Investigating Efficacy of parasitoids as Biological Control Methods against the Agrilus *planipennisi* Invasion in North America

George Ni

Second Year Ph. D. Student

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PH.D. ADVISOR DR. NICHOLAS J. GOTELLI

COMMITTEE MEMBERS   DR. ANTHONY D' AMATO
DR. ALISON BRODY
DR. BRENT LOCKWOOD

BIOLOGY DEPARTMENT UNIVERSITY OF VERMONT BURLINGTON, VT

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Project Summary

As an increasing number of invasive species, especially insect pests, are introduced to landscapes by global trade transport and shifts in habitat range, there is a greater need for research and study on alternative methods of control beyond insecticides and pesticides with many non-target effects. Biological control (biocontrol) is a method of controlling an invasive pest's density through the introduction of another organism, often a predator or parasitoid. One aspect of biological control that still has unanswered questions is how parasitoids may respond to variable climate conditions during winter given their thermotolerance ranges, and whether they are able to succeed in establishing stable populations in more northern regions (Vermunt et al 2012). My overarching goal is to provide a closer look at the efficacy of biocontrol programs through an understanding of physiological and developmental responses driving parasitoid establishment. My proposed reach will focus on the overwintering period of the emerald ash borer's (Agrilus *planipennisi*) parasitoid Tetrastichus *planipennisi* introduced to the North-Eastern region of the U.S through a combination of field, laboratory, and modeling techniques.

I will experimentally manipulate the photoperiod of Tetrastichus via shade treatments during their overwintering periods, and periodically measure supercooling points and eclosion rates. These shade treatment experiments will provide data on how long-term cooling and differing acclimation periods may affect supercooling points, which will also provide data on individual fitness and survival rates. I will also investigate the biochemical and physiological impacts of changes to supercooling points in these Tetrastichus through macromolecule assays, measuring proteins, carbohydrates, and lipids. Doing so will detect significant physiological differences among individuals across cooling periods, and possible explanations or more detailed predictions for overwintering survival of diapausing insects. I will also develop modeling of EAB/Parasitoid interactions through applications of the Nicholson-Bailey model and Species Distribution Models. This will expand the focus beyond individual-level differences to landscape-scale environmental factors that may affect the survival and efficacy of Tetrastichus and generate predictive models to project potential future spread and distributions.

Through these methods, I hope to answer three main questions: **1) How do long-term winter cooling periods and shade treatments affect the eclosion rates and supercooling points of Tetrastichus *planipennisi*? 2) What are the biochemical/physiological mechanisms that affect supercooling point and its impact on parasitoids? 3) How can we improve our understanding of EAB/Parasitoid interactions through Nicholson-Bailey Models and Species Distribution Models to predict future invasive species distributions?**

Merit

Due to live plant imports and increased use of solid-wood packaging materials in global trade, the presence of invasive wood-boring species has increased drastically over the last two decades. The emerald ash borer (EAB) is a phloem-feeding insect that specifically targets ash trees (genus Fraxinus) and is the primary cause of ash tree mortality in North America. In response, parasitoid wasps are now being deployed as biocontrol methods across many states in the U.S and Canada. Despite initial success, there has been some conflicting evidence of whether Tetrastichus are succeeding in establishing stable populations in more northern regions (Duan et al 2011). Not only that, but much of the available research on EAB parasitoids has been on their ability to parasitize the EAB and less on their suitability to introduced habitats (Bauer et al 2015). While laboratory experiments have been run to examine the general thermal limits of Tetrastichus, there are few experiments testing their limits in the field and gather more in-depth
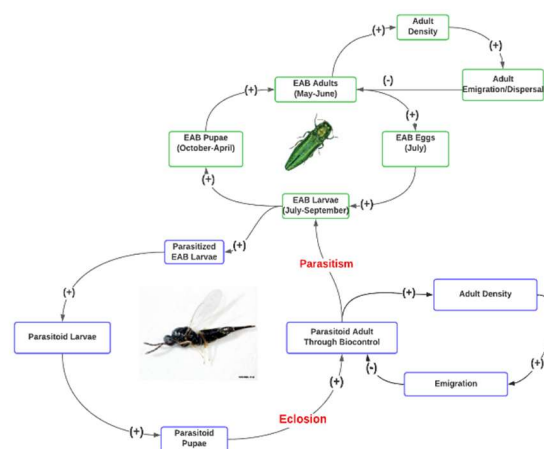
physiological data on their response to cold (Marshall and Sinclair 2015). As such, there is an increased need for field-based methods focused on investigating the physiology and suitability of Tetrastichus *planipennisi* in North America during winter.

My proposed research will measure the physiological and developmental response of Tetrastichus *planipennisi* to variable local environment conditions and lengths of cold exposure. Subsequent data will be applied to modeling techniques to predict future distributions of the EAB parasitoids. This research is especially relevant given the trend for EAB to travel Northwards along a latitudinal gradient and the need for data that explains how its specialist parasitoids will react and establish in the North-East regions under variable climatic conditions. The results of this research will aid in our understanding of invasive species ecology and the effect that climatic conditions may have on insects, as well as provide valuable data to inform forest management agencies on their introduction and release strategies.

Introduction

The Emerald Ash Borer, Agrilus *planipennisi*, is a species of wood-boring beetle native to China that feeds on the phloem, cambium, and shallow sapwood of ash trees. They are present in their adult forms during the early months of May for 20 days, until females deposit eggs inside of ash trees, where they will emerge as larvae from June to April of the following year, approximately 300 days. During this period, they will feed on the phloem of the ash trees, creating long S-shaped galleries that can reach up to 40 cm long, cutting off the transport of water and nutrients in the tree resulting in death. As larvae develop and reach their final instar stage (out of 4 instars), they must bore deeper into sapwood and overwinter starting in late October/early November (Wang et al 2010). As much of the EAB life cycle occurs while hidden under the bark of host trees, common methods of detection and control are ineffective. Effective measures such as systemic insecticides are both economically and environmentally expensive to sustain, leading to a focus on alternative management strategies such as finding more natural enemies to use as biological control measures.

As an invasive species, there are only a few natural enemies in North America that can consistently predate on the EAB, mainly species of woodpecker able to drill through the bark and feed on the larvae (Lindell et al 2008). After extensive exploration and discovery of natural enemies in the EAB's native region of China, there are 4 candidate species of parasitoid wasps that are highly dependent on the life cycle of the EAB and can serve as potential biocontrol agents: Oobius *agrili*, Spathius *galinae,* Tetrastichus *planipennisi*, and Spathius *agrili* (Duan et al 2014). For the purposes of my research, I will focus solely on Tetrastichus *planipennisi* for

Figure 1. Conceptual model and framework for EAB/Larval Parasitoid life cycles and interactions

initial experiments, with the intention of expanding the protocol to the other biocontrol agents over time. Figure 1. illustrates a conceptual model for the interactions of Tetrastichus *planipennisi* with the EAB.

Biocontrol Agents

Spathius *galinae* is a gregarious ectoparasitoid of the EAB first found in Far East Russia and is closely related to another biocontrol agent, Spathius *agrili*. S. *galinae* attacks the EAB in its late instar larval stage, producing 8-12 progeny per host that will develop and feed externally on the EAB until maturation. S. *galinae* is particularly promising as a biocontrol method because of its longer ovipositor length, making it easier for it to drill through the outer bark of host trees and locate the EAB larvae (Duan et al 2014). Its introduction could aid in ash tree recovery and management, as it would help reduce the EAB density in more mature host trees that were previously inaccessible to other parasitoids due to their shorter ovipositors.

Spathius *agrili* is a gregarious larval ectoparasitoid native to regions near Tianjin, China. In its native region it will commonly exhibit 3-4 generations per year, consistently overlapping with the life cycle of the EAB and attacking it during its later instar larval stages. It will produce on average 1-18 progeny, feeding externally on the EAB until full development (Duan et al 2014).

Tetrastichus *planipennisi* is a gregarious larval endoparasitoid found in regions in China. Field studies in North America have recorded 1-2 generations per year that consistently overlap with the life cycle of the EAB. Compared to the other biocontrol agents it is able to produce more progeny, each female producing on average 55 individuals over the course of their life span. As an endoparasitoid, females will oviposit their eggs directly into the EAB larvae, where their progeny will develop internally and feed on the EAB larvae. Tetrastichus *planipennisi* is also smaller in size and ovipositor length than the other biocontrol agents (except for Oobius agrili), but has proven to establish in areas of southern Michigan and the Midwest (Duan et al 2013).

Oobius *agrili* is a solitary egg parasitoid from Northeast China. It is the only egg parasitoid in use as a biocontrol agent and is anticipated to reduce the density of EAB before their harmful larval stage. It has a bivoltine and sometimes even multivoltine life cycle, but is commonly found to parasitize the EAB during the summer months. Current monitoring efforts have found evidence that O. *agrili* has established in some of its introduced ranges (Liu et al 2007).

The above biocontrol agents have all been studied for their ability to parasitize the EAB in their native conditions in North Asia, but there are still many gaps in knowledge related to their efficacy in North America, such as variations in climate and local environmental/landscape effects. One aspect of this is their ability to overwinter and tolerate extreme thermal limits, both for cold and warm periods. An insect's overwintering survival is highly dependent on the climate and local environmental conditions they are in, which can lead to variable success levels as a biological control program. As the temperature is one of the main factors driving overall fitness and survival for insects, more research is needed in studying how insects respond to temperature variation from both spectrums, as well as covariates that may also affect their overwintering survival.

Main Objectives

My research seeks to improve our current understanding of EAB parasitoids as biocontrol agents by integrating field experiments, laboratory analysis, and modeling techniques to **1) understand the effect of overwintering and photoperiod on the eclosion rate, survivorship, and supercooling points (SCP) of parasitoids in Vermont**, **2) provide biochemical and physiological support for the observed SCP changes**, and **3) incorporate these observations along with long-term nation-wide data to predict future invasion ranges and distribution.**

Question I: How do long-term winter cooling periods and shade treatments affect the eclosion rates and supercooling points (SCP) of EAB parasitoids?

Objective I:
1) Determine the effect of micro-habitat and local environmental conditions on Supercooling points of Tetrastichus *planipennisi*
2) Determine the effect of long-term winter cooling and shade treatments on eclosion rates and survivorship of Tetrastichus *planipennisi*,

Hypotheses and Predictions

- ❖ I hypothesize that eclosion rates of Tetrastichus *planipennisi* will differ between shade and control field treatments during its overwintering period.
  - ○ **I predict that shade treatment will have lower eclosion rates as they have decreased levels of photoperiod exposure, which would have significant developmental differences compared to control groups.**
- ❖ I hypothesize that the phenological timing and survival rates of the Tetrastichus *planipennisi* will be different between shade and control field treatments.
  - ○ **I predict that given differences in photoperiod, Tetrastichus *planipennisi* larvae in control groups should eclose earlier than in sheltered groups.**
- ❖ I hypothesize that the supercooling points and cold tolerance of Tetrastichus *planipennisi* will be higher in shaded treatments versus controls.
  - ○ **I predict that as sheltered/shaded groups have decreased photoperiod exposure and a buffer from extreme temperatures, they should have a reduced rate of decrease in SCP compared to control groups.**
- ❖ I hypothesize that across a latitudinal gradient, the supercooling points and cold tolerance of Tetrastichus *planipennisi* will be significantly different due to thermal and environmental variation experienced, and between treatment groups.
  - ○ **I predict that across a latitudinal gradient, Tetrastichus *planipennisi* introduced in the Southern limits will have a significantly higher SCP over the winter season compared to those in the Northern limits.**
  - ○ **I predict that sheltered groups in the southern sites will exhibit similar trends in SCP reduction over time and will have a dampened decrease in SCP compared to control groups.**
  - ○

Background

Insects face many challenges during winter, with local environmental stressors and temperature having large impacts on their overall survival. As ectotherms, they are unable to generate heat on their own-this biology has led to a variety of evolutionary strategies focused on surviving potentially lethal-freezing conditions. As temperatures drop, insects will undergo a process of cooling, initially reaching a reversible stage of muscular neuroparalysis, then freezing when it reaches their supercooling point (SCP). Insects can be classed under two main overwintering strategies: a freeze tolerance or a freeze avoidance strategy. The parasitoid studied in this experiment, Tetrastichus *planipennisi*, are freeze avoidant, but both strategies are explained here.

Freeze tolerance is the process by which insects attempt to tolerate low temperatures for long extended periods by undergoing partial freezing of their body water to internal ice. They accomplish this through a variety of strategies, including the production of ice nucleating agents in extracellular fluid, or a variety of other cryoprotectants. In some cases, insects are even able to convert up to 82% of their body water in order to cope with cold (Toxopeus and Sinclair 2018). The temperature at which ice formation may occur is variable and species-dependent, with documented instances of ice formation ranging from -1 °C to -54 °C, as well as variability across life stages. This is in large part due to variability in how species approach freeze tolerance. While some freeze-tolerant species may accumulate hemolymph antifreeze proteins to maintain the structural integrity of the cells, others produce glycerols, glycolipids, or amino acids (McDonald et al 2000).

Freeze avoidance strategies are defined by the prevention of ice formation and a constant liquid state within the organism, even at freezing temperatures. Freeze avoidance strategies are usually focused on removing ice nucleators through the evacuation of the insect's digestive contents or manipulating the colligative properties of their bodily fluids. Endogenous ice nucleator removal is a common trait within freeze-avoidant species, as doing so results in supercooling, where a relatively small amount of water can maintain a liquid state even when well below its normal freezing point. The point at which freezing occurs is known as the supercooling point (SCP), where crystallization occurs and a state change from liquid to solid occurs (Hanson 2013).

Seasonal patterns have been noted in some insects, where freeze avoidant species enhance supercooling capacities as they prepare for winter, decreasing the chance for lethal freezing of body fluids (Kostal et al 2014). This implies that cold acclimation periods prior to severe cold exposure may have a significant effect on an individual's supercooling point. The exact extent to which this applies is not clear, but some studies have found that fall seasonality can play a role in SCP adjustments (Liu et al 2007). Size and water content of the individuals are also significant factors, as body mass has a strong inverse correlation to supercooling capacity in vertebrate ectotherms (Costanzo and Lee, 1995). During the winter seasons, sunlight and photoperiod are important factors to consider for insects' overwintering ability, as the amount of sunlight/solar loading experienced by their host trees may have a significant effect on their ability to survive in the cold (Vermunt et al 2012).

As these biocontrol agents are introduced into North America, it is important to discuss their efficacy and their anticipated population growth. A key factor in discussing their success during this introduction is their ability to survive in environmental conditions and temperatures different from their native regions. Previous literature on cold tolerance and insect cryobiology has largely focused on the physiological and biochemical mechanisms of surviving and avoiding

freezing through laboratory temperature regimes (Marshall and Sinclair). Because of this, SCP is thought to be synonymous with the lethal limit of low temperature.
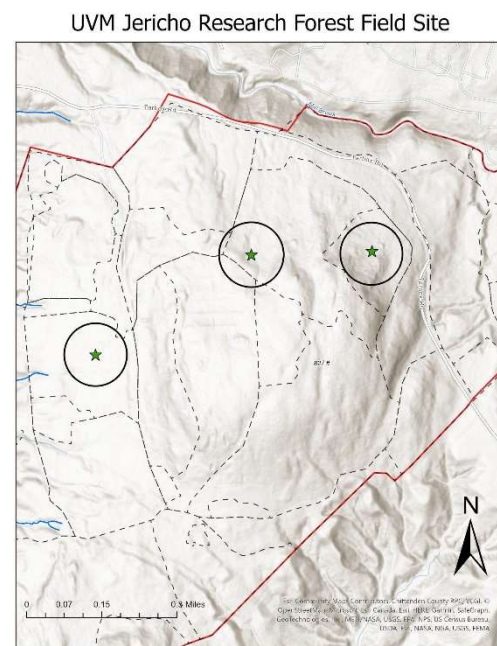
While it is important to understand the specifics of supercooling and its benefits, there are likely more environmental stressors and interaction effects present in nature that play a role in determining an insect's true cold hardiness. I am conducting a field-based press experiment measuring the supercooling points of overwintering Tetrastichus *planipennisi* in jars exposed to variable levels of sunlight/shade, then quantifying their survival rates and development.

Approach
Sampling Design

The initial experiments will only focus on Tetrastichus *planipennisi*, one of the parasitoid wasps used in the US' biological control program for the EAB. It will take the form of a manipulative field experiment during the fall/winter season in Jericho Research Forest, VT. Two experiments are being conducted simultaneously; a SCP measurement experiment and an eclosion rate/survivorship experiment (Figure 2). For the SCP experiment, live EAB larvae that had been parasitized by Tetrastichus *planipennisi* were placed inside 30 sticks and equally distributed into 6 different 3.8 L PET jars. Each stick hosts 3~6 EAB larvae, each of which may have 40 Tetrastichus larvae on average. Each site has two jars with differing treatments, either a "Sheltered" treatment or a "Control" treatment, as depicted in Figures 3 and 4 respectively. Half of the total jars are control groups, uncovered jars attached to ash trees, whereas the other half are sheltered from sunlight by the sides of plastic sheeting covering two sides of the jar, creating a three-sided box dimension (6 in x 6 in x 14 in) (Figure 4). This sheeting reduces total sunlight/solar loading experienced by the logs in the jars while also

Figure 2. Field Site Plot epicenters and boundaries in UVM Jericho Research Forest, VT.

preventing direct contact with precipitation and snow that occurs during the winter. Each month starting in November, a stick from each treatment group at each site is collected and dissected for Tetrastichus larvae, and then measured for their SCP as described in the *Sampling Techniques* section. For the duration of the experiment, HOBO data loggers are monitoring the temperature profiles within each jar every 30 minutes to detect any significant temperature differences between jars.

For the eclosion/survivorship experiment, treatments and sites are the same as the SCP experiment, except there are 6 jars at each site, 3 of which are of the Sheltered treatment and 3 are Controls. Each jar only has one log with 10-12 EAB larvae in it and is not collected until the middle of spring when eclosion is anticipated. At that point, logs will be collected and dissected for Tetrastichus individuals, recording developmental stage, number of eclosed individuals, and number of pre-eclosion individuals (Figure 5). Future additions to this experiment are to

introduce a latitudinal gradient through the establishment of multiple sites along the East coast of the US, with candidate areas being VT, MA, DE, and NC. Treatments and collection methodologies would be the same as described above.

Figure 3. Control Treatment Jar

Figure 4. Sheltered Treatment Jar

Figure 5. Logs housing Tetrastichus planipennisi *larvae for eclosion experiment (left), and stick with larvae for SCP experiment (right)*

Sampling Techniques
Supercooling Point Temperature Assays
Once collected from the field, Tetrastichus larave will be measured in-lab for their SCP. To do so methodology proposed by Hanson in 2013 was adapted for smaller parasitoid insects. A stick from each treatment jar was collected each month. Each stick was dissected for Tetrastichus *planipennisi* larvae (Figure 6) and larvae were placed on a petri dish and supplied water with wet filter paper. Larval samples are placed within size 4 gelatin capsules (Capsuline, Dania Beach, Florida, USA) and inside 1.5 ml microcentrifuge tubes. Each sample is then measured for its supercooling point by a thermocouple connected to a HUATO S220-T8 Datalogger (HUATO ELECTRIC, Shenzhen, China) recording every 2 seconds while chilled in a PolyScience circulating water bath filled with a silicon-180 oil for a lower temperature limit of -50 °C. Samples are chilled from 4 °C to -30 °C over a period of 30 minutes for each run, and the subsequent supercooling point of each individual is recorded as a spike in their temperature (Figure 6). SCP values are then recorded and averaged for each month and treatment group.
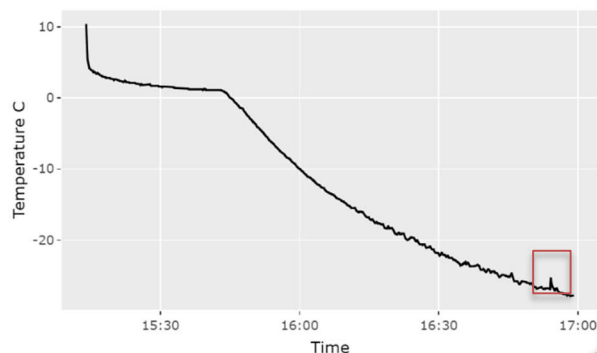
Figure 6. Temperature records of Tetrastichus *planipennisi* from sheltered group during cooling in Polyscience circulating waterbath on January 27, 2022. Temperature readout at point prior to exotherm at ~ -27 °C is marked as its SCP by red box.

Eclosion/Survivorship

For each log replicate, Tetrastichus planipennisi larvae will be collected in-lab. Each log will be dissected and split into 3-inch-long shavings, and then sifted through for individuals. If adult-stage individuals are in free flight, then each jar will be cooled to make transport easier. Each individual will be classed into size, mass, and eclosion vs. pre-eclosion developmental stages.

Figure 7. Dissection of sticks for SCP experiment, depicted in the center is a brood of Tetrastichus *planipennisi* larvae living inside the gallery of its EAB host.

Data Analysis

Data analysis will be done in R (Ver 4.0.3). I will conduct an initial test for normality through visual observation of histograms followed by a comparison of frequency, means, or medians of the different groups. If required, I will then classify the data into "high" and "low" groups depending on bimodality of the distribution. If normal, an initial analysis will be a two-way ANOVA to test for the changes in Supercooling Point between treatment and control groups, and across each month. Afterward, time series analysis and decomposition of trends will be used to remove strong signals such as seasonality and monthly trends, allowing for better forecasting and predictions of SCP change based on temperature factors through Generalized Linear Models (GLZ). To address the possibility of body size and water content variation becoming a confounding factor, body size will be measured, and an analysis of residuals will be conducted for regression of body size on SCP values (Hahn et al 2008).

Initial/Expected Results and Implications

The results from the Supercooling Point experiment will give us a baseline understanding of how Tetrastichus *planipennisi* adjusts its overwintering strategy over time, as well as the effect that adjustments to photoperiod and shade will have on their SCP. I predict that across time, SCP values for both sheltered and control groups will decrease in response to winter temperatures, and an increase in SCP value as temperatures rise during the warmer spring months. Treatment-wise, the sheltered jar groups should see a lag in SCP change due to both reduced photoperiod and precipitation experienced-this means that compared to the control groups, their SCP values should gradually decrease as the temperatures drop but have a significantly different decline/rate.This difference in SCP will also lead to variable eclosion

rates, such that the sheltered group should have significantly lower eclosion compared to the control groups-meaning they are less likely to develop and mature over time. A similar trend is predicted for their developmental stage as their overwintering survival fitness is lower compared to control groups, we should see a decrease in the amount of development in each sheltered jar compared to controls at the same site.

Initial Quantile-Quantile plots of preliminary SCP data test for normality were conducted as seen in Figure 8 (QQ Plot), along with a Shapiro-Wilks test for normality ($p=0.003855$). A histogram was also created to visually inspect any possible bimodality in the distribution (Figure 9-SCP Histogram). Results indicate a statistically significant result for a normal distribution of the SCP values, strengthening confidence in using parametric tests for further data analysis. Time series analysis for the temperature data loggers at each jar will attempt to decompose seasonality and temporal trends/patterns experienced by each jar and give a more accurate analysis of temperature variations across groups (Figures 10 and 11). Initial results of the field experiment have found that variation in photoperiod and available sunlight to experimental plots resulted in significantly different supercooling points exhibited by each treatment (Figure 10). Initial Box plots of SCP values depict changes in SCP values across time and between treatment groups (Figure 12).
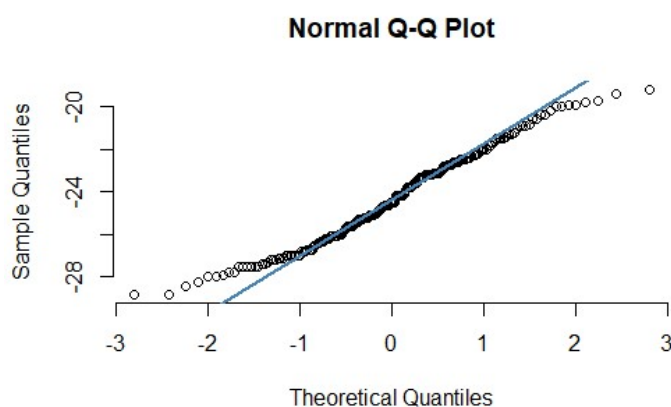
Figure 8. QQ Plot for Normality of SCP Values pulled from records November-February 2021-22.
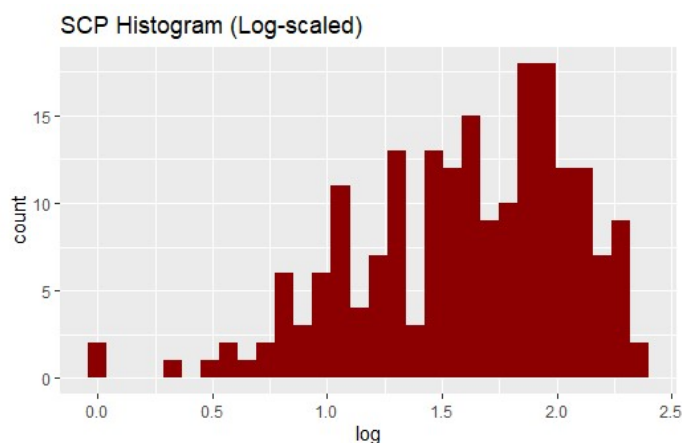
Figure 9. Log-Scaled Histogram of SCP Points example for normality assumptions.
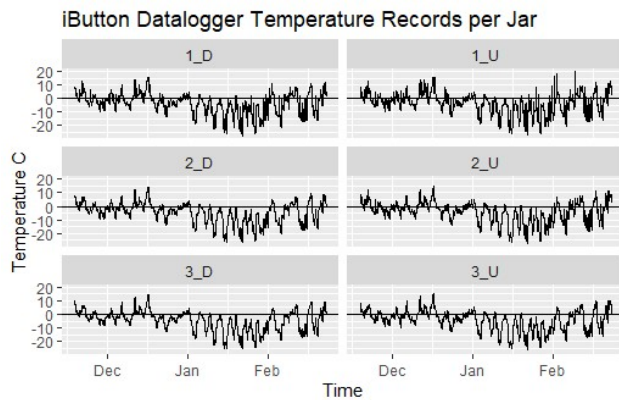
iButton Datalogger Temperature Records per Jar

Figure 10. iButton Datalogger records for each treatment group replicate. "D" groups represent sheltered treatments, while "U" groups represent controls. Recording frequency was every 30 minutes.
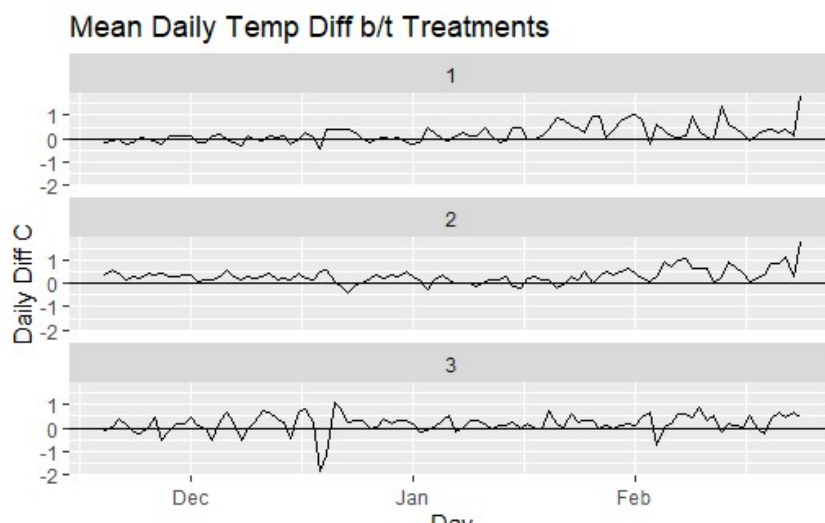

Mean Daily Temp Diff b/t Treatments

Figure 11. Mean Daily Temperature Difference plots for each block group. Temperature records were compressed into daily means. Difference (°C) represents difference between Control and Sheltered groups-positive values indicate higher temperatures experienced by Controls, and negative values indicate sheltered jars were warmer.
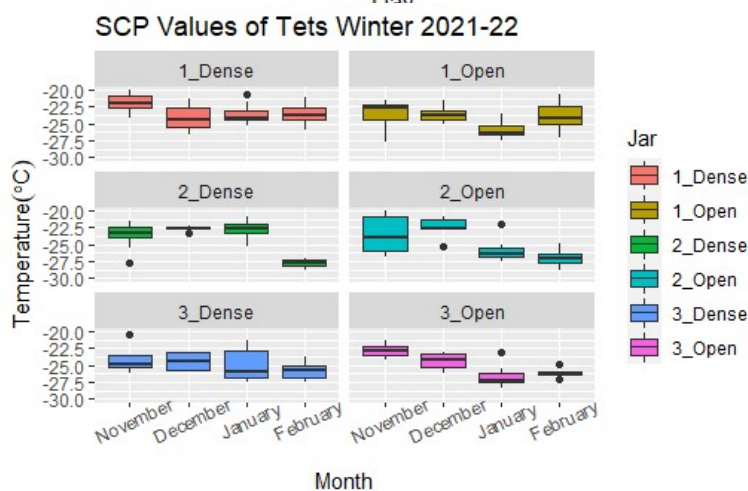

SCP Values of Tets Winter 2021-22

Figure 12. Preliminary results of SCP Field Experiments. Time series boxplot of SCP temperature (°C) values collected each month at sites 1-3 and Open (Control) and Dense (Sheltered) treatments.

Question II: What are the biochemical/physiological mechanisms that affect supercooling point and its impact on parasitoids?

Objective II: Investigate biochemical/physiological impacts of changes to a supercooling point in Tetrastichus *planipennisi*

Hypotheses and Predictions
- ❖ I hypothesize that Tetrastichus *planipennisi* larvae in treatment groups across time will experience significant differences in their lipid content.
  - o **I predict that as Tetrastichus *planipennisi* acclimates to cold temperature, individuals in sheltered groups will decrease their macromolecule content at a slower rate than controls over time.**
- ❖ I hypothesize that Tetrastichus *planipennisi* larvae in sheltered groups will have significantly different levels of lipid content compared to control groups.
  - o **I predict that the lipid content of Tetrastichus *planipennisi* will be significantly decreased in controls compared to sheltered groups, as the controls' microhabitat may result in overall warmer conditions that require increased macromlecule consumption.**
- ❖ I hypothesize that body size will have a significant effect on the exhibited Supercooling Points of Tetrastichus *planipennisi* across the winter season.
  - o **I predict that exhibited SCPs for Tetrastichus *planipennisi* will be lower for larger individuals, and that body size is a strong inversely-correlated factor in overwintering survival.**

SCP is often used as a measure for lethal temperatures of insects, but studies have also shown that cold hardiness can be a spectrum, across which insects may die before or after they reach their SCP. In those cases, it is clear that SCP alone cannot explain whether they will actually survive beyond that initial freeze, requiring the use of further survivorship metrics.

While freeze avoidance and tolerance are two of the most basic strategies and create a clear dichotomy from a foundational perspective, some critics believe that are enough unique cases and situations that necessitate more distinct categories beyond just two. Bale asserts that while the clear dichotomy of freeze-tolerant vs. freeze avoidant is useful for polar and alpine climatic zones, in less severe areas, the risk of chilling injury and death outweighs the risk of freezing injury and death. It is in these cases that he argues the simplified paradigm of cold tolerance fails to properly convey their differences, and further research on the mechanisms behind it may be useful. As the EAB parasitoids are largely freeze avoidant, it becomes important to understand how chilling may affect their overwintering mortality in more temperate regions.

One way to gather more information about their survival/recovery from freezing or near-freezing is through a collection of biochemical and physiological characteristics. Doing so will give us additional information about its survival chances after the initial cold shock, as well as links to energetics and its potential for development afterward.

As briefly stated in Chapter 1, during the early months of fall/winter, Ice-nucleating agents (INAs) initiate extracellular freezing at temperatures around -10 C. This freezing results

in an osmotic shift and transfer of water from inside the cell, preventing intracellular freezing and potential death.

During the winter season, insects accumulate polyols such as glycerol or sorbitol, and sugars such as fructose, sucrose, or trehalose. Polyols assist in reducing the amount of freezing damage experienced through a variety of mechanisms, including increased water binding capacity and stabilization of protein structure, which can reduce the rate of ice growth and ice content. Anti-Freeze Proteins (AFPs) will lower the freezing point of water relative to its melting point, valuable in conditions when INAs have not yet been synthesized, such as the early months of fall, or during the beginning of spring when warmer temperatures result in loss of INAs. AFPs are most valuable in their ability to prevent damaging effects of secondary recrystallization: when they experience warmer temperatures in the winter (Toxopeus and Sinclair 2018).

Freeze avoidant strategies in insects often take place in two stages: first being physiological changes through either reduction of body water content, increased fat content, and evacuation of its digestive system for any potential ice nucleators, and then second, the synthesis of polyols and AFPs that work to depress the SCP to avoid freezing. The production of polyols and AFPs will often ramp up as seasonal temperatures decrease, along with a corresponding decrease in SCP. These trends noted from other insects are valuable baselines to use as estimates for further investigation in the EAB's parasitoids, most of which are freeze avoidant (Li et al 2020).

Approach
Sampling Design

This experiment will conduct a biochemical composition analysis of Tetrastichus *planipennisi* and will be run in conjunction with the SCP field experiments from Chapter 1, either as a subset of the individuals used in the experiment or nearby neighbors. The study area will remain in the Jericho Research Forest for the full late-fall to spring duration as the above field experiment, as well as manipulation of the jar designs (i.e., sheltered vs. control treatments). As freeze avoidant insect species rely on different biochemical responses and mechanisms to survive cooling, a general analysis of biochemical/physiological characteristics of Tetrastichus may provide explanations for their SCP or survivorship.

Sampling Techniques

Each month, a subset of the parasitoid wasp larvae will be collected and sent into the lab, weighing their dry mass using a microbalance, then dissecting each individual into 3 separate parts. Each piece will be evaluated for a specific assay, of proteins, lipids, or carbohydrates.

Protein Assay

For proteins, a Pierce bicinchoninic acid assay (BCA) will be used to measure protein concentration. This assay utilizes the reduction of Cu^{2+} to Cu^+ by a protein in an alkaline medium and colorimetric detection of the cuprous cation Cu^+ by bicinchoninc acid, and the more intense the color detected through a spectrophotometer, the more peptide bonds that participated in the reaction.

Using a 96 well plate, 25 µL of either standard or sample will be pipetted into the wells with 200 µL of working reagent and mixed. Then, plates will be covered and incubated at 37 ℃ for 30 minutes, cooled to room temperature, then measured for absorbance at 562 nm.

Carbohydrate Assay

In order to measure carbohydrates, an anthrone assay will be used. This assay is based on the phenol-sulfuric acid method in which polysaccharides are hydrolyzed and converted into furfural or hydroxyl furfural. When combined with a Developer (Anthrone Assay Kit), a chromagen is generated and can be detected spectrophotometrically, similar to the BCA assay. Glucose standards for colorimetric detection will be created in a 96 well plate, first adding 0, 2, 4, 6, 8, and 10 µL of 2 mg/mL standard solution to create 0, 4, 8, 12, 16, and 20 µg/well standards with enough water to fill each well to 30 µL. Tissue samples will be homogenized with 200 µL Assay buffer and centrifuged at 13,000 x g for 5 minutes. Samples should be at a final volume of 30 µL of water. For the Assay reaction, 150µL of concentrated sulfuric acid (H_2SO_4) is added to each well, incubated at 90 ℃ for 15 minutes, and covered and protected from light. 30 µL of Developer is added to each well, mixed for 5 minutes at room temperature, and then measured for absorbance at 490 nm.

Lipid Assay

Lipid content can be measured using a gravimetric assay similar to the previous assay. Dry mass of homogenized tissue will be weighed, then soaked within non-polar solvent to dissolve the lipid. Centrifugation will separate the remaining undissolved portion, remove the dissolved lipid, dry again, and then weigh the remaining. Difference in masses between these will equal the lipid content.

Data Analysis

Data analysis of macromolecule assays will first assess the difference in means between control and sheltered groups through analysis of variances (ANOVA), followed by an analysis of covariances to determine the impact of each biochemical component as a covariate. A generalized linear model will also be used to determine the relationships between body size (mass) vs. SCP, and total protein/lipid/carb content vs. SCP.

Expected Results and Implications

Previous research has found evidence that body size is a significant factor in determining the SCP of ectotherms (Hahn 2008). As there should be an inverse correlative relationship between the two due to larger individual larvae having increased protein/lipid/carb/water content we should expect to also see that relationship here in Tetrastichus *planipennisi*, with individuals of larger mass having a lower SCP.

We should also see that biochemical components should differ between groups, such that individuals with higher protein/lipid/carb content should exhibit a lower SCP as well. Overtime, all overwintering individuals should decrease their overall lipid stores as it is a primary source of overwintering fuel. Evidence from previous studies found that the effect of microhabitat and variability in temperature may result in differences in the quantity of macromolecule/energy consumption (Irwin and Lee 2003). Studies done on gall flies and wooly bear caterpillars found that individuals exposed to winter air temperatures consumed less energy compared to those insulated beneath the snow. It is possible that microhabitat selection could result in increased periods of warm conditions and subsequently higher levels of fat or lipid consumption (Layne et al 1999).

Macromolecule content can also help inform us on post-overwintering survival and eclosion of individuals. Given that individuals after overwintering are still not actively feeding,

their remaining fat stores are often used for development, dispersal, or other post-winter activities. A metabolomics approach to this may also be useful in determining more factors important to overwintering survival, as analysis of cryoprotectants such as trehalose and sorbitol were often found to have a strong inverse relationship with decreasing SCPs. An untargeted metabolomics approach may identify key cryoprotectants used by Tetrastichus *planipennisi*, or a targeted approach with the most common cryoprotectants could confirm their role in overwintering survival.

Question III: How useful are population dynamic models of EAB/Parasitoid interactions and Species Distribution Models for predicting future invasive species distributions?

Objective III: Develop EAB/Parasitoid population dynamic models through life table analyses and integrate field data from nation-wide surveys and laboratory experiments. Assess performance of Species Distribution Models for predicting future invasion spread given climate change projections.

Hypotheses and Predictions

- ❖ I hypothesize that parasitoids of the EAB and their interactions are heavily impacted by spatial heterogeneity, resource availability, and climate, which have the potential to reduce the parasitoids' efficacy as a biocontrol program.
  - o **I predict that with the inclusion of density dependence and spatially explicit systems, the NB model can better model host/parasitoid systems and be applied to this specific model system (EAB/Parasitoid).**
- ❖ I hypothesize that changes in global climate will result in suitable habitat range shifts for both EAB and its parasitoids, with an increased trend northwards given ash tree distributions.
  - o **I predict that short-term invasion spread will be impacted more by availability of habitats, resulting in shifts southwards.**
  - o **I predict that given global climate projections, future projections of EAB habitat ranges will indicate shifts northwards.**
  - o **I predict that as parasitoid local populations reach the edge of the EAB invasion boundary, low densities of the EAB may prevent establishment for its biocontrol agents and lower their efficacy as a biocontrol method.**

Background

Population models can serve as useful tools for wildlife and forest managers interested in how species of interest may interact with each other and grow. As insects often have very distinct life stages that are synchronized to seasonality, initial literature utilized discrete-time models, with initial iterations of this being the Nicholson-Bailey model. It was originally designed to describe the population dynamics of a coupled host-parasitoid systems, which can also be used for host-parasite and predator-prey systems.

Nicholson-Bailey Model

The Nicholson-Bailey (NB) model assumes that both host and parasitoid are highly synchronized and operate under distinct life cycle stages, where parasitoids lay eggs into their host which feed and kill the host, continuing the cycle (May et al 1981). Mathematically, assuming the host and parasitoids operate under a univoltine generation time, the base NB model consists of two difference equations, one for host and one for parasitoid:

1) $H_{t+1} = RH_t e^{-aPt}$
2) $P_{t+1} = cH_t(1 - e^{-aPt})$

Where H_T and P_T represents the density of the host and parasitoid respectively at year t, R is the offspring of the host that are unparasitized and survive to the next year, c is the number of parasitoids hatched from a parasitized host, and a as a proportionality constant. In its base form, the NB model's equilibrium is unstable as densities increase in amplitude until a crash. It also fails to include spatial patterns/trends, which is an important concern for invasion biology (Murdoch et al 2005). Since its creation, there have been a number of modifications to the NB model to induce stability, including the addition of spatial heterogeneity, density dependence, and age-structure.

Integro-Difference Models

Building on these initial models, Kot and Schaffer in 1986 introduced integrodifference equations, a class of dynamical systems models that are spatially explicit. These equations are built on the premise of having distinct growth and temporal dispersal phases. Assuming $N_t(x)$ as the population density at discrete time (t) at a continuous location in space (x), the integrodifference equation can be written as:

3) $N_{t+1}(x) = \int K(x - y)F(N_t(y))dy$

With K as the probability density of function of offspring location, and F as the growth phase. By applying this to the NB models, we change H_T and P_T to serve as population densities depending on spaces $H_t(x, y)$ and $P_t(x, y)$. Likewise, we include $K_H(x, y)$ and $K_P(x, y)$ to represent species specific dispersal probabilities. Therefore, the integrodifference approach to the NB model will see two stages:

1) $H_{t+1/2(x,y)} = f(H_t, P_t) = H_t(x, y)e^{r(1-(H_t(x,y)/K))-aP_t(x,y)}$
2) $P_{t+1/2(x,y)} = g(H_t, P_t) = nH_t(x, y)(1 - e^{-aP_t(x,y)})$

And:

1) $H_{t+1}(x, y) = K_H * H_{t+1/2}(x, y)$
2) $P_{t+1}(x, y) = K_P * P_{t+1/2}(x, y)$

With the first stage being the growth phase, where the hosts and parasites interact based on the initial population values, and then a dispersal phase, where the organisms travel through discrete patches. Expansions on this by researcher such as Hassell and May found that spatial patchiness

of ecosystems can lead to stability, as some patches serve as refuges from which hosts are protected from parasitism (Hassell and May 1988).

Species Distribution Models

Species Distribution Models (SDMs) predict potential future range shifts of species and are a popular framework for quantitative ecological modeling. Through georeferenced observations such as individual location or presence data, coupled with environmental variables such as climate, land cover, or soil attributes, a statistical model is used to estimate the species-environment relationship, which is then used to predict potential geographic distributions. While SDMs are quite common, there are a variety of methodologies and algorithms that can be used, each with its own limitations and benefits that require careful consideration before use (Mainali 2015). In general, SDMs can fall under two categories: correlative and mechanistic models. Correlative models attempt to link georeferenced climatic predictor variables to observed presences and absences of a particular species to predict the most suitable environmental ranges. Mechanistic models utilize physiological data and understanding of a specie's biological mechanisms to determine suitable environmental ranges. Mechanistic models have been observed to perform better when modeling species with active range shifts, such as invasive species, but are often more data-intensive, as it requires comprehensive records of physiological mechanisms and life history characteristics that are not often available (Jarnevich et al 2015).

Approach
Sampling Design

To develop a variety of models for both hosts and parasitoids, the conceptualization of the model framework will guide the search for data, followed by model fitting and predictions. To begin, a model of population dynamics built on the NB model will investigate the effects of spatial variables on parasitoid spread. This includes spatially dynamic systems with heterogenous environments which could lead to partial refuge effects, as well as demographic effects from invulnerable host life stages. I will also model any potential Allee effects experienced by the host, including spatial heterogeneity that may increase dispersal requirements and mate limitations experienced by the EAB. I will manipulate the initial parameters of the model-to-model potential management strategies-including inoculative release strategies of parasitoids in response to early detection of EAB, and inundative releases within areas with already present high levels of EAB density. Currently, the model is being run using MatLab, but one of the first objectives is converting it into R.

I will also use Species Distribution Models (SDMs) to predict future invasion spreads of EAB and parasitoids. I will begin with Niche Models, a subset of correlative SDMs that determines the most suitable environmental ranges a species can live in given a set of observed occurrences and climate maps. As there are many algorithms currently available to use, several different candidate models will be used and assessed for the best fit. The majority of EAB and parasitoid occurrence datasets conducted in the U.S are compiled into the MapBioControl dataset available online, so I will verify and assess the datasets there for potential use and pull them into the models. For habitat suitability, I will include data on Ash tree distributions, pulling from state-wide ash tree inventories and national surveys. Algorithms such as MaxEnt, GARP, GLM, and Random Forest will be used and compared.
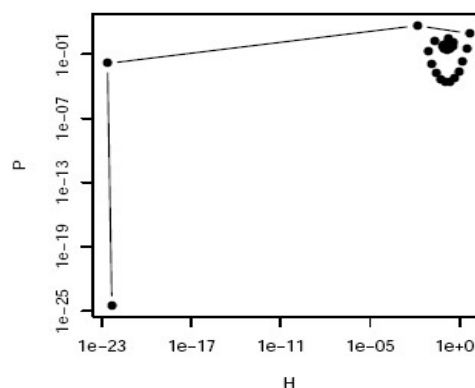
Data Analysis

Initial steps for modeling will be to conduct a literature review and meta-analysis of current EAB and parasitoid life history characteristics and demographic data. Parameters will include demographic data from life table analyses of EAB and its parasitoids, occurrence data points from MapBioControl as well as recorded nearby ash tree characteristics (DBH, age-class, among others), state-wide ash tree inventories, and climatic variables from BioCLIM. Modeling and visualization of Nicholson-Bailey population dynamic models and SDMs will be done in R (Ver. 4.0.3). Cross-validation for model performance will occur through inspection of the area under the receiver operating characteristic (ROC) and Aikake Information Criterion (AIC) measures (Sobek-Swant et al 2012).
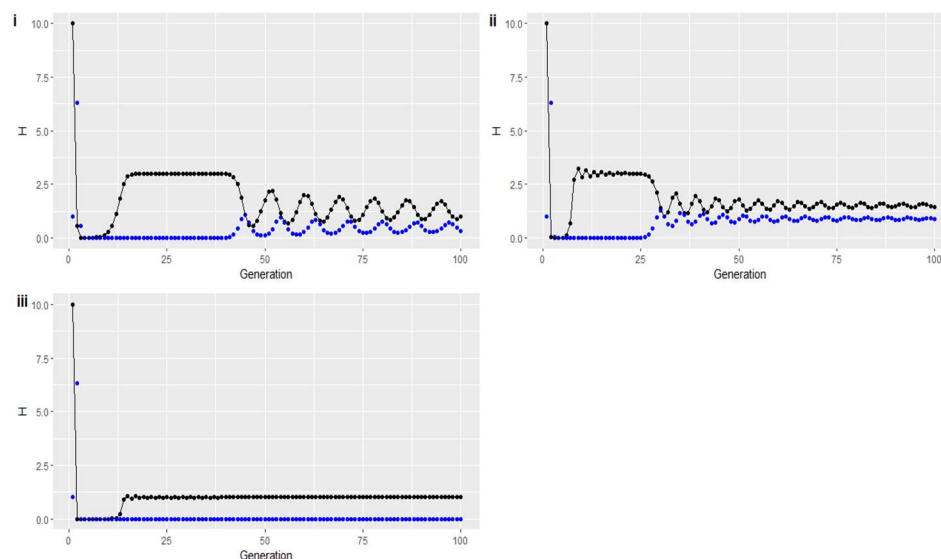
Expected Results and Implications

As there are gaps in knowledge about invasive species biology and the use of modeling for forestry management issues, I hope to demonstrate how population dynamic models such as the Nicholson-Bailey model can still be improved and serve as useful tools for host-parasitoid systems. I also hope to identify key inputs/parameters that are critical points for managers to consider in the future. Initial work on NB Host-Parasitoid dynamic models was done both in MATLAB and R, with generic parameter estimates simply to showcase model behavior and feasibility. The basic version of the NB Model along with an enhanced version including density dependence in R is shown in Figures 13 and 14. Figure 13 illustrates the main issues of the basic model, as it is unstable,

Figure 13. Basic Nicholson Bailey Model in R. Dynamics are characterized by oscillations with increasing amplitude until a population crash.

inevitably resulting in a population crash. The inclusion of density dependence in Figure 14 can result in cyclical trends of host-parasitoid interacting with each other and reducing their density before reaching stability. Inclusion of spatial dynamics was done in MatLab through the use of two distinct phases; a dispersal phase and a growth phase where the hosts and parasitoids travel through a landscape represented by cells on a 100 x 100 lattice, and then grow given the population dynamics of the NB model. Figure 15 depicts an example of this model.

To predict species distributions of both parasitoids and hosts, I will focus for now on the North-East region of the U.S with correlative SDMs. I will apply climatic and landscape variables to generate potential areas of risk of invasion from the EAB, and also the potential spread of parasitoids given biocontrol release strategies and current distributions of EAB. Assessment of models will help identify key areas of knowledge and data currently missing that should be included in future iterations.

Figure 14. Nicholson Bailey Model with Density Dependent Effects in R and differing initial inputs. From left to right and top to bottom: i) Coexistence between H/P (r=0.8, a=1.0, c=1.0, K=3.0) ii) Damped Oscillations until coexistence (r=1.8, a=1.0, c=1.0, K=3.0) iii) Extinction of parasitoid (blue points) (r=1.8, a=1.0, c=1.0, K=1.0)
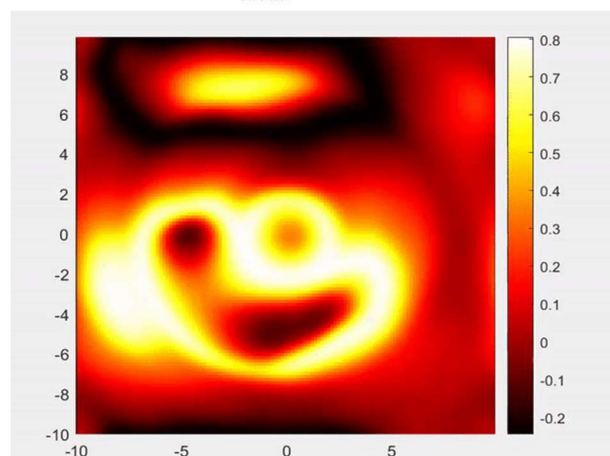
Figure 15. Spatially Explicit Nicholson Bailey Model Time-Lapse done in MatLab for host/parasitoids, both capable of diffusive movement. X and Y axes represent spatial grid lattice. Each point on plot represents density of host and parasitoid at that location.

Broader Impacts

These experiments will lead to a clearer understanding of the physiological mechanisms driving parasitoid establishment and cold tolerance. They will expand the currently available research on parasitoid wasps by creating field experiments with ecological relevance and applicability for biological control programs. This project will assist forest managers in identifying key environmental conditions and habitat characteristics that may affect introduction effectiveness and the use of more comprehensive predictive Species Distribution Models can identify regions of concern and even anticipate future distribution patterns. In the future, I hope to expand these initial experiments to the other 3 parasitoids currently in use as EAB biocontrol agents, as well as include latitudinal gradient as a possible factor for insect cold tolerance, applying this protocol to 3 other locations across the East Coast, namely NC, DE, and MA. This will provide more data on how latitudinal gradients may affect the parasitoids' deployment efficacy. This project can answer key unanswered questions about the biology and physiology of these parasitoid species that we now rely on for biocontrol in new environments and create useful predictive tools for forest managers and policymakers concerned with the impact of invasive insect species.

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