors. Adult EAB self-marked as they emerged from protein-treated trees, with higher protein concentrations persisting for longer on the cuticle when exposed to sun and rain. This technique offers a convenient, inexpensive and durable means of marking woodborers and circumvents the need for human handling, allowing for more natural behavior and more realistic estimates of dispersal. Protein self-marking marking may find application in studies of woodborer dispersal within natural forest environments.

2.2 Introduction

Woodboring beetles, especially invasive species, are among the most important pests to threaten the health and productivity of forests worldwide (Muirhead et al., 2006; Holmes et al., 2009; Haack et al., 2010; Kovacs et al., 2010). Recent introductions of destructive species such as the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), into North America, and the translocation of the Arizona (USA) native goldspotted oak borer, *Agrilus auroguttatus* Schaeffer (Coleoptera: Buprestidae) into California (USA) illustrate the need to effectively manage incipient populations before they become established (Haack, 2006; Haack et al., 2010; Kovacs et al., 2010; Hishinuma et al., 2011; Coleman et al., 2012). Invasive woodborers are typically managed through a multifaceted effort consisting of eradication of small localized populations, containment of spread, and population-level suppression strategies (Liebhold & Tobin, 2008). The success of these approaches is dependent on understanding the biological characteristics and geographical distribution of the pest within an invaded area (Muirhead et al., 2006; Poland & McCullough, 2006; Haack et al., 2010). An understanding of dispersal patterns of invasive species can inform management efforts by predicting rates of spread, factors affecting dispersal, and risks associated with the establishment of a particular pest.

Studies aimed at understanding long-distance and within-stand dispersal characteristics of woodboring pests employ a variety of techniques, including computerized flight mills, harmonic radar, mark-release-recapture, and destructive sampling of host trees surrounding outlier infestations (Smith et al., 2004; Williams et al., 2004; Siegert et al., 2010; Taylor et al., 2010; Lopez et al., 2014). Within-stand dispersal of woodborers is often measured using mark-release-recapture techniques that require mass collection of insects for marking (typically with paints,

dyes, dusts, hydrocarbons, or elemental marks) and subsequent release back into the environment. Marked insects are then recaptured at specified distances from the release point over time (Zumr, 1992; Turchin & Thoeny, 1993; Smith et al., 2001; Ginzel & Hanks, 2002). This approach is best suited to species that are relatively abundant and easy to collect or inexpensive to rear in large numbers. Alternatively, mark-capture is an approach for investigating within-stand dispersal and involves directly marking individuals of a naturally occurring insect population in the field and then measuring their dispersal from the point of marking to the point where they are captured (Hagler & Jackson, 2001). This approach has advantages over mark-release-recapture techniques because it circumvents the need for capturing or rearing large numbers of insects. However, the greatest benefit of the mark-capture approach over mark-release-recapture is that it does not require handling of test subjects before capture, which can potentially influence dispersal behavior (Taylor et al., 2010).

Immunomarking, a technique that uses vertebrate derived proteins (e.g., ovalbumin and bovine casein) as markers for invertebrates, is well suited for use in mark-capture studies (Jones et al., 2006; Hagler & Jones, 2010). These markers can be easily applied to the insect cuticle, incorporated into insect diet to be ingested, or applied to various surfaces in the environment, which enables insects to mark themselves when they contact the substrate (Hagler, 2019). Protein markers are cost-effective, easy to apply, durable, reliable to detect, and inexpensive to analyze by an enzyme-linked immunosorbent assay (ELISA). Protein-based markers have been used in numerous mark-capture studies evaluating field dispersal of insect pests in agricultural systems (Jones et al., 2006; Boina et al., 2009; Horton et al., 2009; Basoalto et al., 2010; Hagler et al., 2011; Krugner et al., 2012; Sivakoff et al., 2012; Swezey et al., 2013, 2014; Peck et al., 2014). However, no studies to date have evaluated the utility of mark-capture immunomarking to

characterize dispersal abilities of woodborers. Protein marking techniques can be especially useful for understanding woodborer dispersal within natural areas because they are non-toxic, inexpensive, readily available, do not require special permits for use, and direct application to a substrate in the field eliminates the need for mass acquisition of study individuals, typically a major obstacle to research on many woodborers (Hagler & Jackson, 2001; Slosky et al., 2012; Hagler, 2019).

In this study, I investigate the extent to which woodborers self-mark when emerging from immunoprotein treated trees. This approach involved two experiments which investigated: 1) the efficacy with which adult beetles acquire and retain a protein mark obtained by emerging from infested host material which had been directly sprayed with ovalbumin, and 2) the long-term persistence of ovalbumin adhering to insect cuticle when fully exposed to sun and rain. Information gained from this study can be applied to provide a realistic assessment of woodborer dispersal in order to inform management efforts and reduce their negative impacts on forest ecosystems.

2.3 Materials and Methods

2.3.1 Experiment 1 – Investigating ovalbumin acquisition through emergence from protein treated logs

Between March and May 2017, I felled approximately 30 white and green ash trees (*Fraxinus americana* L. and *F. pennsylvanica* Marshall), and cut boles (≤28 cm diameter) into approximately ≤72 cm long bolts. Trees were located at Purdue University Richard G. Lugar Forestry Farm (40.4223543, -86.9649226, 203 m elevation) and a privately-owned woodlot (40.5041460, -86.9005280, 202 m elevation) in Tippecanoe County, IN, USA, and each displayed signs and symptoms of EAB infestation. Emerald ash borer was selected for these

experiments because it is common in the study area and there are established rearing protocols (Stack et al., 2019). Bolts were then held in cold storage (4°C) to keep beetles in diapause until the beginning of experiments.

In June 2017, I removed bolts from cold storage and adult beetles emerged from logs approximately 14 days later. Prior to EAB emergence, bolts were randomly divided into four treatment groups with approximately 20 logs per treatment. Chicken ovalbumin was selected as the protein marker because it is relatively inexpensive (~$5/L of egg whites vs ~$2300/L of rabbit IgG (Slosky et al., 2012)), available at local grocery stores, persistent in the environment, and can be reliably detected by ELISA (Jones et al., 2006; Hagler et al., 2010, 2014; Slosky et al., 2012). Treatments consisted of a water control (0% v/v egg whites) and three concentrations of chicken ovalbumin (10%, 50% and 100% v/v egg whites) prepared by combining ovalbumin (AllWhites® 100% liquid egg whites; Michaels Foods, Minnetonka, MN, USA) with deionized water. Treatment solutions were applied onto the surface of each log approximately 3-4 days before anticipated EAB emergence using a 1 L hand sprayer at the rate of 1 mL/ 5 cm^2 to ensure even coverage. To reduce risk of cross contamination between different concentrations of ovalbumin, logs were sprayed at separate locations. Treatments were allowed to dry overnight before transporting logs to the laboratory at Purdue University (West Lafayette, IN, USA).

Marked logs were placed into capped 30 cm diameter by 75 cm long cardboard tubes with a clear plastic collection cup affixed to one end. Logs were stored at 21°C and 55% r.h. on a L15:D9 cycle. I checked tubes daily, collected all emerged EAB (sex was not determined), and placed them into individual cages. Cages were constructed from 16 oz. (~473 mL) clear plastic cups (Solo®, Dart Container Corporation, Mason, MI, USA) with a 15x15 cm square of plastic window screen held in place over the opening by a rubber band. To reduce cross contamination

between collected insects, I handled emerged beetles only using forceps washed with 95% ethanol before and after each use. Emerged beetles were caged individually in separate containers to prevent marked beetles from transferring protein to unmarked beetles. Beetles were provisioned with white or green ash leaves and water in 10-dram (37 mL) vials *ad lib.* until they died. Cages were labeled by treatment and emergence date and kept inside a greenhouse. I checked cages for dead EAB daily, are replaced leaves and water every 3-5 days or as needed. All expired EAB were removed from their cages, using alcohol washed forceps as above. Beetles were placed individually into labeled microcentrifuge tubes and immediately frozen at -20°C until ELISAs were performed.

2.3.2 Experiment 2 – Testing ovalbumin persistence after exposure in the field

To test the persistence of ovalbumin on beetle cuticle, I captured live adult EAB using unbaited green 12-funnel Lindgren funnel traps with dry collection cups (ChemTica Internacional, Heredia, Costa Rica). Traps were deployed from May to July 2017 at the same locations infested trees were harvested for Experiment 1 (see coordinates above) and serviced twice per week. All EAB were transported to Purdue University, freeze-killed, and stored in a single plastic container at -20°C until the beginning of the experiment. Field-captured EAB (n=280) were divided into four treatment groups of 70 beetles each. A 0%, 1%, 5%, or 100% v/v concentration ovalbumin solution was sprayed (prepared as above and using same sprayers) directly onto the cuticle of beetles. The amount of ovalbumin that adheres to beetles during self-marking has not been quantified and likely varies considerably depending on many factors including rate of application, emergence location, and time spent on log (pers. obs.). Therefore, I applied ovalbumin directly to the cuticle instead of logs to ensure that the amount of protein was consistent across beetles within treatments. The highly dilute concentrations of 1% v/v and 5%

v/v were selected to approximate the small amount of ovalbumin an emerging insect might acquire as it bores out of the host tree. These concentrations applied directly to the cuticle may not reflect the amount of ovalbumin an insect acquires through emergence from marked material, but it demonstrates the persistence of extremely small amounts of ovalbumin on cuticle over a gradient of concentrations. The 100% v/v concentration served as the positive control and the 0% v/v concentration was the negative control. Beetles were coated evenly with respective treatment concentrations, then pinned to four quadrants of a cork board (3 x 5 feet) which was placed outside (40.427804, -86.948846, 204 m elevation) where beetles had full exposure to sunlight and rain from June 29, 2017 to August 10, 2017. Once per week (days 0, 7, 14, 21, 28, 35, and 42) for six weeks, I randomly removed 10 EAB from each treatment and placed them into labeled microcentrifuge tubes using forceps washed with 95% ethanol after each use. Local weather measurements were acquired from Weather Underground (Weather Report Purdue University, IN July 2017). After collection, EAB were stored frozen at -20°C until ELISAs were performed.

2.3.3 Protein ELISAs

A total of 484 adult EAB emerged from marked logs and were used in the first experiment: 137 from 100% v/v ovalbumin treated logs, 125 from 50% v/v ovalbumin treated logs, 152 from 10% v/v ovalbumin treated logs, and 70 from 0% v/v ovalbumin treated logs. Beetles were shipped frozen in individual 100 µL microcentrifuge tubes to the U.S. Arid Land Agricultural Research Center (Maricopa, Arizona, USA) for ELISAs. Two different ELISAs (indirect and sandwich) were performed on each beetle to compare the ability of these techniques to detect trace amounts of ovalbumin on woodborers. Indirect ELISAs are excellent for detecting ovalbumin on insect cuticle, while sandwich ELISAs are generally best at detecting ingested

ovalbumin in the gut (Hagler et al., 1992; Hagler, 1997, 2019; Jones et al., 2006). All ELISAs were performed according to protocols reported in Hagler (2019). Every beetle was first rinsed in buffer to remove ovalbumin on the cuticle and these washes were analyzed by indirect ELISA. Then, each beetle was placed into a new aliquot of buffer and pulverized using a disposable pipette to homogenize the sample and release ingested ovalbumin from the gut. These pulverized samples were then tested by sandwich ELISA. In addition, 160 pulverized samples were tested for the presence of ovalbumin using an indirect ELISA. After 160 samples were tested, it became apparent that the indirect ELISA was ineffective, and this method was abandoned. For the persistence study, only indirect ELISAs were performed on whole beetles. Finally, an additional 88 unmarked EAB were acquired from the USDA APHIS EAB Biological Control Facility (Brighton, MI, USA) and were tested for the presence of endogenous proteins which may cause a color change in an indirect ELISA.

We quantified ovalbumin concentration in each sample by measuring the optical density (i.e., light scattering caused by the presence of reactive proteins) using a 96 well plate reader (SPECTRAmax^{TM} 250; Molecular Devices, Sunnyvale, CA, USA) and converted optical density scores to binary data. Samples were scored for the presence or absence of ovalbumin based on a threshold calculated as three standard deviations above the mean optical density for negative controls (i.e., non-marked beetles, n=8/plate), as described in Hagler (1997). Eleven 96 well plates were required to accommodate all samples used in these two experiments and positive thresholds were calculated separately for each plate to account for differences in well-plate density (Table 1).

2.3.4 Statistical analysis

To determine the most effective method for detecting ovalbumin, I compared the proportion of marked beetles between ELISAs using a Pearson's chi-squared test followed by pairwise comparisons between the three methods. The indirect ELISA performed on whole beetles was found to be the most effective at detecting marked beetles and subsequent statistical analyses were performed using data from the indirect ELISA with whole beetles only. Mean optical densities were calculated for marked and unmarked beetles from each treatment. These data were not normally distributed nor were variances homogenous; therefore, a non-parametric one-way Kruskal-Wallis test was used to compare the effect of ovalbumin concentration on median optical density scores. Pairwise comparisons were performed using a Dunn's test. The proportion of positive beetles between treatments was compared with a Pearson's chi-squared test followed by pairwise chi-squared independence tests between all treatment combinations. In order to test mark retention over the course of the study, I compared the log odds ratios of detection of three ovalbumin concentrations against the water control with a logistic regression using a logit link function. The model used the number of positive and negative scoring beetles as the binary response variable, with treatment concentration used as a categorical predictor variable and number of days since mark application (i.e., emergence) used as a continuous predictor variable. The interaction effect was not significant and therefore removed from the model. Two *a priori* contrasts were conducted between the log odds ratios of the 10% v/v ovalbumin and 50% v/v ovalbumin treatments, and the 50% v/v and 100% v/v treatments. All analyses were repeated using the data from samples used in the persistence study. All analyses were conducted in RStudio (version 3.5.1) using an alpha value of 0.05. R script is available upon request.

2.4 Results

2.4.1 Experiment 1 – Investigating ovalbumin acquisition through emergence from protein treated logs

These experiments demonstrate that EAB acquire ovalbumin as they emerge from protein treated logs, suggesting that immunomarking can be an effective tool for field-marking woodborers for dispersal research. The indirect ELISA on whole beetles outperformed both the sandwich and indirect ELISAs on pulverized beetles. Across all treatment concentrations, 51% (249 of 484) of whole beetle samples tested positive for the presence of ovalbumin using the indirect ELISA, 4% (19 of 484) of ground samples tested positive for ovalbumin using the sandwich ELISA, and 5% (8 of 160) of ground samples tested positive for ovalbumin using the indirect ELISA. Pairwise comparisons indicate that the indirect ELISA using whole samples was significantly more reliable at detecting ovalbumin at any concentration than either indirect or sandwich ELISAs using pulverized samples (χ^2 (2) = 333.93, $p < 0.001$). Therefore, results presented below represent analyses performed only on data from whole beetle indirect ELISAs.

Marked beetles were detected in all treatments including two from the control, but higher proportions of marked beetles were recovered from higher ovalbumin concentrations (Table 2). Optical density scores and proportions of marked beetles both differed between treatments (χ^2 (3) = 168.23, $p < 0.001$ and $\chi^2(3) = 149.43$, $p < 0.001$, respectively) and pairwise comparisons showed both measures correlated with higher ovalbumin concentration (i.e., 100%>50%>10%>0% v/v, all Bonferroni-adjusted p-values < 0.001) (Figures 2.1-2.4). Ovalbumin was detected on approximately 85% of all beetles from the 100% v/v treatment, 62% of beetles from the 50% v/v treatment, and only 35% of beetles from the 10% v/v treatment. The mean optical density score for unmarked beetles from all treatments was 0.043 ± <0.001. Mean

optical density scores for the marked beetles from the 100% v/v treatment was ~3x higher, the 50% v/v treatment was ~2x higher, and for the 10% v/v treatment ~4x higher than unmarked beetles (Table 2).

As expected, more ovalbumin marks were detected as the concentration of applied ovalbumin increased (Table 2). The 100% v/v ovalbumin treatment resulted in the highest frequency of detection across the life of the marked beetles, and each successively lower concentration corresponded with lower frequencies of detection throughout the study period (Figure 2.5). The odds of ovalbumin detection for a beetle that emerged from logs in the 10% v/v treatment was 38 times higher than the control, 119 times higher than control for 50% v/v treatment, and 432 times higher for the 100% v/v treatment (Table 3). The odds of detecting ovalbumin decreased by approximately 0.89 each day, regardless of treatment concentration. Contrasts revealed a significant difference in odds of detection between the 10% v/v and 50% v/v ($z = 4.16$, $p < 0.001$) ovalbumin treatments and between the 50% v/v and 100% v/v ($z = 4.11$, $p < 0.001$) treatments.

2.4.2 Experiment 2 – Testing ovalbumin persistence after exposure in the field

We recovered 272 of the original 280 EAB used in this experiment. Four beetles were lost from the 100% v/v, 1 from the 1% v/v, and 3 from the 0% v/v concentrations. These missing beetles were likely dislodged from pins by scavengers or extreme weather. Both optical density scores and proportion of marked beetles differed between treatments (χ^2 (3) = 141.78, $p < 0.001$ and $\chi^2(3) = 83.27$, $p < 0.001$, respectively). The 100% v/v ovalbumin treatment had greater relative protein concentrations than the 1% v/v and control treatments but were no different than the 5% v/v treatment. The 5% v/v treatment samples had greater relative protein concentrations than the control treatment but were no different from the 1% v/v treatment and the relative

protein concentrations between 1% v/v and control treatments were the same. The proportion of positive samples was higher in the 100% v/v treatment than all other treatments. The proportion of positive samples in the 5% v/v treatment was higher than the control treatment, but the same as the 1% v/v treatment. There was no difference in proportion of marked beetles between the 1% v/v treatment and control treatment (Table 4).

Ovalbumin was detected on beetles in all treatments (Table 5). Higher concentrations of ovalbumin correlated with higher frequency of detection as well as longer persistence (Figure 2.6 & 2.7). More beetles retained the protein mark from the 100% v/v treatment than the 5% v/v treatment ($z = 4.42$, $p < 0.001$) and the protein mark was retained better in the 5% v/v treatment than the 1% v/v treatment ($z = 2.77$, $p < 0.01$).

2.4.3 Test for endogenous proteins

In both studies, two beetles from the untreated logs tested positive for the presence of ovalbumin. To ensure this color change was not caused by endogenous beetle proteins, I conducted ELISAs on 88 additional unmarked beetles. All 88 beetles tested negative for ovalbumin, suggesting that the false positives were caused by contamination, not by endogenous proteins interfering with the ELISA.

2.5 Discussion

Our study demonstrates that woodboring beetles can acquire a vertebrate protein marker as they emerge from treated host material. The highest concentration of ovalbumin was the most effective at marking beetles and the most persistent on cuticle. This technique could be utilized for dispersal studies of insects beyond EAB, which would provide useful information for

developing strategies to manage pest populations (Muirhead et al., 2006; Robinet & Liebhold, 2009; Chivers & Leung, 2012; Koch et al., 2012).

In the efficacy study, three ELISA techniques were tested, and an indirect ELISA using whole beetle washes performed significantly better than either an indirect or sandwich ELISA with pulverized samples. This poor performance is likely due to two reasons. First, homogenizing an insect releases a large amount of other proteins which compete for the non-specific binding sites, therefore proteins found in low concentrations were likely washed away (Hagler, 2019). Second, the sandwich ELISA was designed to function with chicken IgG, rather than ovalbumin, which is only present in vanishingly small quantities in egg whites (Hagler, 2019). Although ovalbumin was detected on beetles from each treatment throughout the study, there was a steep decline in the number of positives recovered in the 10% v/v and 50% v/v treatments as the beetles aged. However, the mark was detectable even up to 25 days after marking for over 75% of beetles that emerged from logs treated with the 100% v/v ovalbumin solution. Overall, 65% (n= 99) of beetles from the 10% v/v solution marked logs, failed to either acquire or retain the protein mark, mostly in aged beetles. The percentage of unmarked beetles was reduced to just 37% (n = 47) by increasing the concentration of solution to 50% v/v ovalbumin and was further reduced to only 15% (n = 21) through the use of the 100% v/v ovalbumin solution. This occurrence could likely be further reduced by improving mark application techniques, however, simply applying the protein more frequently (e.g., reapplying the marker every week) could improve marker detectability, especially in humid or wet environments (Hagler et al., 2014).

During the persistence study, there were five days of heavy storms (>2.54 cm of rain) including the first evening after application of the mark. Despite the heavy rainfall, ovalbumin

was detected on 7 of 10 beetles marked with the 5% v/v ovalbumin solution one week later. However, no positives could be detected from the 1% v/v ovalbumin treatment after one week. The protein marked persisted for the entire experiment on a relatively high percentage (>60%) of beetles from the 100% v/v ovalbumin treatment. The concentrations selected for treatments do not necessarily represent the amount of ovalbumin that a beetle would acquire while emerging but represent a range of concentrations and demonstrate that higher concentrations persist longer on insect cuticle. This experiment also suggests that ovalbumin eventually degrades over time on the insect cuticle, but nonetheless the highest concentrations can persist for several weeks, even if exposed to heavy rain and direct sunlight. Also, beetles in our study were fully exposed to both rain and sunlight, however, a live beetle would likely seek refuge from both, potentially further prolonging the persistence of the mark. Therefore, to maximize longevity of the mark on insects, I recommend using undiluted, 100% v/v egg whites to mark trees or logs used in mark-capture studies investigating woodborer dispersal. Because ovalbumin is an inexpensive marker, spraying host trees until the surface is visibly wet and, depending on environmental conditions, re-applying the mark as often as weekly for the duration of the study will maximize the likelihood of an insect acquiring the mark.

Throughout the course of the efficacy study, the mark was undetected on 167 beetles that emerged from logs treated with ovalbumin, suggesting that these beetles did not acquire the mark upon emergence or that it was removed during their adult life. The failure to acquire the mark may have resulted from an inconsistent application of ovalbumin to the logs. However, the decrease in the number of marked beetles throughout the course of the study suggests that the mark was abraded from the cuticle rather than beetles avoiding the mark when emerging. If beetles failed to acquire the mark, a more uniform distribution of unmarked beetles across the

study period would be expected, rather than the majority of unmarked beetles being found in the later days of the study. Of the 140 control beetles across both studies, ovalbumin was detected by ELISA on four individuals (~3% of total). The ELISA is sensitive and small quantities of ovalbumin can elicit a positive optical density score. To ensure that these false positives were not caused by an endogenous protein reacting during the ELISA, we tested 88 additional unmarked beetles and all tested negative for the presence of ovalbumin. The optical density scores from the false positives were comparable to those of the true positives, suggesting that they likely resulted from contamination during handling and processing – underscoring the need for rigorous cleaning protocols to prevent such errors.

Immunomarking is an excellent alternative technique to traditional markers. It requires relatively few inexpensive reagents and supplies, samples can be processed quickly and cheaply using ELISAs, and proteins show similar marking efficacy and persistence to other common broadcast markers such as dusts (Boyle et al., 2018). By applying the protein directly to the tree, this technique has several advantages over traditional marking techniques by eliminating the time-consuming and costly process of rearing or trapping large quantities of beetles. Additionally, self-marking eliminates the need for human handling, preserves natural behavior and gives more accurate estimates of dispersal. These studies will enable researchers to rapidly and inexpensively assess the dispersal capabilities of future invasive woodborers and make better informed management decisions. Results from this work support the use of vertebrate protein markers (e.g., chicken ovalbumin) for single and multiple tree studies measuring woodborer dispersal within natural forests environments. Vertebrate protein markers potentially could be used in dispersal studies ranging from short-range movement from single, marked trees to long-range dispersal from hectare-sized plots. The flexibility, simplicity, and low cost of this protein

marking method makes it a potentially important tool for better understanding dispersal capabilities and patterns of invasive wood-boring beetles in order to better inform management decisions.

2.6 Conclusions

The results of this study demonstrate that an indirect ELISA using cuticular rinses from whole beetles is the optimal method for detecting self-marked beetles. When compared to the indirect method, the sandwich ELISA was far less effective at detecting marked beetles. Moreover, when logs were treated with higher concentrations of ovalbumin, more beetles acquired the mark as they emerged. In the persistence experiment, higher concentrations of protein persisted longer on treated beetles that were exposed to heavy rains and full sunlight, suggesting that ovalbumin is a durable mark.

In sum, this study demonstrates that ovalbumin applied directly to the bark of logs is an effective tool for marking emerging woodborers. This technique may find application in understanding the dispersal ability of forest pest insects and their natural enemies, estimating the active space of semiochemical attractants, and building population density and distribution models. To realize the full utility of this novel marking technique, future work should focus on determining the extent to which ovalbumin is transferred by contact between beetles, evaluating whether reapplication can extend the persistence of the mark, and assessing trapping designs for optimal mark recovery.

2.7 References

Basoalto K, Miranda M, Knight AL & Fuentes-Contreras E (2010) Landscape analysis of adult codling moth (Lepidoptera: Tortricidae) distribution and dispersal within typical

agroecosystems dominated by apple production in Central Chile. Environmental Entomology 39:1399-1408.

Boina DR, Meyer WL, Onagbola EO & Stelinski LL (2009) Quantifying dispersal of *Diaphorina citri* (Hemiptera: Psyllidae) by immunomarking and potential impact of unmanaged groves on commercial citrus management. Environmental Entomology 38:1250-1258.

Boyle NK, Machtley SA, Hagler JR & Pitts-Singer T (2018) Evaluating the persistence of fluorescent and protein powders on adult blue orchard bees, *Osmia lignaria* (Hymenoptera: Megachilidae), for mark-capture studies. Apidologie 49:378-385.

Chivers C & Leung B (2012) Predicting invasions: alternative models of human-mediated dispersal and interactions between dispersal network structure and Allee effects. Journal of Applied Ecology 49:1113-1123.

Coleman TW, Graves AD, Hoddle M, Heath Z, Chen Y, Flint ML & Seybold SJ (2012) Forest stand composition and impacts associated with *Agrilus auroguttatus* Schaeffer (Coleoptera: Buprestidae) and *Agrilus coxalis* Waterhouse in oak woodlands. Forest Ecology and Management 276:104-117.

Ginzel MD & Hanks LM (2002) Evaluation of synthetic hydrocarbons for mark-recapture studies on the red milkweed beetle. Journal of Chemical Ecology 28:1037-1043.

Haack RA (2006) Exotic bark- and wood-boring Coleoptera in the United States: recent establishments and interceptions. Canadian Journal of Forestry Research 36:269-88.

Haack RA, Hérard F, Sun J & Turgeon JJ (2010) Managing invasive populations of Asian longhorned beetle and citrus longhorned beetle: a worldwide perspective. Annual Review of Entomology 55:521-546.

Hagler JR, Cohen AC, Bradley-Dunlop D & Enriquez FJ (1992) A new approach to mark insects for feeding and dispersal studies. Environmental Entomology 21:20-25.

Hagler JR (1997) Field retention of a novel mark-release-recapture method. Environmental Entomology 26:1079-1086.

Hagler JR (2019) Super Mark It! A review of the protein immunomarking technique. *Annals of the Entomological Society of America* 112:200-210.

Hagler JR & Jackson CG (2001) Methods for marking insects: current techniques and future prospects. Annual Review of Entomology 46:511-543.

Hagler JR & Jones VP (2010) A protein-based approach to mark arthropods for mark-capture type research. Entomologia Experimentalis et Applicata 135:177-192.

Hagler JR, Mueller S, Teuber LR, Machtley SA & Van Deynze A (2011) Foraging range of honey bees, *Apis mellifera*, in alfalfa seed production fields. Journal of Insect Science 11:1-12.

Hagler JR, Naranjo SE, Machtley SA & Blackmer F (2014) Development of a standardized protein immunomarking protocol for insect mark–capture dispersal research. Journal of Applied Entomology 138:772-782.

Hishinuma S, Coleman TW, Flint ML & Seybold SJ (2011) Goldspotted oak borer: Field identification guide. University of California Agriculture and Natural Resources, Statewide Integrated Pest Management Program, 1-6. viewed 2 July 2012. <www.ipm.ucdavis.edu/PDF/MISC/GSOB_field-identification-guide.pdf>.

Holmes TP, Aukema JE, Von Holle B, Liebhold A & Sills E (2009) Economic impacts of invasive species in forests. Annals of the New York Academy of Sciences 1162:18-38.

Horton DR, Jones VP & Unruh TR (2009) Use of a new immunomarking method to assess movement by generalist predators between a cover crop and tree canopy in a pear orchard. American Entomologist 55:49-56.

Jones VP, Hagler JR, Brunner J, Baker C & Wilburn T (2006) An inexpensive immunomarking technique for studying movement patterns of naturally occurring insect populations. Environmental Entomology 35:827-836.

Koch FH, Yemshanov D, Magarey RD & Smith WD (2012) Dispersal of invasive forest insects via recreational firewood: a quantitative analysis. Journal of Economic Entomology 105:438-450.

Kovacs KF, Haight RG, McCullough DG, Mercader RJ, Siegert NW & Liebhold AM (2010) Cost of potential emerald ash borer damage in US communities, 2009–2019. Ecological Economics 69:569-578.

Krugner R, Hagler JR, Groves RL, Sisterson MS, Morse JG & Johnson MW (2012) Plant water stress effects on the net dispersal rate of the insect vector, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae), and movement of its egg parasitoid, *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae). Environmental Entomology 41:1279-1289.

Liebhold AM & Tobin PC (2008) Population ecology of insect invasions and their management. Annual Review of Entomology 53:387-408.

Lopez VM, McClanahan MN, Graham L & Hoddle MS (2014) Assessing the flight capabilities of the goldspotted oak borer (Coleoptera: Buprestidae) with computerized flight mills. Journal of Economic Entomology 107:1127-1135.

Muirhead JR, Leung B, Overdijk C, Kelly DW, Nandakumar K, Marchant KR & MacIsaac HJ (2006) Modelling local and long-distance dispersal of invasive emerald ash borer *Agrilus planipennis* (Coleoptera) in North America. Diversity and Distributions 12:71-79.

Peck GW, Ferguson HJ, Jones VP, O'Neal SD & Walsh DB (2014) Use of a highly sensitive immunomarking system to characterize face fly (Diptera: Muscidae) dispersal from cow pats. Environmental Entomology 43:116-122.

Poland TM & McCullough DG (2006) Emerald ash borer: invasion of the urban forest and the threat to North America's ash resource. Journal of Forestry 104:118-124.

Robinet C & Liebhold AM (2009) Dispersal polymorphism in an invasive forest pest affects its ability to establish. Ecological Applications 19:1935-1943.

Siegert NW, McCullough DG, Williams DW, Fraser I, Poland TM & Pierce SJ (2010) Dispersal of *Agrilus planipennis* (Coleoptera: Buprestidae) from discrete epicenters in two outlier sites. Environmental Entomology 39:253-265.

Sivakoff FS, Rosenheim JA & Hagler JR (2012) Relative dispersal ability of a key agricultural pest and its predators in an annual agroecosystem. Biological Control 63:296-303.

Slosky LM, Hoffmann EJ & Hagler JR (2012) A comparative study of the retention and lethality of the first and second generation arthropod protein markers. Entomologia Experimentalis et Applicata 144:165-171.

Smith MT, Bancroft J, Li G, Gao R & Teale S (2001) Dispersal of *Anoplophora glabripennis* (Cerambycidae). Environmental Entomology 30:1036-1040.

Smith MT, Tobin PC, Bancroft J, Li GH & Gao RT (2004) Dispersal and spatiotemporal dynamics of Asian longhorned beetle (Coleoptera: Cerambycidae) in China. Environmental Entomology 33:435-442.

Stack SC, Sadof CS & Ginzel MD (2019) Effects of grafting on host plant resistance in ash (*Fraxinus* spp.) to emerald ash borer (*Agrilus planipennis* Fairmaire). Agricultural and Forest Entomology 21:180-189.

Swezey SL, Nieto DJ, Hagler JR, Pickett CH, Bryer JA & Machtley SA (2013) Dispersion, distribution and movement of *Lygus* spp. (Hemiptera: Miridae) in trap-cropped strawberries. Environmental Entomology 42:770-778.

Swezey SL, Nieto DJ, Pickett CH, Hagler JR, Bryer JA & Machtley SA (2014) Spatial density and movement of the *Lygus* spp. parasitoid *Peristenus relictus* (Hymenoptera: Braconidae) in organic strawberries with alfalfa trap crops. Environmental Entomology 43:363-369.

Taylor RAJ, Bauer LS, Poland TM & Windell KN (2010) Flight Performance of *Agrilus planipennis* (Coleoptera: Buprestidae) on a flight mill and in free flight. Journal of Insect Behavior 23:128-148.

Turchin P & Thoeny WT (1993) Quantifying dispersal of southern pine beetles with mark-recapture experiments and a diffusion model. Ecological Applications 3:187-198.

Weather Report Purdue University, IN July 2017, Weather Underground, viewed 1 March, 2019, <https://www.wunderground.com/history/monthly/us/in/rensselaer/KRZL/date/2017-7>

Williams DW, Li GH & Gao RT (2004) Tracking movements of individual *Anoplophora glabripennis* (Coleoptera: Cerambycidae) adults: application of harmonic radar. Environmental Entomology 33:644-649.

Zumr V (1992) Dispersal of the spruce bark beetle *Ips typographus* (L.) (Coleoptera: Scolytidae) in spruce woods. Journal of Applied Entomology 114:348-352.

Table 2.1 Mean optical density and positive thresholds for each plate used in analysis.

Plate #	Mean Control OD (±SEM)	Positive Threshold
1	0.038 (±0.007)	0.057
2	0.037 (±0.007)	0.057
3	0.038 (±0.007)	0.057
4	0.042 (±0.007)	0.061
5	0.041 (±0.007)	0.061
6	0.037 (±0.007)	0.057
7	0.042 (±0.007)	0.062

Table 2.2 Descriptive statistics for efficacy study.

Concentration	n	# positive	% positive	Mean Pos OD (±SEM)	Mean Neg OD (±SEM)	Median OD (±IQR)
0% v/v	70	2	2.86%	0.066 (±0.006)	0.039 (±0.000)	0.039 (0.037 – 0.041)
10% v/v	152	53	34.87%	0.181 (±0.020)	0.042 (±0.001)	0.046 (0.040 – 0.076)
50% v/v	125	78	62.40%	0.112 (±0.007)	0.049 (±0.001)	0.070 (0.054 – 0.099)
100% v/v	137	116	84.67%	0.150 (±0.009)	0.046 (±0.002)	0.110 (0.072 – 0.172)

Table 2.3 Logistic regression model parameters for efficacy study.

Model Variable	Odds of ovalbumin detection	ln(odds ratio)	95% CI ln(odds ratio)	P value	odds ratio	CI odds ratio
0% v/v	0.073	-2.62	-4.44, -1.42	0.000334	0.07	0.01, 0.24
10% v/v	38.208	3.64	2.39, 5.49	1.27E-06	38.21	10.93, 242.77
50% v/v	118.909	4.78	3.51, 6.64	3.03E-10	118.91	33.47, 762.26
100% v/v	431.946	6.07	4.76, 7.95	5.59E-15	431.94	116.44, 2834.86
Days Alive	0.897	-0.11	0.14, -0.08	4.87E-11	0.89	0.87, 0.93

Table 2.4 Descriptive statistics for persistence study.

Concentration	n	# positive	% positive	Mean Pos OD (±SEM)	Mean Neg OD (±SEM)	Median OD (±IQR)
0% v/v	70	2	2.857	0.088 (±0.007)	0.038 (±0.000)	0.038 (0.036 - 0.039)
1% v/v	70	13	18.571	0.150 (±0.023)	0.045 (±0.001)	0.044 (0.042 - 0.052)
5% v/v	70	26	37.143	0.290 (±0.050)	0.047 (±0.001)	0.055 (0.044 - 0.091)
100% v/v	70	50	71.429	0.338 (±0.048)	0.047 (±0.002)	0.100 (0.060 - 0.275)

Table 2.5 Logistic regression model parameters for persistence study.

Model Variable	Odds of ovalbumin detection	ln(odds ratio)	95% CI ln(odds ratio)	P value	odds ratio	CI odds ratio
0% v/v	0.11	-2.20749	-4.02, -0.98	0.00288	0.1099768	0.02, 0.37
1% v/v	9.566	2.25823	0.86, 4.17	0.0051	9.5661122	2.36, 65.01
5% v/v	32.839	3.49162	2.13, 5.40	1.17E-05	32.839018	8.41, 221.16
100% v/v	241.865	5.48838	4.03, 7.47	9.06E-11	241.8639406	56.12, 1758.44
Days Alive	0.909	-0.09568	-0.13, -0.07	7.39E-10	0.9087551	0.88, 0.94

Figure 2.1 Mean (+ SEM) ELISA optical density values for marked and unmarked beetles from the 100% v/v ovalbumin treatment over study period. Numbers at the bottom of each bar are numbers of marked and unmarked beetles. Percent of marked beetles is shown above grey bars.

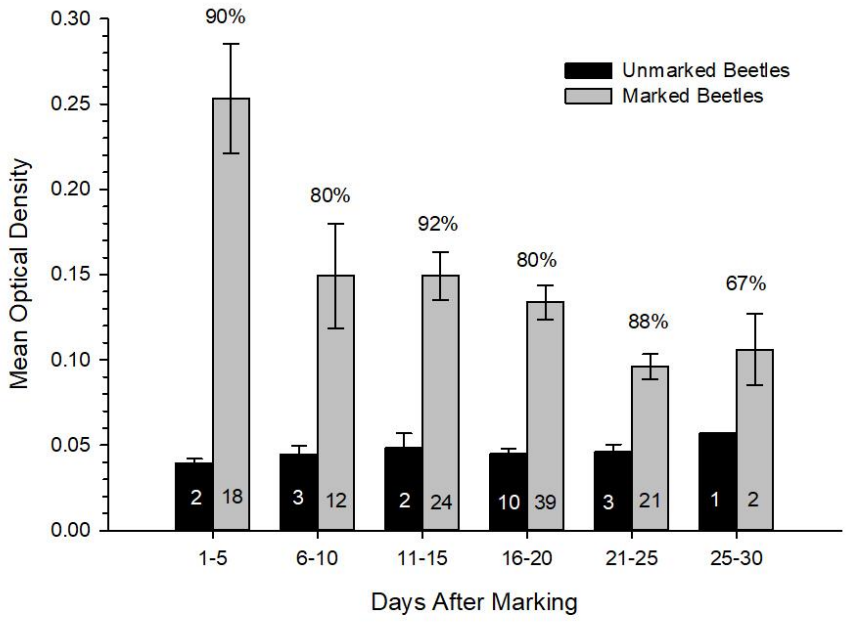


Figure 2.2 Mean (+ SEM) ELISA optical density values for marked and unmarked beetles from the 50% v/v ovalbumin treatment over study period. Numbers at the bottom of each bar are numbers of marked and unmarked beetles. Percent of marked beetles is shown above grey bars.

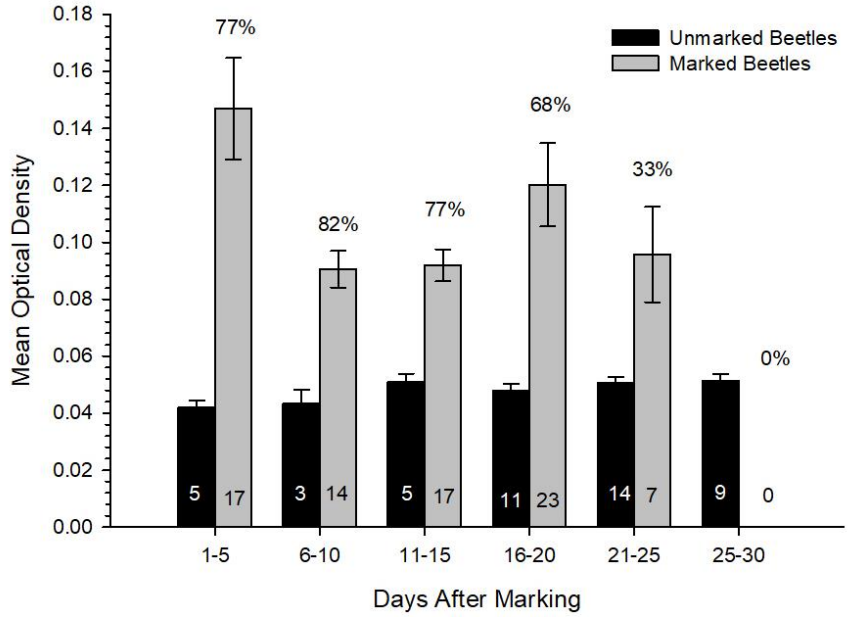


Figure 2.3 Mean (+ SEM) ELISA optical density values for marked and unmarked beetles from the 10% v/v ovalbumin treatment over study period. Numbers at the bottom of each bar are numbers of marked and unmarked beetles. Percent of marked beetles is shown above grey bars.

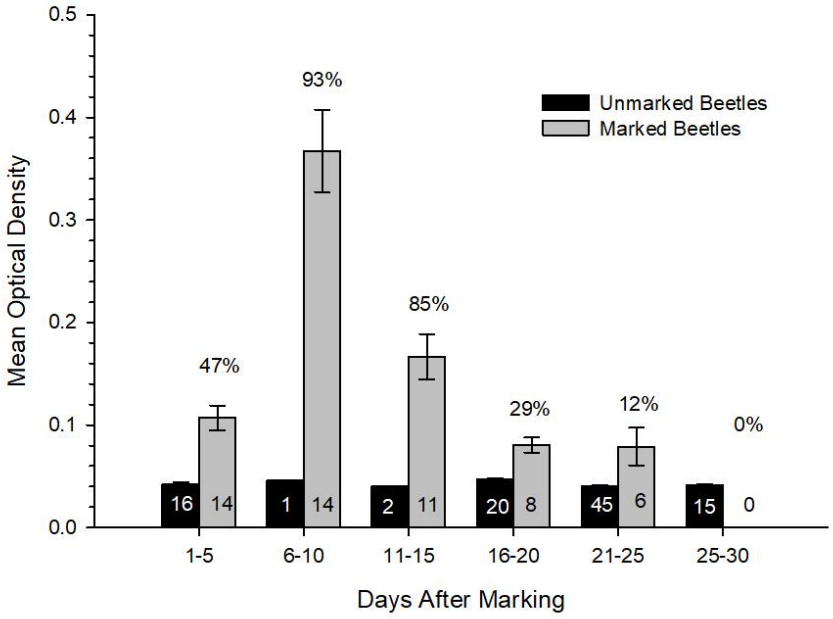


Figure 2.4 Mean (+ SEM) ELISA optical density values for marked and unmarked beetles from the 0% v/v control treatment over study period. Numbers at the bottom of each bar are numbers of marked and unmarked beetles. Percent of marked beetles is shown above grey bars.

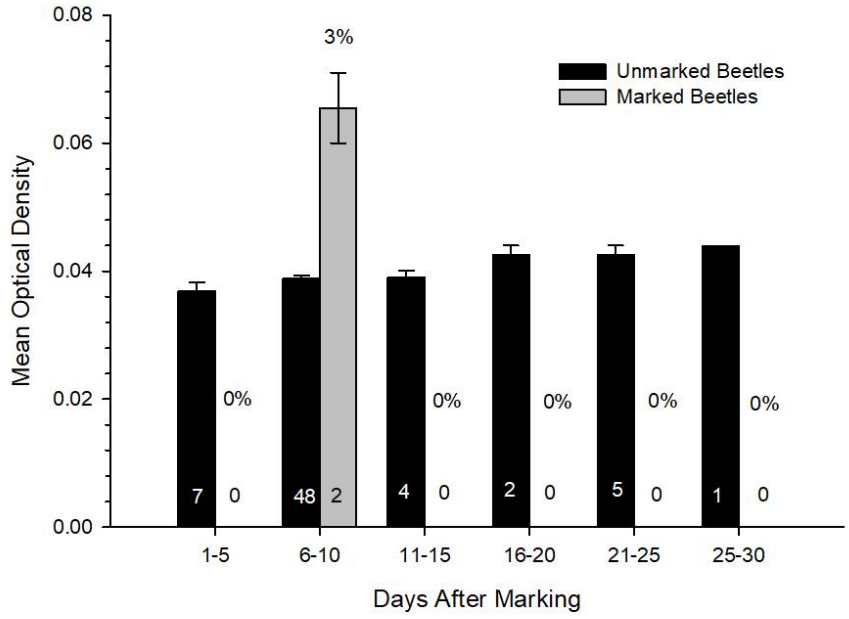


Figure 2.5 Probabilities of detecting ovalbumin on the cuticle of beetles housed in greenhouse. Ovalbumin detected with indirect ELISA. Shaded areas represent 95% confidence intervals.

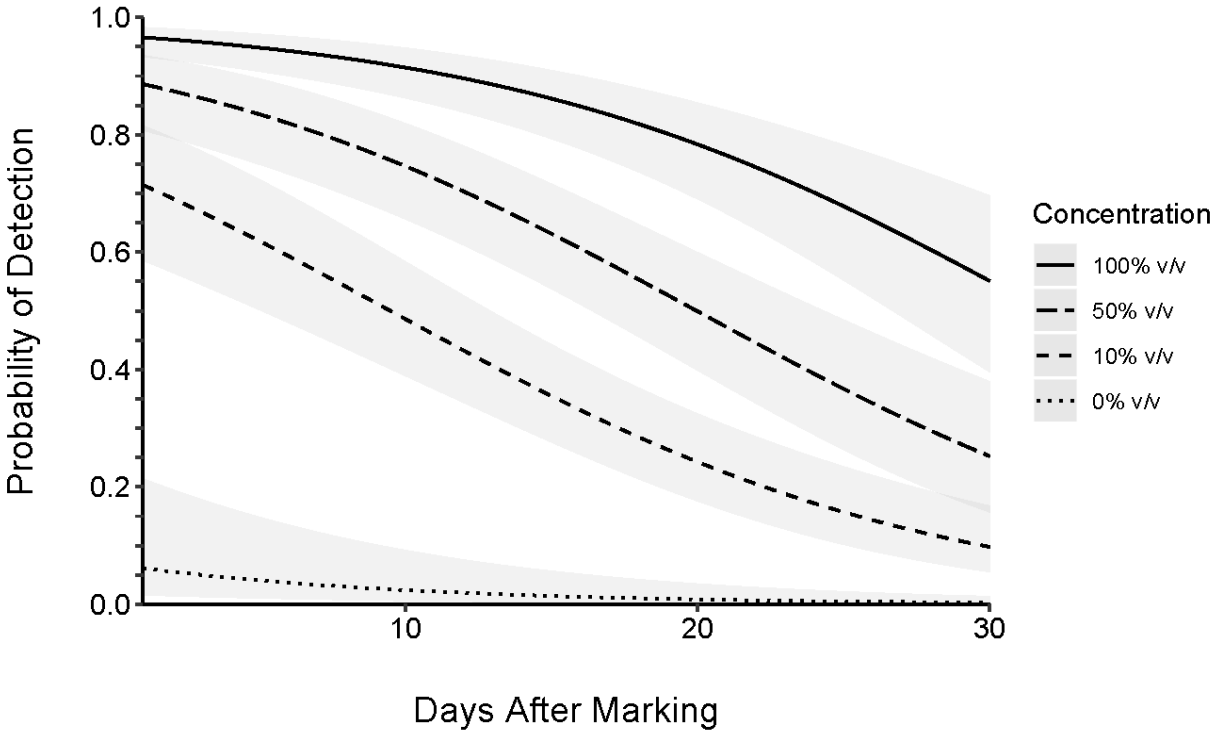


Figure 2.6 Percentage of pinned EAB with detectable ovalbumin mark each week of persistence study.

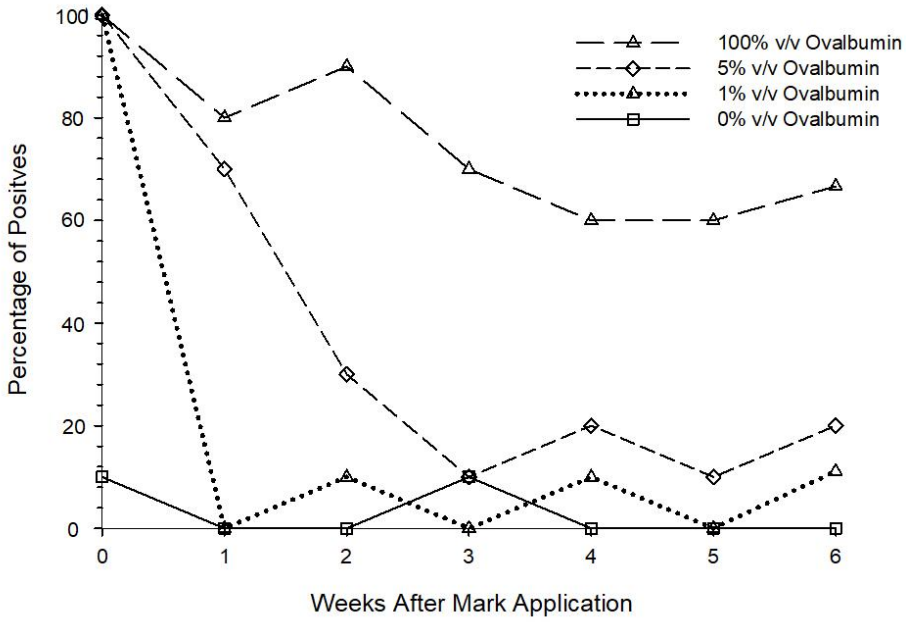
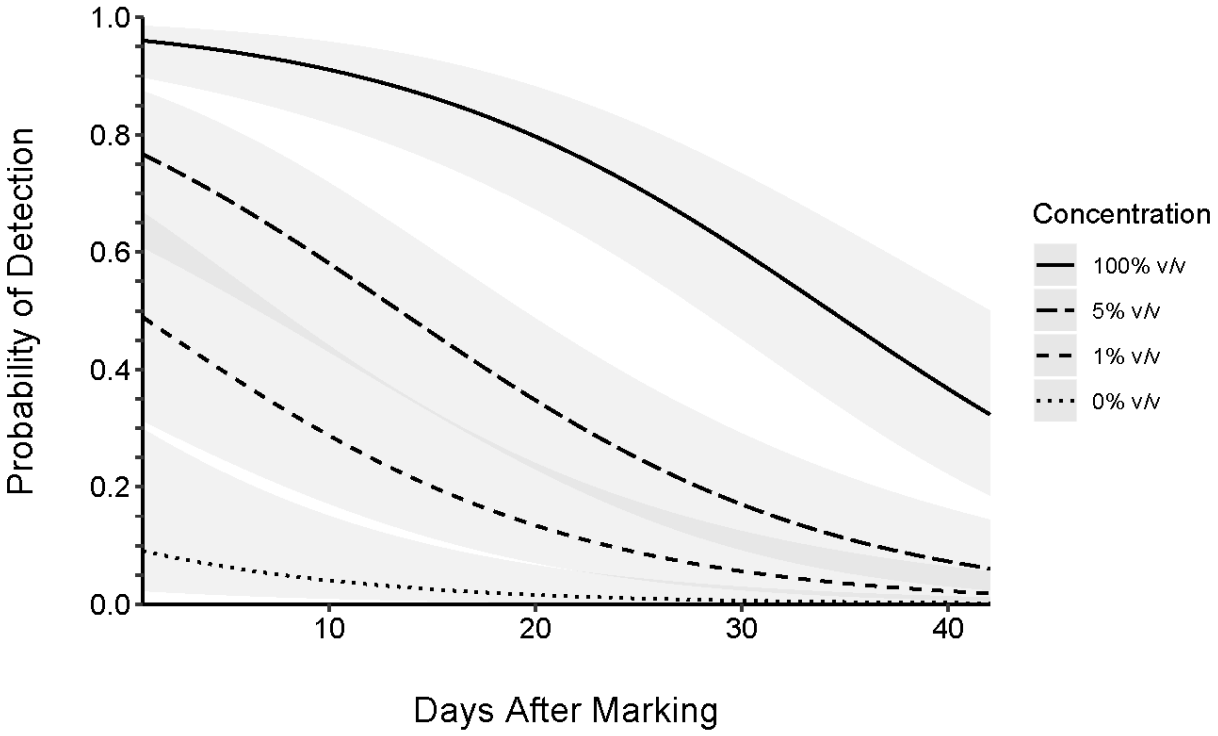


Figure 2.7 Probabilities of detecting ovalbumin on the cuticle of beetles when exposed to full summer weather conditions. Ovalbumin detected with indirect ELISA. Shaded areas represent 95% confidence intervals.



VITA

Scott W. Gula was born in Cincinnati, Ohio in 1990. He graduated from Xavier University in Cincinnati, Ohio in 2013 with a B. A. in History. After graduation, he worked for Dr. Ann Ray at Xavier University as a technician on a collaborative project with the USDA researching the chemical ecology of the Asian longhorned beetle. Following completion of his Master of Science degree in the Department of Entomology at Purdue University, Scott will be joining the Department of Forestry and Natural Resources at Purdue University to continue work with Drs. Matthew Ginzel and John Couture studying the chemical ecology of woodboring beetles and the tree species they infest.