2011 Project Abstract
For the Period Ending June 30, 2014

PROJECT TITLE: Emerald Ash Borer Biocontrol Research and Implementation
PROJECT MANAGER: Monika Chandler
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06b

APPROPRIATION AMOUNT: $ 500,000

Overall Project Outcome and Results

We made great progress with the biological control for emerald ash borer (EAB) in Phase 1 of this project. We simultaneously released wasps that parasitize EAB while we studied them. EAB can kill ash trees quickly (within 6 years). We have responded rapidly to EAB finds so that we might avoid large numbers of EAB over extensive areas, a situation that would be difficult to manage effectively. At the same time, we studied the parasitoid wasps to understand their cold tolerance and dispersal capability. Our studies improved our implementation strategies.

Over 127,000 parasitoid wasps were released at 21 sites in the Twin Cities and southeastern Minnesota. Recovery of immature parasitoids in the field demonstrated that these agents are dispersing then finding and parasitizing EAB. We will continue releases in Phase 2. Research efforts demonstrated that the egg parasitoid, *Oobius agrili*, is the most cold tolerant and the larval parasitoid, *Tetrastichus planipennisi*, is the least cold tolerant. Therefore, we began releasing *T. planipennisi* earlier in the season to allow multiple generations to build a population sufficient to withstand anticipated cold induced mortality losses. We learned that *T. planipennisi* is capable of dispersing almost 5 miles within 24 hours but that most will fly ¾ miles in 24 hours. Therefore, we began releasing *T. planipennisi* over a large area at a release site rather than at a central cluster to enable faster *T. planipennisi* dispersal. Research efforts trained a total of six graduate students, five undergraduate students, and three technicians in whole or in part on these projects.

We will continue a study of ash health, EAB, and parasitoid wasps in the Twin Cities area where EAB was first found in 2009. To date, ash mortality within the study area has been substantially lower than anticipated.

Project Results Use and Dissemination

Information about this project has been and will continue to be disseminated to the public, land managers and researchers. Media releases (3) and social media were utilized to inform the public of major developments. There were 15 scientific presentations to researchers and land managers. Additional training presentations (24) were given to the public, professional land managers, and tree care professionals at many venues. Outreach at public events (20) helped us to connect with people about our activities. Two research papers on parasitoid cold tolerance were published. An additional two papers on parasitoid dispersal are anticipated. In addition, we participate in the EAB Forum, a multi-agency/organization venue for discussing EAB management. We maintain a website www.mda.state.mn.us/plants/pestmanagement/eab/eabbiocontrol.aspx with project information.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2011 Work Plan

Date of Final Report: **08/15/2014**
Date of Work Plan Approval: 6/23/2011
Project Completion Date: 6/30/2014 **Is this an amendment request?** Yes

Project Title: Emerald Ash Borer Biocontrol Research and Implementation

Project Manager: Monika Chandler
Affiliation: Minnesota Department of Agriculture
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City: St Paul **State:** MN **Zipcode:** 55155

Telephone Number: (651) 201-6537
Email Address: Monika.Chandler@state.mn.us
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Location:

Counties Impacted: Statewide

Ecological Section Impacted: Lake Agassiz Aspen Parklands (223N), Minnesota and Northeast Iowa Morainal (222M), North Central Glaciated Plains (251B), Northern Minnesota and Ontario Peatlands (212M), Northern Minnesota Drift and lake Plains (212N), Northern Superior Uplands (212L), Paleozoic Plateau (222L), Red River Valley (251A), Southern Superior Uplands (212J), Western Superior Uplands (212K)

Total ENRTF Project Budget:		
	ENRTF Appropriation $:	500,000
	Amount Spent $:	500,000
	Balance $:	0

Legal Citation: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06b

Appropriation Language:
$250,000 the first year and $250,000 the second year are from the trust fund to the commissioner of agriculture to assess a biocontrol method for suppressing emerald ash borers by testing bioagent winter survival potential, developing release and monitoring methods, and piloting implementation of emerald ash borer biocontrol. This appropriation is available until June 30, 2014, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Emerald Ash Borer Biocontrol Research and Implementation

II. PROJECT SUMMARY: Biological control is currently the only promising long-term management strategy for emerald ash borer (EAB), a beetle that is native to Asia. It was first detected in North America near Detroit in 2002 and has killed millions of ash trees. In May 2009, Minnesota's first EAB was detected in St. Paul and has since been found in Minneapolis and in a natural ash stand on the Mississippi River in southeastern Minnesota. The loss of Minnesota's nearly 1 billion ash trees, more ash on forestland than any other state, would be catastrophic. Ash-dominated sites are essential to many native plants and wildlife.

Biological control is the only potential tool to save ash that can be implemented at a forest scale. Biological control reunites the target pest with the insects or diseases that control the pest in its native range. In this case, parasitoids that control EAB in Asia would be released to control EAB in Minnesota.

The project goal is to establish biological control agent populations that suppress EAB and minimize EAB damage. Our objectives are to assess biological control agent winter survival potential, and assess establishment and spread of biological control agents after release in order to successfully implement EAB biocontrol in Minnesota.

III. PROJECT STATUS UPDATES:

Project Status as of May 31, 2012:
Biological control of EAB is making good progress. There is a synergistic collaboration among project partners. Since we started our project in July of 2011, we have made strong progress on testing biological control agent cold-hardiness (Activity 1), developed methods for laboratory studies to measure biological control agent dispersal (Activity 2), and field-released thousands of biological control agents (Activity 3). We jointly initiated an ash health, EAB, and EAB biological control agent study in the Twin Cities.

Outreach to date has included the web, media coverage, events, and presentations.

To accomplish the above:
- Students and staff were hired.
- The capital equipment purchase was made of precision controlled freezers that are in use.
- A contract was written between the University of Minnesota (U of M) and the Minnesota Department of Agriculture (MDA) to transfer ENRTF project funds from MDA to the U of M.
- Federal 526 permits to receive and research or release biological control agents were obtained.

ENRTF dollars were leveraged to secure additional USDA APHIS CPHST funding at $70,162 to provide quantities of the exact lifecycle stages of biological control agents needed for Activities 1 and 2.

Project Status as of November 30, 2012:
The biological control of EAB project is proceeding well. Data collection for testing biological control agent cold-hardiness (Activity 1) is near completion and ready for analysis. Data collection is well underway for studying parasitoid dispersal with the construction of a flight mill (Activity 2). We continue to field release biological control agents and collect data (Activity 3). Data collection is continuing for the ash health, EAB, and EAB biological control agent study in the Twin Cities.

Project Status as of May 31, 2013:
Project progress continued smoothly. All biological control agent cold-tolerance experiments are finished and a preliminary analysis completed (Activity 1). The impact of mating status, gender, size, age and feeding on parasitoid flight were measured and additional studies on impacts on temperature and humidity planned (Activity 2). Field monitoring of EAB and parasitoids continued over the winter

and plans made for the upcoming field season (Activity 3). Data collection continues for the ash health, EAB, and EAB biological control agent study in the Twin Cities.

Project Status as of November 29, 2013:
Data collection is nearly completed for research activities of parasitoid cold tolerance (Activity 1) and dispersal capacity (Activity 2). Data analysis will continue in preparation for our final report. Continuation of our ash health, EAB and EAB bioagent monitoring study was recommended for funding for an additional 3 years. Over 50,000 parasitoids were released during the 2013 field season at sites in Twin Cities and southeastern Minnesota. We are beginning to recover parasitoids in the field and received a definitive species confirmation. Continuation of EAB biocontrol implementation was recommended for funding for an additional 3 years.

Amendment Request November 29, 2013
Amendment request from 11/29/13 was approved
Activity 1 budgetary changes: This part of the project is on track to finish on budget. We are requesting permission for three variances between categories:

1. Move $3,000 from Supplies to Salaries. The original proposal included funds to raise the parasitoids in our laboratory. This effort proved to be difficult and inefficient. USDA APHIS provided all parasitoids needed for this portion of the project and created a project savings for this line item.
2. Move $2,500 from Travel to Salaries. Travel has been lower than originally estimated because most field work has been accomplished without the need for overnight stays.
3. Move $1,640 from Equipment to Salaries. The original equipment estimates did not account for discounts that the University receives from some suppliers.

We are requesting the additional funding for graduate-student, undergraduate-student, and technician salaries for the processing of samples from the in-field overwintering study, data analysis, and write-up of the final report.

Please note that technician salary was reported in the Activity 1 column of the May 2013 status report but should have been in the Activity 2 column as it appears in this Nov 2013 report.

Activity 2 budgetary changes: This part of the project is on track to finish on budget. We are requesting permission for two variances between categories:

1. Move $2,000 from Supplies to a new category, Services. Services include repair of environmental growth chamber (condenser breakdown during temperature flight trials) ($1900) and scientific poster printing for research dissemination at local meetings ($100).
2. Move combined $8,000 from Travel to Salaries due to increasing benefit rates over past three years. Travel has been lower than expected due to a) travel scholarships of students on project, b) purchasing a vehicle with non-project funds as cost-savings vs. annual leasing, and c) proximate work in metro area.

Activity 3 budgetary changes: We are requesting permission for the following variances between categories.

1. Move $4,500 from Travel meals and lodging to Travel vehicle/mileage ($1,000), Supplies ($500) and Salaries ($3,000).
2. Move $300 from Equipment to Salaries.

We anticipated that EAB would spread quickly throughout the state and much overnight travel would be needed. Fortunately, EAB has not been found throughout the state so we request to spend the funds on salary for monitoring/branch sampling.

Retroactive Amendment Request: September 17, 2014
Activity 1 budgetary changes: We are requesting permission for the following changes to reflect exact amounts spent in each category.

1. Increase personnel from $134,740 to $136,102.

2. Decrease capital equipment from $12,360 to $12,131.
3. Decrease supplies from $3,000 to $1,994.
4. Decrease travel meals and lodging from $500 to $373.

Activity 2 budgetary changes: We are requesting permission for the following changes to reflect exact amounts spent in each category.

1. Decrease personnel from $153,100 to $152,937.
2. Decrease services (equipment repair) from $2,000 to $1,917.
3. Increase supplies from $4,000 to $4,068.
4. Decrease travel vehicle/mileage from $900 to $650.
5. Increase travel meals and lodging from $2,000 to $2,429.

Activity 3 budgetary changes: We are requesting permission for the following changes to reflect exact amounts spent in each category.

1. Increase personnel from $173,300 to $175,685
2. Decrease supplies from $2,000 to $1,574.
3. Decrease travel vehicle/mileage from $9,100 to $7,858.
4. Increase travel meals and lodging from $3,000 to $2,282.

Amendment Approved: 10/07/14

Final Report Summary:

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IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Assess biological control agent winter survival potential

Description: Cold hardiness of *S. agrili* and *T. planipennisi* will be assessed using established laboratory methods to measure the insect supercooling point, lower lethal temperature, and lower lethal times and field studies to measure actual agent survival. Temperatures experienced by the bioagents will be measured with thermocouples beneath the bark on various parts of the tree. This research will be completed by a graduate student, Anthony Hanson, and one undergraduate student advised by Dr. Robert Venette with the Forest Service and the University of Minnesota. This study complements Dr. Venette's research on EAB larval cold weather survival potential.

Summary Budget Information for Activity 1:

ENRTF Budget:	**$ 150,600**
Amount Spent:	**$ 156,600**
Balance:	**$ 0**

Activity Completion Date: 06/30/2014

Outcome	Completion Date	Budget
1. Measure bioagent cold hardiness for two species	06/30/2014	$108,100
2. Develop predictive model and map of expected bioagent survivorship	06/30/2014	$42,500

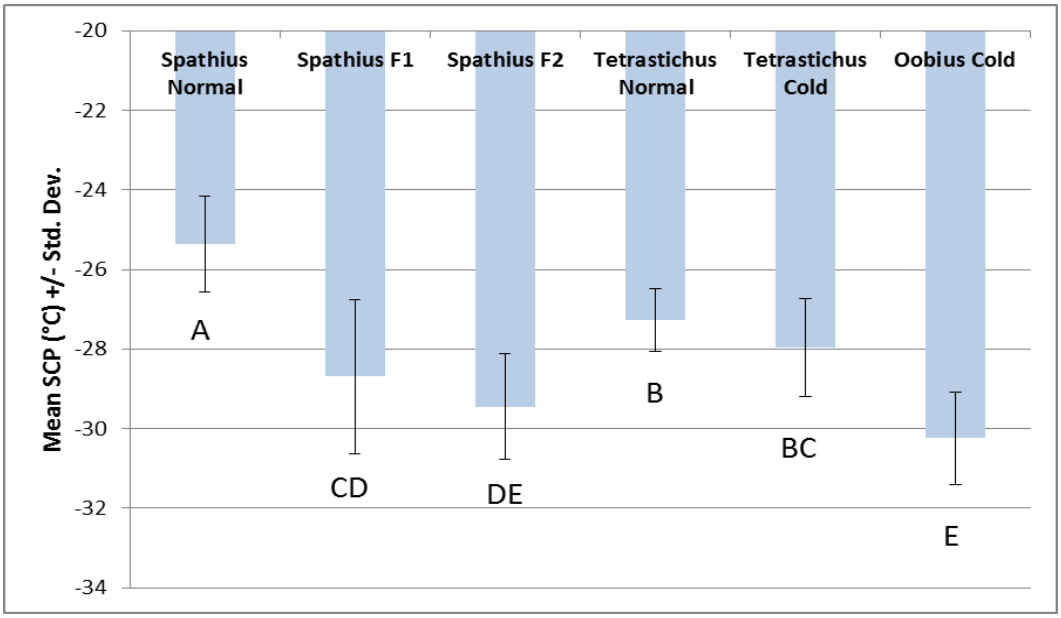
Activity Status as of May 31, 2012:
Parasitoid cold tolerance testing is progressing well. To determine cold tolerance, the following tests are almost completed.

1. Supercooling Point = the temperature at which an insect freezes
2. Lower Lethal Temperature = the lowest temperature that the insect can survive
3. Lower Lethal Time = the duration that an insect can survive at a specific temperature

For each species, comparisons are made between parasitoid larvae that were acclimated to cold before testing vs. parasitoid larvae that were not acclimated. Additional tests were performed on *S. agrili* to determine whether cold tolerance varies as individuals are put into diapause. A combination of these factors will used to determine cold tolerance.

The methodology is working well and the tests are approximately 75% completed. The developmental biology of the biological control agents slows progress. For example, it takes 16 weeks after cold exposure to break diapause. Data will be analyzed after test completion.

Figure 1. Preliminary average supercooling points (± standard deviation) of emerald ash borer parasitoids that were acclimated (cold) or not (normal) to cold temperatures before testing. Bars with the same letter are not significantly different (Tukey's HSD at α=0.05).



The supercooling point of three parasitoid species (*Spathius agrili*, *Tetrastichus planipennisi*, and *Oobius agrili*) is one factor that will be used to determine cold tolerance.

We are in the process of analyzing temperature records from the winter of 2011-2012 beneath the bark of cut logs and trees.

Budget note: Salary funds for the graduate and undergraduate students working on Activity 1 were covered to date from another funding source. Use of ENRTF funds for their salaries will begin in the fall of 2012.

Activity Status as of November 30, 2012:
Parasitoid cold tolerance testing is completed for both larval parasitoid species and lifecycle stages with the exception of S. agrili emergence from their 16 week diapause (see Lower Lethal Temperature). Results of these studies indicate *S. agrili* may be more cold tolerant than *T. planipennisi*.

Supercooling Point: Acclimation to shorter days and cooler temperatures decreases the likelihood that *S. agrili* will freeze at very cold temperatures (50% freezing at -29°C and 100% freezing at -34°C for the sampled individuals). In contrast, *T. planipennisi* did not show a difference after exposure to shorter days and cooler temperatures (50% freezing at -28°C and 100% freezing at -29°C for the sampled individuals).

Lower Lethal Temperature: Initial determinations of non-survival after exposure to cold temperatures were made by visually inspecting the larvae or pre-pupae (overwintering stage for each species) for discoloration or lack of movement. Final determinations of survival will also include adult emergence after exposure to warm temperatures for a sufficient period. The measure of adult emergence for *S. agrili* is ongoing until December, 2012. For *S. agrili*, survival of pre-pupae was increased by cold acclimation (50% mortality at -27.5°C and >99% mortality at -32.5°C). The close correlation of supercooling point and lower lethal temperature for *S. agrili* indicates that unlike some insect species, *S. agrili* is not freeze tolerant. It was difficult to cold acclimate *T. planipennisi* larvae as evidenced by high control mortality. For this reason, cold acclimated larvae were not used for these tests, but it is possible that cold acclimation could increase cold tolerance. The adult emergence measure was completed for this test and incorporated into results (50% mortality at -24.5°C and >99% mortality at -27°C). High mortality levels at temperatures above the supercooling (freeze) point indicate that *T. planipennisi* is chill-intolerant.

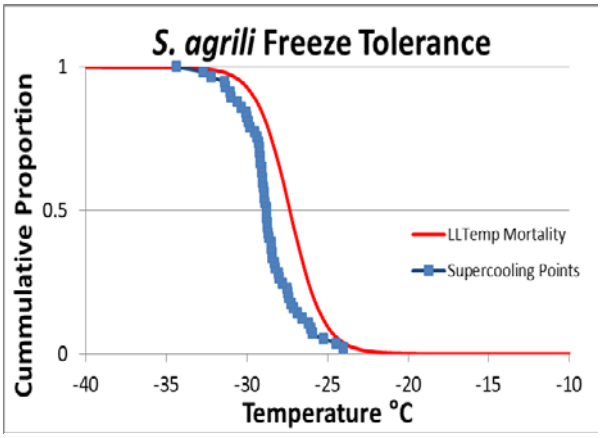


Figure 2. *S. agrili* lower lethal temperature 3 days after cold exposure and supercooling point distribution of pre-pupae reared under 1 generation diapause conditions.

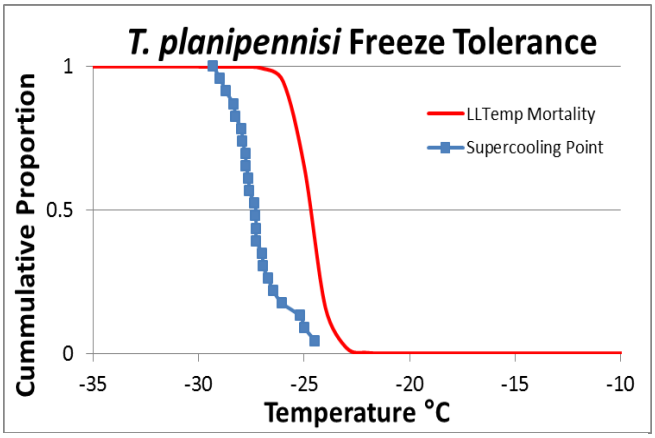


Figure 3. Lower lethal temperature based on adult emergence and supercooling point distribution of *T. planipennisi* reared under warm/long conditions.

Lower Lethal Time: Larvae and pre-pupae (overwintering stage for each species) were held at 0, -5, -10, or -15°C for 3, 14, 28, 56, or 84 days.

Table 1: Mortality induced by prolonged exposure to cold temperatures as measured by the number of days at specific temperatures.

Temperature	***Spathius agrili***		***Tetrastichus planipennisi***	
(°C)	**25% Mortality**	**50% Mortality**	**25% Mortality**	**50% Mortality**
0	83 days	>84 days	79 days	>84 days
-5	83 days	>84 days	76 days	>84 days
-10	83 days	>84 days	41 days	61 days
-15	61 days	84 days	23 days	32 days

The next step is to analyze these results in the context of long term climate data and recorded temperatures beneath bark.

Activity Status as of May 31, 2013:
Both species of larval parasitoids appear to be sufficiently cold tolerant for southern Minnesota but may be less suitable to northern Minnesota. In all parts of the state there is likely to be mortality during cold winters.

The impact of winter climate on population dynamics is related to population growth rate. Although S. agrili appears to be more cold tolerant than *T. plannipennisi*, *S. agrili* has only 1-2 generations per year and produces 1-18 progeny per EAB larva. The population growth rate is slow. In contrast, *T. planipennisi* has 3-4 generations per year and produces 4-172 progeny per EAB larva. The higher population growth rate of *T. planipennisi* may allow it to withstand winter mortality losses better than S. *agrili*.

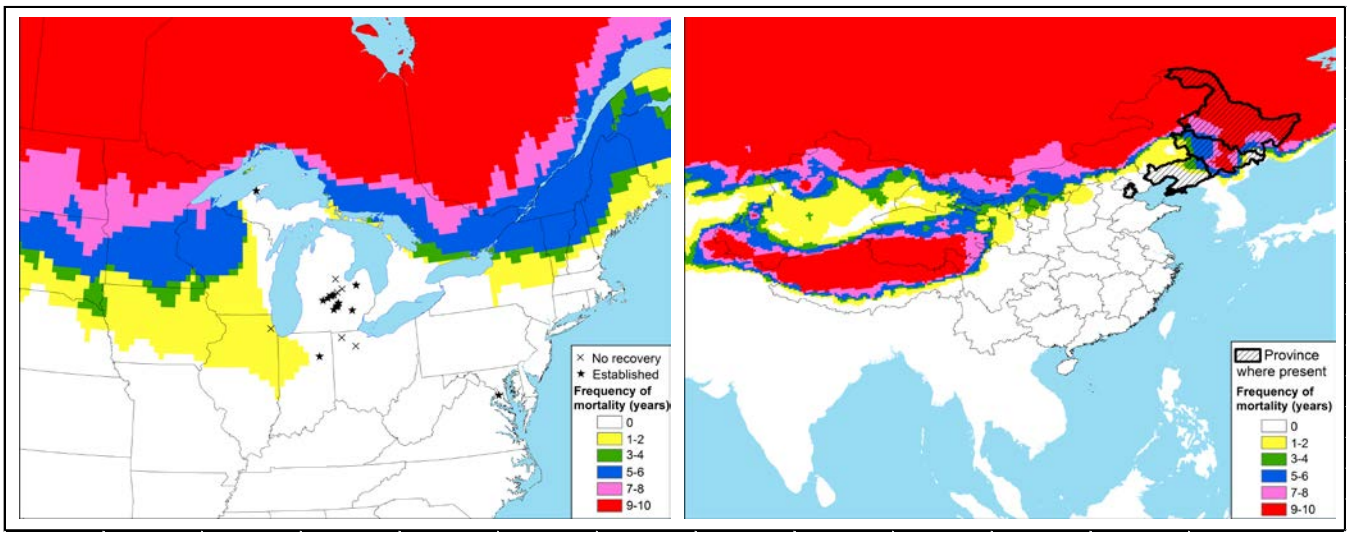


Figure 4. Frequency of North American (left) and Chinese (right) winters from 2002-2012 cold enough to cause 90% mortality of overwintering *Tetrastichus planipennisi* larvae. The native range of *T. planipennisi* in China is marked in hatch.

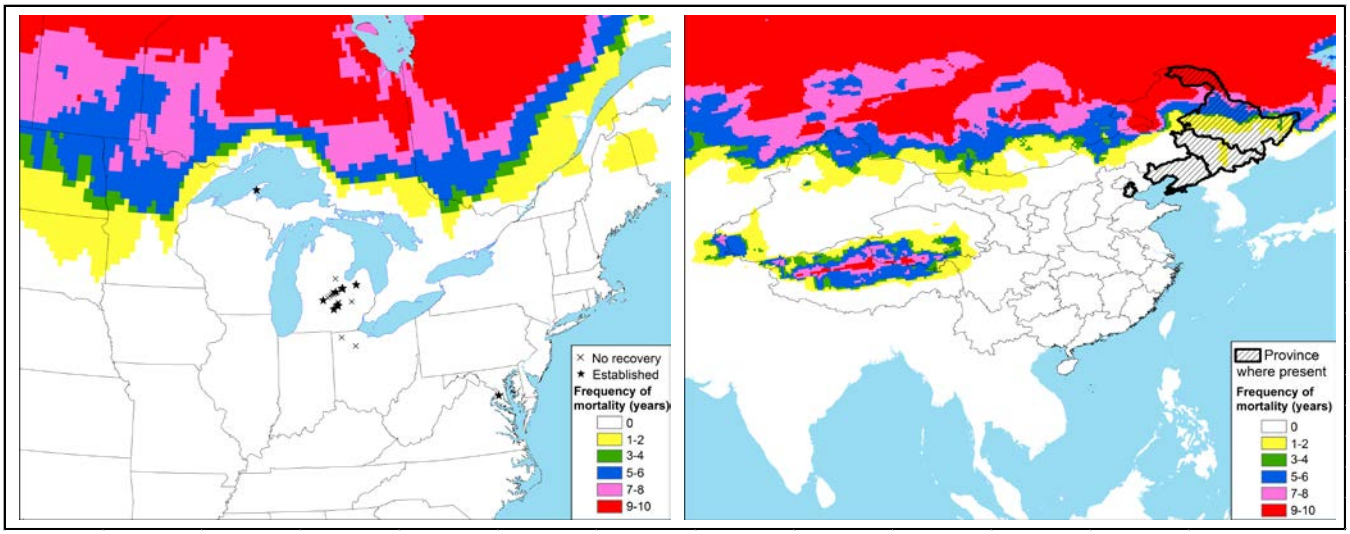


Figure 5. Frequency of North American (left) and Chinese (right) winters from 2002-2012 cold enough to cause 90% mortality of overwintering *Spathius agrili* pre-pupae. The native range of *S. agrili* in China is marked in hatch.

An important management recommendation is to prioritize early season releases of biological control agents to allow as many generations as possible to reproduce and the population to grow. Winter mortality of the overwintering generation will not have as large of an impact on the overall population.

Upcoming activities include studying cold tolerance of the egg parasitoid*, Oobius agrili*, and enclosing parasitoids on EAB infested branches to research their overwintering in the field.

Activity Status as of November 29, 2013:
Unfortunately, there was not a sufficient population of the egg parasitoid*, Oobius agrili*, available for cold tolerance research. This research is above and beyond our work plan commitments, but we had hoped to complete this work and may be able to at a future date.

The larval parasitoid, *Tetrastichus planipennisi*, was enclosed with EAB larvae in mesh covered cages on ash trees at Great River Bluffs State Park to study overwintering. Twenty-four cages were set up. Samples were collected from 12 cages this fall to determine the condition of the parasitoids going into winter. Data will be collected from these samples over the winter. Samples will be collected from the remaining 12 cages in spring 2014 to assess parasitoid condition after winter.

Final Report Summary:

Major conclusions

- Of the three parasitoid species that have been approved to control emerald ash borer, the egg parasitoid *Oobius agrili* appears to be the most cold-tolerant, while the larval parasitoid *Tetrastichus planipennis* is the least cold-tolerant. *Oobius agrili* had lower supercooling points than any other species tested (i.e., colder temperatures were required before this insect began to freeze). *Tetrastichus planipennisi* did not appear to acclimate to colder temperatures or shortened day-lengths. The third parasitoid species, *Spathius agrili,* did acclimate to cold temperatures as it went into diapause in response to shortened day-lengths and cooler temperatures. Both *T. planipennisi* and *S. agrili* were classified as chill intolerant (i.e., individuals may succumb to cold before they freeze). These results appear in the publication: Hanson, A.A., R.C. Venette, and J.P. Lelito. 2013. Cold tolerance of Chinese emerald ash borer parasitoids: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). Biological Control 67: 516-529.
- New tools were needed to measure the temperatures of tiny insects during cold tolerance testing. A new thermocouple design was developed and tested during the course of this project. The new thermocouple design, known as a 'cradle', proved to be more sensitive than traditional thermocouples. Thus, we are more confident in our assessments of the cold tolerance of emerald-ash-borer parasitoids, particularly measurements of supercooling points, than we otherwise would have been. The design and testing of the new thermocouple are described in the publication: Hanson, A.A., and R.C. Venette. 2013. Thermocouple design for measuring temperatures of small insects. Cryoletters 34: 261-267.

Management implications

- Our results suggest that *O. agrili* is least likely to freeze at temperatures that are common during Minnesota winters. This species might be best suited to overwinter in Minnesota and might be preferred for future releases (but see 'Future Research').
- *Tetrastichus planipennisi* is vulnerable to winter cold. As such, for this species to survive the winter, releases should be conducted in spring. This strategy will allow populations of the parasitoid to build to large numbers. If large populations are present in the fall, the probability that at least some individuals will survive the winter improves.
- Although *S. agrili* is more cold-tolerant than *T. planipennisi*, the Animal and Plant Health Inspection Service of the US Department of Agriculture is no longer rearing *S. agrili* for release at northern latitudes.

Future research

- At the start of this project, individual *O. agrili* were scarce, primarily because laboratory rearing techniques were still rudimentary. As a result, we were only able to focus on one dimension of insect cold hardiness. During the course of this project, rearing of *O. agrili* has improved. We would like to evaluate the lower lethal time of these insects (i.e., the effects of brief exposure to sub-freezing temperatures on survival) and lower lethal temperature (i.e., the extent of mortality at different periods of time when held at constant low temperatures). These two measures would substantially improve our ability to forecast the winter survival of this insect and would provide stronger support for the conclusion that this species is, indeed, the most cold tolerant.
- An initial attempt to measure winter survival of *T. planipennisi* in the field failed. The main stems of 24 green ash trees in Great River Bluffs State Park were artificially infested with emerald-ash-borer eggs. Cages were placed on the trees and *T. planipennisi* were released into the cages. That fall, half of the trees were harvested to confirm establishment of the parasitoids. The plan was to harvest the remaining trees in the spring and to evaluate the extent of parasitoid winter mortality. However, we recovered no parasitoids from the fall samples. Insect predators (especially ants and earwigs) were common on the trees and may have impacted the eggs that were put into trees or the parasitoids that were put in the cages. A recent report also suggests that *T. planipennisi* is unable to parasitize larvae in trees that are greater than 3 inches in diameter because the bark becomes too thick. Our pilot taught us how to refine the study for future trials. We would like to repeat this study, but on smaller-diameter

branches or younger trees, with barriers to exclude the insect predators. Ultimately, we would like to elucidate the true overwintering capacity of *T. planipennisi* and subsequent emergence patterns of this insect in the subsequent spring.

- USDA-APHIS continues foreign explorations and testing of new parasitoid species to control emerald ash borer. Species currently being tested, such as *Spathius galinae* from Russia or an unidentified *Oobius* species from South Korea, may be more cold tolerant than the three species from China. It would be useful to evaluate the cold tolerance of these new species. Our laboratory is the only facility to have provided pre-release cold tolerance testing of emerald ash borer parasitoids.

ACTIVITY 2: Examining parasitoid establishment and dispersal
Note: Activity 2 was revised from the submitted proposal based upon peer review comments to the research addendum.

Description: The goal is to determine movement and potential establishment of parasitoids of EAB from the release site. Biological control agent traps will be placed at incremental distances of up to 1km from the sites where biological control agents have been or are being released (e.g., 0, 100, 200, 500, 1000m in four cardinal directions, or at points along an arc). These traps will serve to monitor EAB parasitoids emerging from the release site over time. This research will be conducted by a graduate student and one undergraduate student advised by Dr. Brian Aukema at the University of Minnesota. This study complements Dr. Aukema's existing work on landscape ecology, movement patterns, and spatiotemporal modeling of insects undergoing range expansion events.

Summary Budget Information for Activity 2:	**ENRTF Budget:**	**$ 162,000**
	Amount Spent:	**$ 162,000**
	Balance:	**$ 0**

Activity Completion Date: 06/30/2014

Outcome	Completion Date	Budget
1. Identify monitoring points within/around release sites	10/01/2013	$ 63,800
2. Determine dispersal gradient of parasitoids	06/30/2014	$ 98,200

Activity Status as of May 31, 2012:
A graduate student, Samuel Fahrner, has been recruited to work on this project. In June 2011 after consultation with Drs. Julie Gould and Leah Bauer (USDA Forest Service), an array of yellow pan traps at increasing distances around two local release sites were deployed to detect dispersal of biological control agents. It is thought that yellow pan traps can be more efficient at sampling larval parasitoids than sentinel logs. A month of monitoring yielded negative results. This was disappointing but not unexpected. Biological control agents frequently require months to establish and reproduce, and we know little about their density-dependent dispersal. Over the winter we developed laboratory studies to understand reproduction of different densities of biological control agents on different densities of immature EAB. These results, integrated with better understandings of dispersal capacities and lower lethal temperature limits, will ultimately inform site selection and release strategies.

To further address dispersal of biological control agents, project partners initiated a field study of ash health, EAB, and EAB biological control agents in the Twin Cities. This is a large, collaborative project with multiple partners including USDA Forest Service, the Department of Natural Resources, and the cities of Falcon Heights, Minneapolis, Lauderdale, Roseville, and St. Paul. Three hundred ash trees were selected in the late summer of 2011. Each tree will be monitored for three years. We collect data on tree size and health. We are using branch sampling methodology to subsample ash trees for evidence of EAB and EAB biological control agents. Two branches per year are removed from each tree for three years. A length is cut from each branch then peeled. Detailed information is collected on each EAB gallery, EAB larvae, biological control agent parasitoids, and native parasitoids. Data collection for this study is coordinated by Jonathan Osthus with MDA (Activity 3), and will be analyzed

by Drs. Aukema (Activity 2) and Venette (Activity 1). The first year of branch sampling was completed in mid-April. We learned that this method, though labor-intensive, can detect EAB in asymptomatic trees and improve data resolution in areas where EAB are known to occur. We did not find any surprise infestations outside of the known infested area to date.

Activity Status as of November 30, 2012:
Parasitoid flight capability will inform decisions about where to release and possibly recover parasitoids and increase the accuracy of estimates of population spread in the field. We are studying the dispersal potential of *Tetrastichus planipennisi* using 24 custom, computer-monitored flight mills. This sophisticated system collects robust data on a large number of individuals. We learned that *T. planipennisi* can fly long distances – up to 7 km (4.3 miles) within a 24 hour period with a mean flight speed of 0.8 km per hour. *Tetrastichus planipennisi* has the potential to move very far over its adult lifespan (mean adult lifespan of 45 days in the lab) and multiple generations per year. We will continue flight studies to determine the effects of age, feeding, mating status, sex, size, and temperature on the flight patterns. We plan to correlate these data with field release and recovery data.

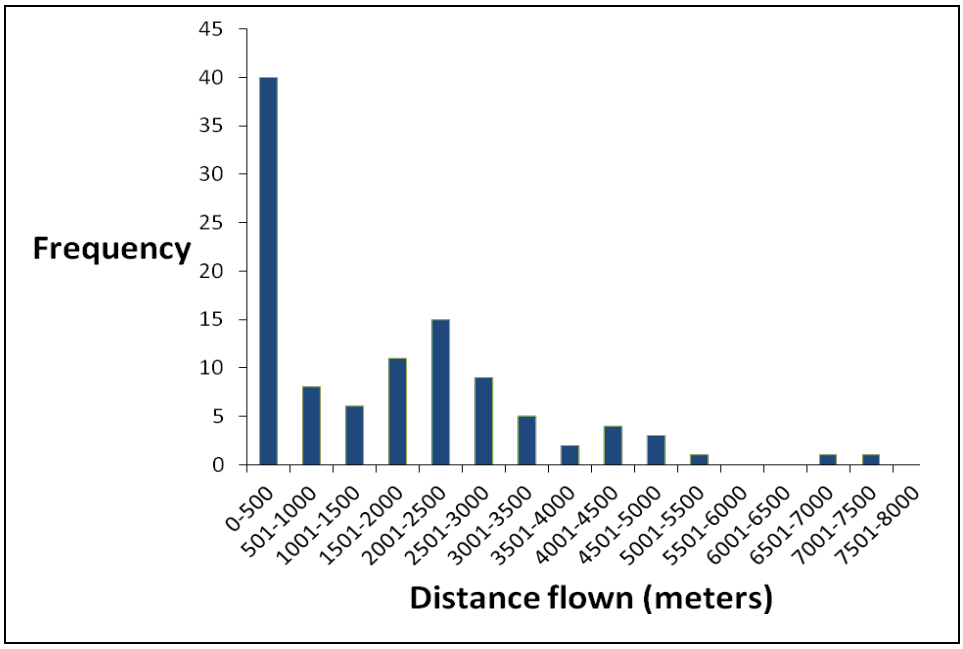


Figure 1. Measured distance frequency that *Tetrastichus planipennisi* flew in a 24 hour period on a flight mill.

Unlike the other EAB biocontrol parasitoid species*, T. planipennisi* is well-suited to flight mill studies. Although a strong flier in the field, *Spathius agrili* will not fly when attached to a flight mill. *Oobius agrili* is simply too small to attach to a flight mill.

In the previous status report we stated that we planned an EAB larval density dependence study on the flight pattern of larval parasitoids. We decided to study flight capability instead after learning that other researchers had studied parasitoid flight response to EAB larval density. We will use their findings in addition to our flight studies to better understand parasitoid dispersal.

Budget note: In lieu of an undergraduate student worker, a recent graduate with applicable experience was hired on this project. The total salary and fringe budget amounts are not changed.

Project partners continue to work jointly on the ash health, EAB, and EAB bioagent monitoring study. Ash health data were collected last summer. Approximately 1/4 of the branches have been cut, peeled and data collection for the second year of sampling. Of the 300 tree initially selected, 298 were sampled in the winter of 2011/2012 (2 trees were removed before sampling began). 260 trees will be sampled again in the winter of 2012/2013 (39 trees were removed since initial study tree selection). 20 infested trees were identified through branch sampling in 2011/2012.

Activity Status as of May 31, 2013:
The studies of flight behavior of *T. planipennisi* were expanded to study the effects of several factors on dispersal capacity (e.g., sex, size, age, feeding status). Our goal is to understand what conditions will result in the best coverage upon release. On average, females flew farther than males. Further studies revealed this is likely because females are larger and larger *T. planipennisi* fly farther. Age did not affect flight distance until the parasitoids were very old – they appear to be viable into their second month post hatching from their pupal cases. Mating status also did not affect flight distance. Feeding the parasitoids sugar enabled them to fly much farther than starved parasitoids. The starved parasitoids barely flew. This highlights the importance of having a nectar source for the adults to feed on. The parasitoids that we receive and release are fed honey before release. Our work confirms that this pre-release step is critical and suggests that the parasitoids may perform well in field settings where there are available nectar sources.

Future studies on the effect of temperature and humidity on flight will be conducted.

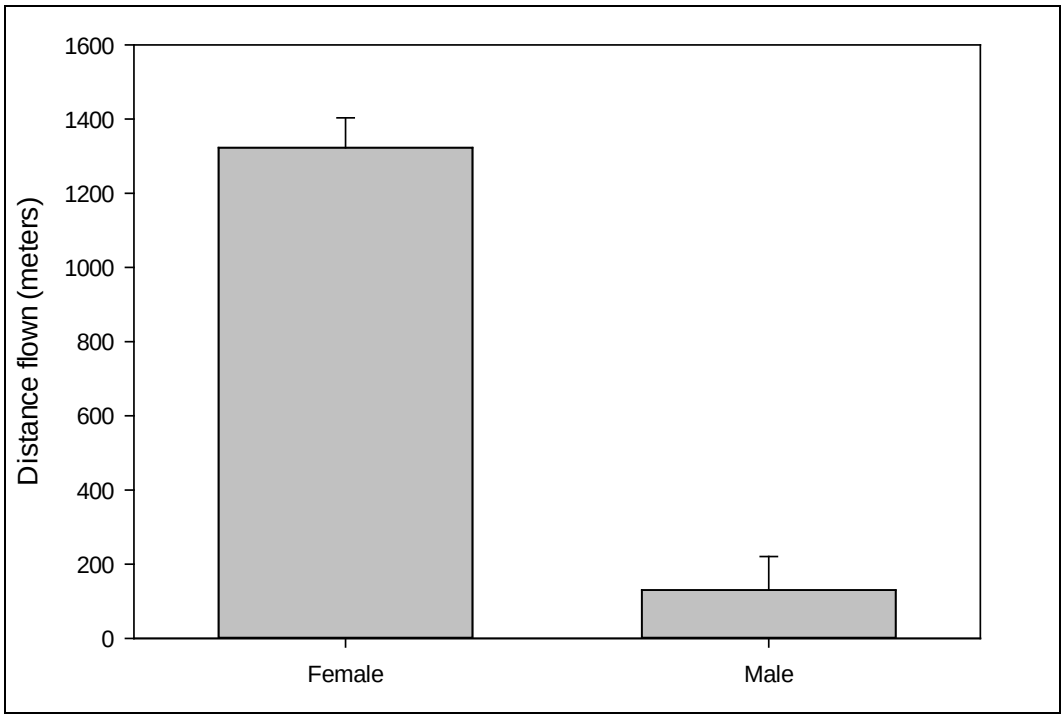


Figure 2. Distance flown by female and male parasitoids. Each parasitoid used in this study was flown once for a 24-hour period. Flight distances were measured using 24 custom-built computer-monitored flight mills. Parasitoids were fed a dilute honey solution and water prior to flight.

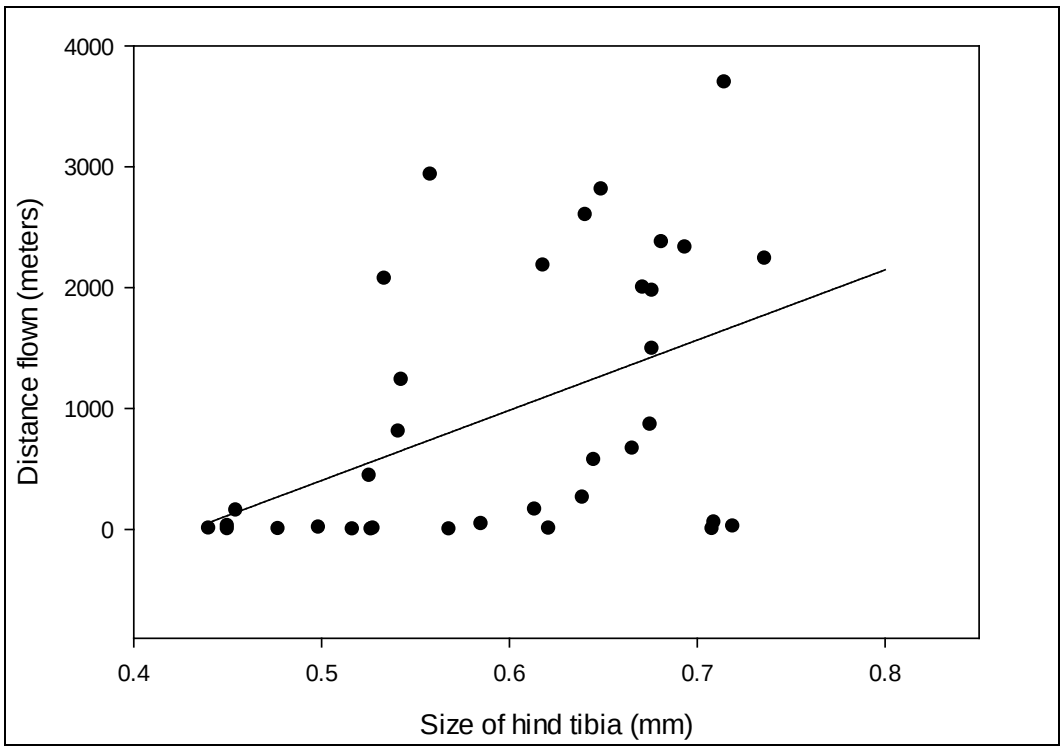
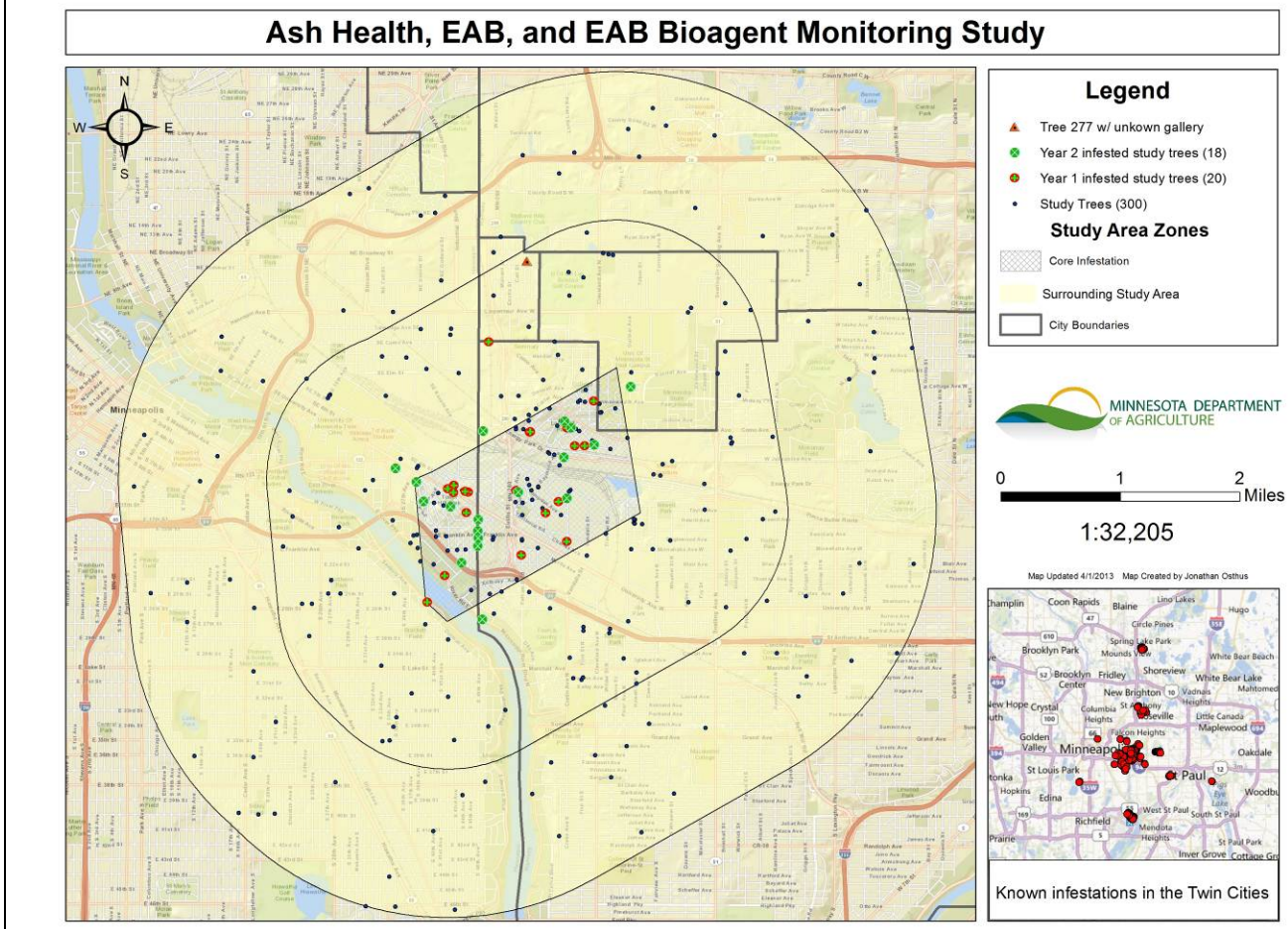


Figure 3. Parasitoid size (as measured by the size of a hind leg segment) as an indicator of flight distance potential. Each parasitoid used in this study was flown once for a 24-hour period. Flight distances were measured using 24 custom-built computer-monitored flight mills. Parasitoids were fed a dilute honey solution and water prior to flight. Parasitoid size was determined by measuring the mean of the hind tibias.

Project partners continue to work jointly on the ash health, EAB, and EAB bioagent monitoring study. Branch sampling has been completed for the year with overall positive results. 18 infested trees were identified or about 7% of the study trees sampled. Also, EAB gallery densities remained quite low in the majority of the branch samples. The results highlight the impact that management is having in the core infestation area where much higher rates of infestation were expected with how long EAB has been established.



Activity Status as of November 29, 2013:
Studies of EAB and the larval parasitoid, Tetrastichus planipennisi, flight continue and will be completed in early 2014. Mean flight speed increases with temperature for EAB. EAB appears to have higher mortality rates after flying at high speed. We will study mortality as it relates to temperature and flight speed.

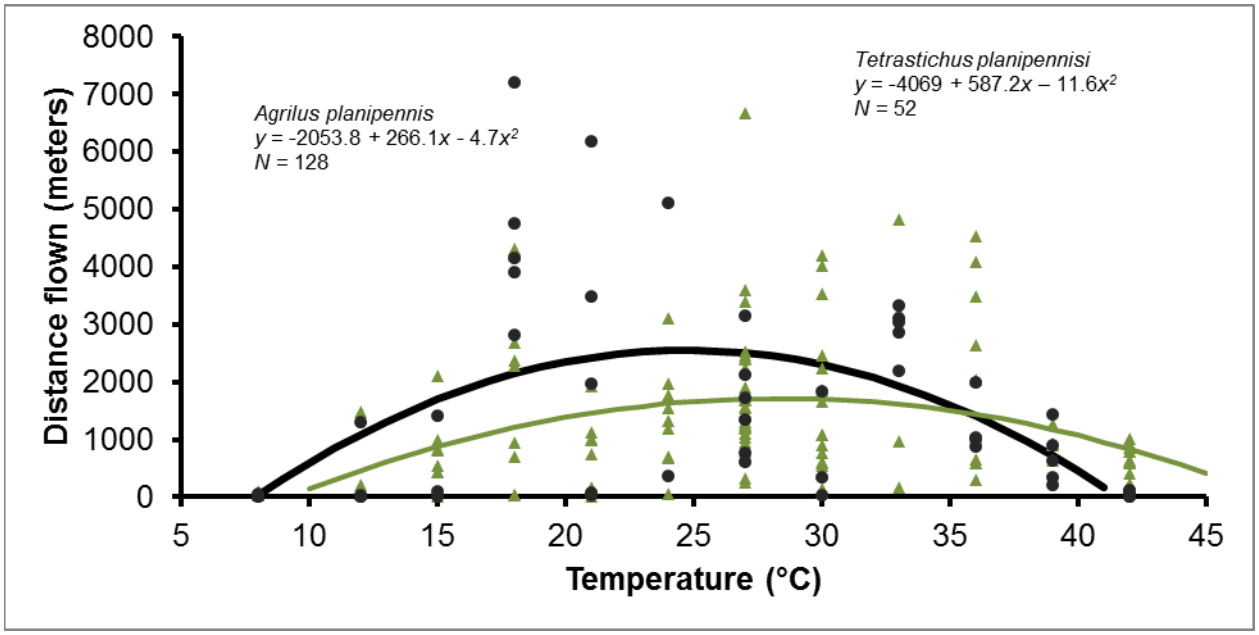


Figure 4. Dispersal capacity of emerald ash borer, *Agrilus planipennis*, and the larval parasitoid, *Tetrastichus planipennisi*. Insects were flown at increments of temperature between 8 and 42° C. All insects were tethered to computer-monitored flight mills and were flown continuously for one 24-hour period. Flight mills were placed inside of environmental chambers and insects were not provided water or nutrition during flight trials.

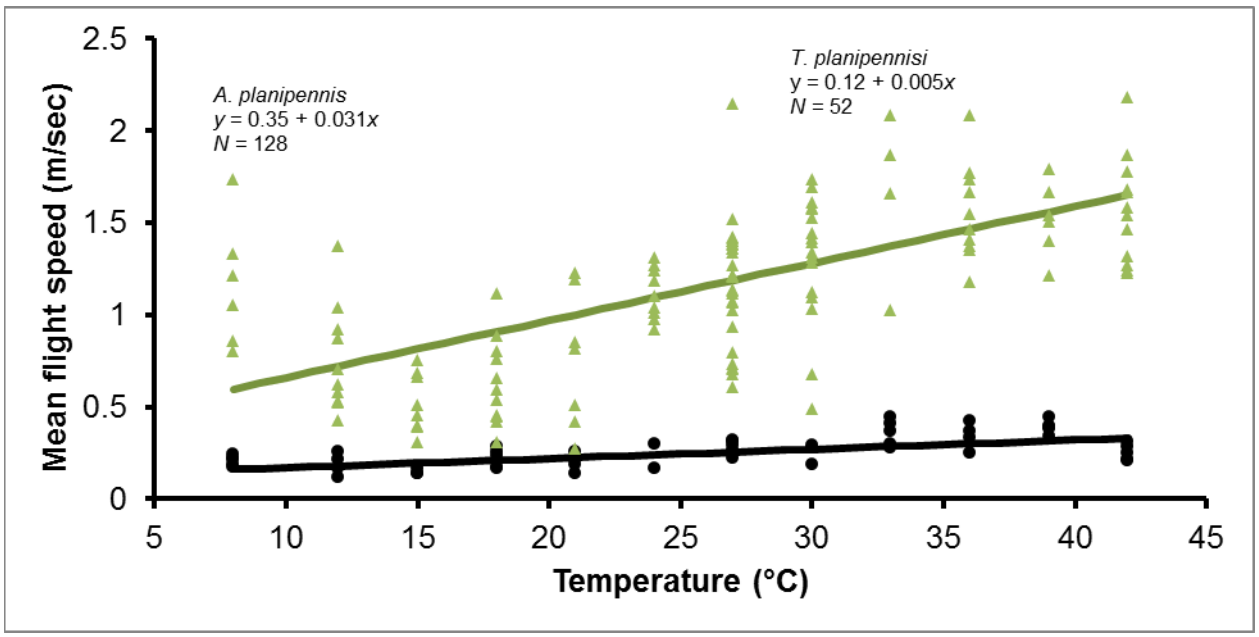


Figure 5. Flight speed of emerald ash borer, *Agrilus planipennis*, and the larval parasitoid *Tetrastichus planipennisi*. Insects were flown at increments of temperature between 8 and 42° C. All insects were tethered to computer-monitored flight mills and were flown continuously for one 24-hour period. Flight mills were placed inside of environmental chambers and insects were not provided water or nutrition during flight trials.

Ash health data were collected from all ash health, EAB and EAB bioagent monitoring study trees. Branch sampling has started and will continue over the winter.

Final Report Summary:
We have learned a great deal about dispersal capacity of *Tetrastichus planipennisi* (aka "Tets") during the last three years of opportunity provided by ENRTF funds. Below we provide an overview of challenges in field studies with Tets and subsequent adjustments to study flight capacity across a range of environmental conditions on a state-of-the-art laboratory flight mill.

When we commenced this project, initial studies of dispersal of biological control agents on the urban and natural landscape provided little information. Despite several lines of traps and recapture attempts in as little as six hours post-release, we were not able to recapture any biological control agents using standard yellow pan traps. Indeed, to date in Minnesota, very few Tets have been recovered post-release in comparison to other states (see Activity 3). This is likely indicative of low pest densities to date in Minnesota. For example, the state detected EAB very early in the Twin Cities urban environment (2009), and densities of the pest have not yet built to tree-killing levels where biological control agents would typically cluster. We suspect there may be an advantage to our strategies of "carpet-bombing" these stingless wasps into low density infestations, from whence they spread (see Activity 3).

We have concentrated our efforts on laboratory studies over the past three years. We contributed additional funds from a McKnight Land-Grant faculty award to construct a laboratory flight mill. This award, distinguishing Prof. Aukema as a top early-career faculty within the University of Minnesota system, allowed us to study flight energetics of the Tets and EAB across a range of situations. Research findings have included:

- Flight distances of female Tets representative of populations released in the biological control program average 3/4 miles in 24 hours. Half of them do not fly farther than 450 yards, however. The farthest a female will fly is almost 5 miles.
- Larger females fly farther than smaller ones.
- Females who feed on a honey-water solution prior to flight fly 40X farther than those who do not fly. Tets that do not receive honey-water do not fly very far at all. Clean, uncontaminated nectar sources in the field may be critical to success of these biological control agents.
- Females fly very well for 10 weeks after they hatch. We do not know if their eggs are viable for that long, but these data strongly suggest that Tets are viable biological control agents for two months after emerging.
- Both Tets and EAB fly fastest at warmer temperatures (95-100F). However, on the flight mill, they tended to die quickly at these warmer temperatures. This may change in field situations where they can stop and feed on dew or rainwater or nectar.
- Overall, Tets and EAB fly the farthest at temperatures between 80-85F. They can engage in flight all the way down to 55F, however.
- In Minnesota, our temperature data suggest that Tets should be able to fly through early to mid-October. This estimate is based upon temperature equations developed in the laboratory and the past 10 years of meteorological data from the MSP airport.

These studies formed the basis of a MS thesis for Samuel Fahrner, who received the 2014 Peterson Award for MS thesis research in the Department of Entomology at the University of Minnesota. Both thesis chapters have been or are being disseminated to reputable scientific peer-reviewed journals. Final copies of these papers will be submitted when available.

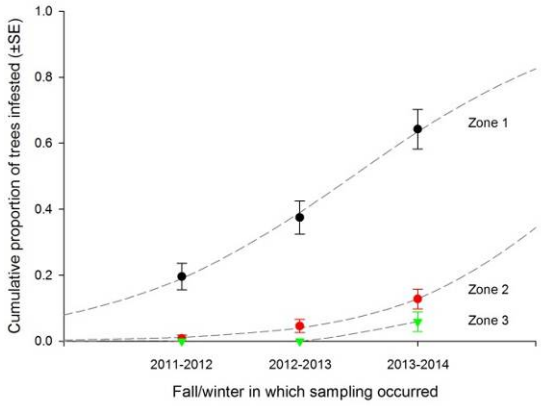
Fahrner, S.J., Lelito, J.P., Blaedow, K., Heimpel, G.E., and B.H. Aukema. (2015) Factors affecting the flight capacity of *Tetrastichus planipennisi* (Hymenoptera: Eulophidae), a classical biological control agent of emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae). *Environmental Entomology*. Accepted pending minor revisions.

Fahrner, S.J., Lelito, J.P., and B.H. Aukema. (2015) The effect of temperature and humidity on the flight capacity of *Tetrastichus planipennisi* (Hymenoptera: Eulophidae) and its host, *Agrilus planipennis* (Coleoptera: Buprestidae). To be submitted to *Biocontrol*.

Tracking the EAB infestation core

Major conclusions

- Mortality of ash in the study area has been substantially less than anticipated. Earlier research suggested that 100% of ash in Hennepin and Ramsey counties would be killed by emerald ash borer five years after the insect was first detected in 2009. Of the 300 trees in this study, none have been killed by emerald ash borer. A total of 68 trees were found to be infested, and of those 58 were removed or treated with insecticide. At the time of detection, often only one or two galleries were present in branch samples. This small number of galleries suggests that many infestations were found early.



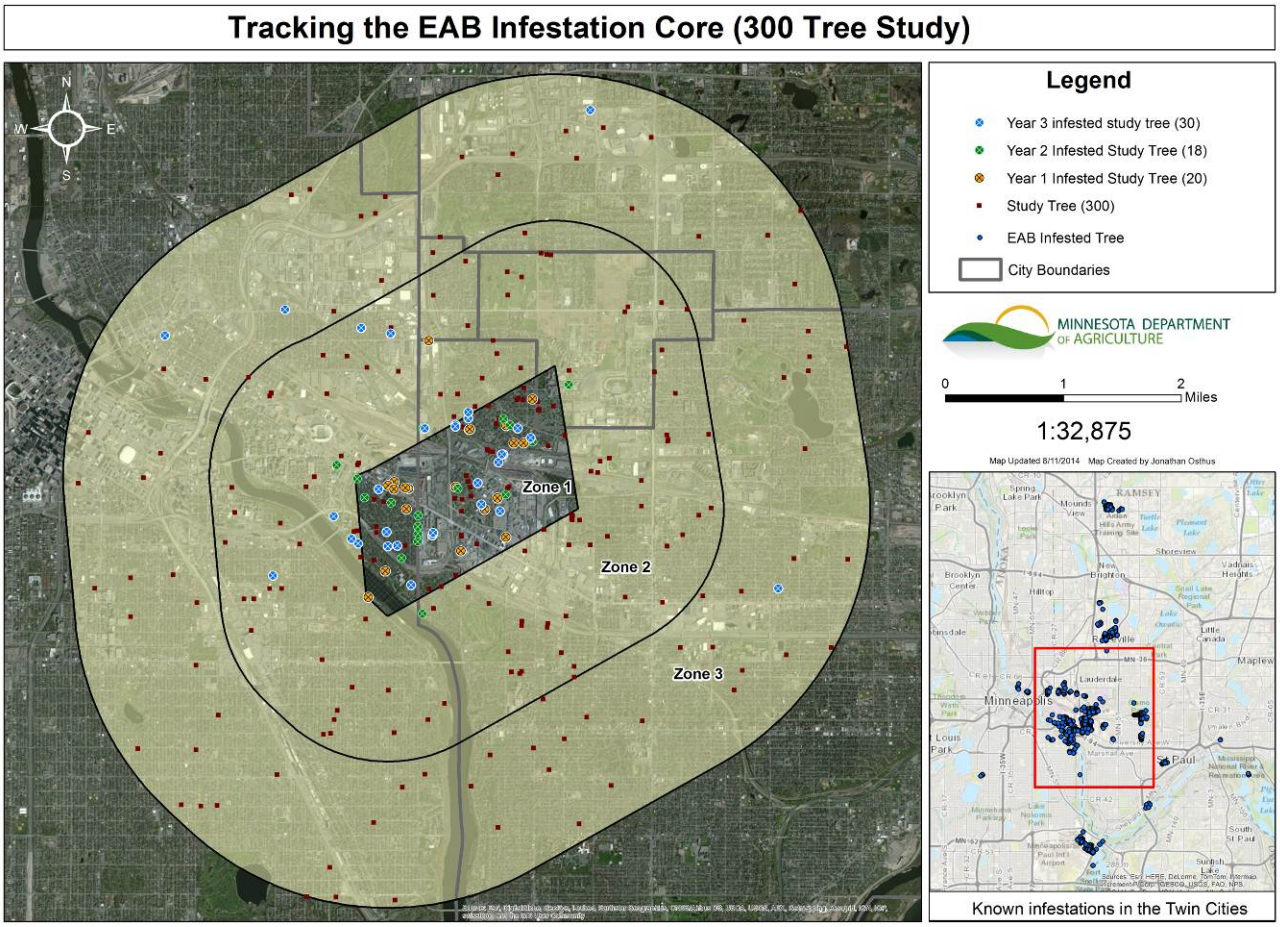
- Approximately 64% of the ash trees in the core area (Zone 1) are infested, while only 13% are infested in Zone 2, and 6% are infested in Zone 3.
- The rate at which the proportion of infested trees is increasing is the same in the core area (Zone 1) and the adjacent land (Zone 2). Fewer trees were initially infested in Zone 2 than Zone 1. None of our study trees in Zone 3 were found to be infested until the 2013-2014 sampling was completed.
- No parasitoids were recovered from these trees. This result suggests that the parasitoids are either not established or population densities are still below detectable levels.

Management implications

- The similar rate of increase in Zone 1 and Zone 2 suggests that emerald-ash-borer management in the core area is not slowing the spread in adjacent areas. Cities may need to intensify emerald-ash-borer management.
- Releases of parasitoids in and around the Twin Cities continue to be justified.

Future research

- Observations of these 300 trees will continue under Phase II of the biological control implementation project. New trees will be added to replace those that were treated or removed to maintain the sensitivity of the monitoring network.
- This study provides one of the most rigorous in-field assessments of the efficacy of emerald-ash-borer management techniques in the nation. Our approach provides a reliable estimate of the proportion of ash trees that are infested in each zone. We are currently using volunteers to estimate the number of publically-owned ash trees in each zone. With that information, we can estimate the number of publically-owned ash trees that are likely to be infested in each zone. We can then compare this number with records of tree removals or insecticide treatments to determine the proportion of infested trees that were managed. These values provide a basis to intensify management activities as needed.



ACTIVITY 3: Coordinate Minnesota's biological control implementation

Description: Strategic implementation of EAB biocontrol will require coordination, communication, and facilitation with other agencies, private landowners, and the general public. Potential release sites will be assessed and information related to field releases will be tracked. A new position will be created within the Plant Protection Division at MDA to coordinate implementation.

Summary Budget Information for Activity 3:

ENRTF Budget:	**$ 187,400**
Amount Spent:	**$ 187,400**
Balance:	**$ 0**

Activity Completion Date: 06/30/2014

Outcome	Completion Date	Budget
1. Webpage developed for outreach	12/30/2011	$ 500
2. Phase one implementation strategy for Minnesota developed	06/30/2012	$ 50,000
3. Potential release sites delimited and assessed	04/30/2014	$ 77,800
4. Field data collected and entered into database	06/30/2014	$ 59,100

Activity Status as of May 31, 2012:
EAB biological control implementation scaled up in response to new EAB finds in 2011 and the hiring of Jonathan Osthus as the EAB Biocontrol Coordinator at MDA. Jon has years of experience working with EAB and was up to speed quickly. There were new EAB finds in Shoreview, at the Summit and Dale area of St. Paul, at several sites in Winona County, and a single trap catch in La Crescent. Biological

control agents were released at all of these sites in addition to existing sites in Houston County and the Twin Cities. The one exception is La Crescent. We received permission from the city to release but we have not found the EAB infestation pocket yet. This will determine where we release.

Bioagent releases were conducted throughout the 2011 field season for a total of 30,717 released.

	Biological Control Agent Species			
Site Name	Spathius agrili	Tetrastichus planipennisi	Oobius agrili	All Species
E. River Pkwy 1	269	973	260	1,502
E. River Pkwy 2	745	2,531	660	3,936
Great River Bluffs State Park 1	521	1,103	0	1,624
Great River Bluffs State Park 2	899	2,663	0	3,562
Houston Release 1	138	842	200	1,180
Lamoille	234	282	0	516
Langford Park	809	2,398	394	3,601
Shoreview 1	888	1,536	338	2,762
Summit & Dale	736	1,983	0	2,719
Tower Hill Park	1,050	2,970	774	4,794
W. River Pkwy 1	1,307	2,199	1,015	4,521
Totals	7,596	19,480	3,641	30,717

Data were collected according to USDA APHIS guidelines and entered into a MDA database. Data were sent to the national database for EAB biocontrol. In addition, 10 release trees in Minneapolis were felled because they were confirmed infested. These trees were peeled, but no bioagents were recovered. This is in keeping with bioagent recoveries in other states at least two years after release. We are still at one year after release at our sites.

Releases have begun for the 2012 field season. We anticipate releasing bioagents at all sites and will consider new sites as additional EAB detections are reported.

EAB detection and defining the leading edges of infestations are critical to site selection for bioagent releases. Therefore much effort is put into coordinating data collection for the ash health, EAB, and EAB bioagent monitoring study (details in Activity 2 section). Cutting and peeling 600 branches was a lengthy and labor intensive task completed over the winter. Visual surveys of infested areas were completed to better understand the extent and severity of infestations. In addition, we started a pilot study called EAB STUC. Male EAB beetles fly over ash leaves searching for female beetles then land nearby to investigate. We exploit this behavior by setting traps of dead female beetle decoys covered with sticky spray to catch the live males – they get STUC (Stick Traps Using Cadavers). Results of this preliminary study will determine whether this method is employed on a larger scale.

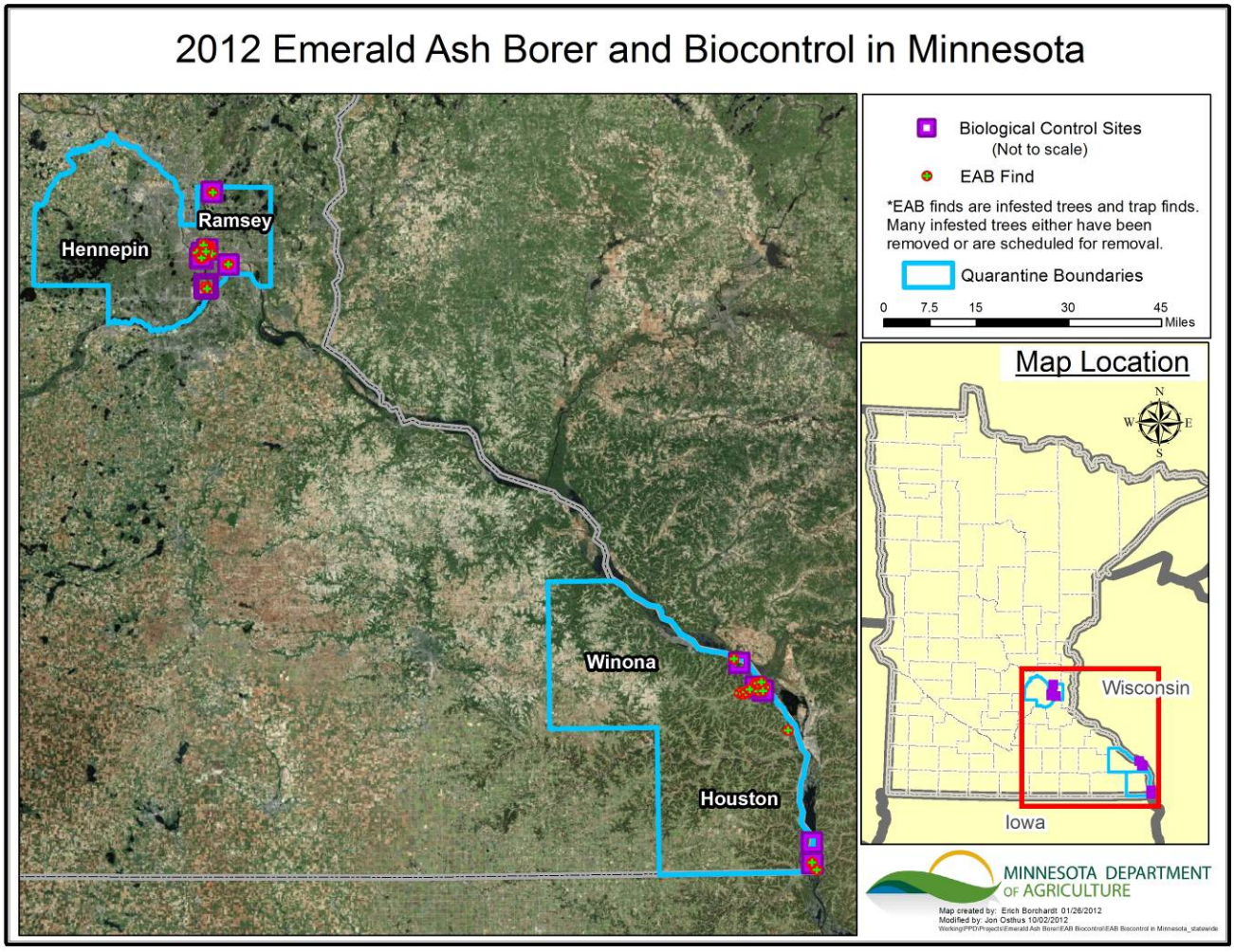
This study will be conducted during EAB's peak flight season. We selected 125 trap trees in Falcon Heights, Lauderdale, Minneapolis and St. Paul. Five dead beetles are placed on different areas of the tree and covered with Tanglefoot®, a sticky substance. STUC is modeled on field research by Penn State University conducted in Michigan. This will be the first implementation test of this methodology. This is a collaborative project with the University of Minnesota, USDA, and cities of Minneapolis and St. Paul.

In March 2012, we hired an undergraduate student worker to help with all of the branch and log peeling and the EAB STUC pilot study. He worked part-time during the school year and started full-time in mid-May.

Activity Status as of November 30, 2012:
Biological control agent releases continued at all established sites and were initiated at the Fort Snelling golf course in response to a new EAB find.

Release Site	2010 Total Released	2011 Total Released	2012 Total Released	All Years Site Total
E. River Pkwy 1	0	1,502	2,532	4,034
E. River Pkwy 2	0	3,936	6,788	10,724
W. River Pkwy 1	0	4,521	5,861	10,382
Tower Hill Park	0	4,670	4,176	8,846
Summit & Dale	0	2,719	4,223	6,942
Langford Park	0	3,601	5,016	8,617
Shoreview 1	0	2,762	3,429	6,191
Fort Snelling	0	0	2,557	2,557
Houston Release 1	3,326	1,180	2,948	7,454
Lamoille	0	516	441	957
GRB SP 1	0	1,624	1,346	2,970
GRB SP 2	0	3,686	3,389	7,075
GRB SP 3	0	0	2,615	2,615
All Sites Total	**3,326**	**30,717**	**45,321**	**79,364**

GRB SP = Great River Bluffs State Park



Data were collected for biological control agent releases. Monitoring data collection will continue throughout the winter with intensive branch and tree sampling.

We completed our test of EAB STUC (Sticky Traps Using Cadavers) and determined that it was not an effective method for detecting and delimiting EAB populations at low levels. Neither the STUC nor adjacent purple traps caught a single EAB beetle. Therefore, we will not employ this method unless there is a significant increase in the EAB population.

Activity Status as of May 31, 2013:

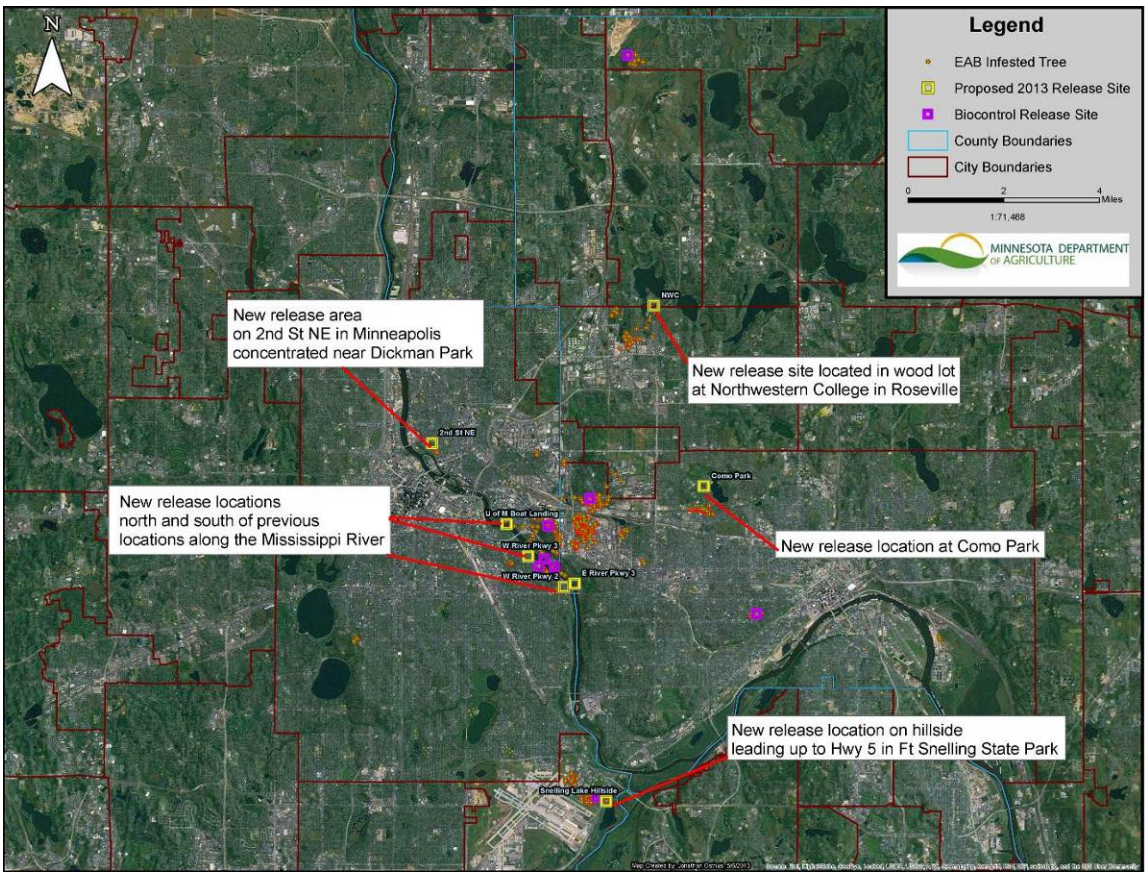
Sampling and Visual Survey: During the winter, 48 metro and 6 southern Minnesota trees from bioagent release areas were sub-sampled to gauge EAB density and look for EAB parasitoids. Visual survey was also used at all sites. EAB density was low in most trees with a few notable exceptions of medium to high density at Great River Bluffs State Park.

Logs from two sample trees (not release trees but near release trees) at Great River Bluffs State Park were brought back to MDA to be peeled carefully in the lab. Four parasitoid samples were recovered from EAB pupal chambers in one of the logs. Two different types of cocoons were observed and were sent to Dr. Juli Gould with USDA APHIS PPQ in Massachusetts and a parasitoid expert. One type appears to be a native parasitoid in the genus Atanycolus. Atanycolus has been documented attacking EAB in other states but has not been documented in Minnesota. The other type has not been found associated with EAB in other states. Multiple tiny wasps of an undetermined species emerged from one of these samples and a wasp in the family Crabronidae (species undetermined to date) emerged from a cocoon. These are solitary wasps that nest in wood cavities so are unlikely to have anything to do with EAB except use the galleries. No parasitoid samples of the species released have been recovered to date.

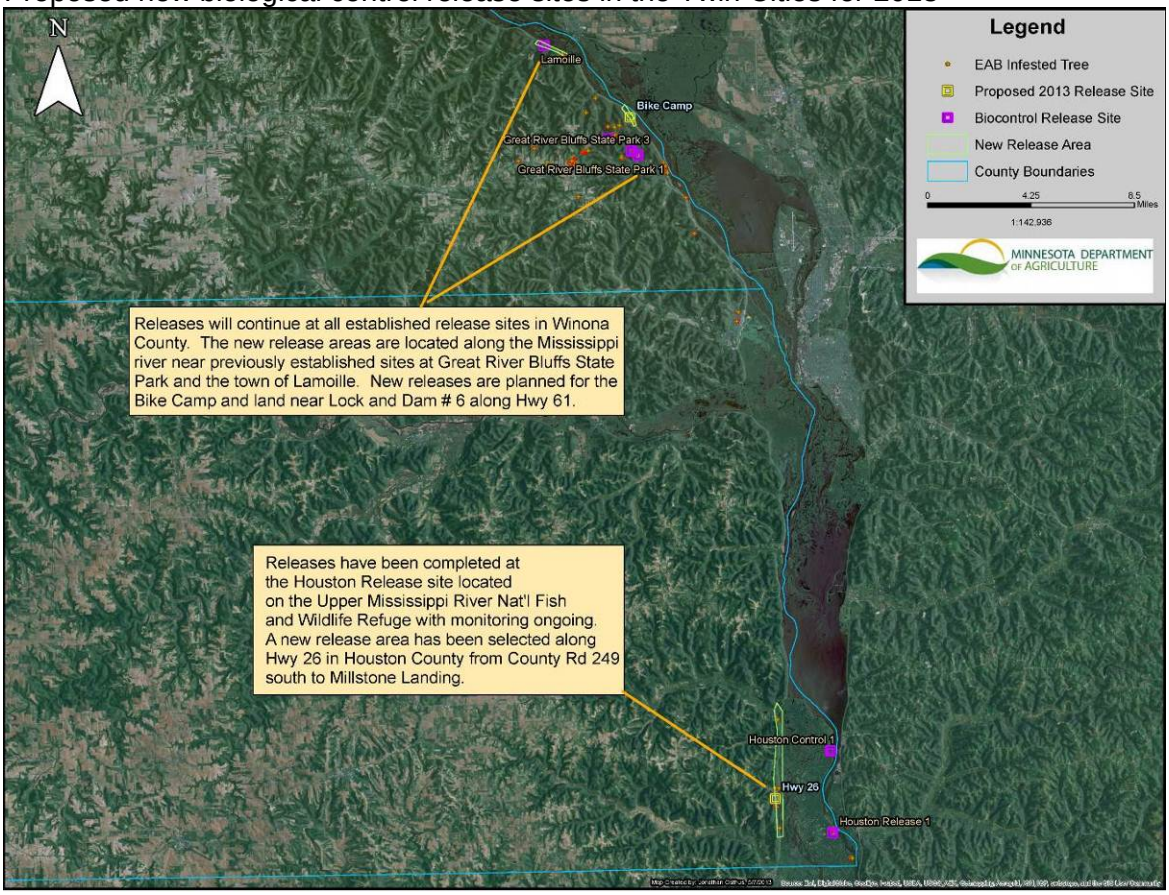
Planning: Meetings were held with individual project partners to discuss progress, sampling results and plan for the upcoming field season. This included planning for infestations that were detected over the winter. The EAB infestation density was greater at new finds in the metro than at known sites where the infestation had been detected at an earlier stage and managed.

- **New Sites:** Five metro and three southeastern Minnesota areas were selected to initiate releases in 2013. Bioagent availability for releases may be a limiting factor.
- **Continuation Sites:** Releases at three metro (Shoreview, Summit and Dale and Fort Snelling) and four southeastern (Lamoille and 3 sites at Great River Bluffs State Park) will continue in 2013.
- **Completed Release Sites:** Federal guidelines for releases are to release for two years, wait an additional year then monitor for parasitoid recovery. The E. River Pkwy 1 & 2, W. River Pkwy 1, Tower Hill Park, Langford Park and Houston 1 sites will not receive additional releases but will be monitored.

We are working with federal and state partners to increase releases at sites over broad areas rather than in tight clusters of ash trees.



Proposed new biological control release sites in the Twin Cities for 2013



Proposed new biological control release sites in southeastern Minnesota for 2013

Activity Status as of November 29, 2013:
Over 51,000 parasitoids were released at 14 sites over the field season. Releases were made on a weekly basis alternating between southeast Minnesota and the Twin Cities. Ash health data were collected from both new and existing release sites (20 sites total). Data were entered into state and federal databases.

Region	2013	2012	2011	2010	All Years
Twin Cities	16,703	34,582	23,711	0	74,996
SE MN	34,473	10,739	7,006	3,326	55,544
Total	**51,176**	**45,321**	**30,717**	**3,326**	**130,540**

We receive excellent support from USDA APHIS and MN DNR for our parasitoid release efforts. 40,000 (30% of statewide total) wasps were released at Great River Bluffs State Park from 2011-2013. This is one of the few sites selected nationwide for "carpet bombing" the site with high release numbers. The site was selected because of its location in the heart of a large EAB infestation in SE MN and its appeal to EAB (sunny and open with ash trees stressed from prescribed fire). The site is strategically located with EAB movement corridors of the Mississippi River to the east and Interstate 90 to the south.

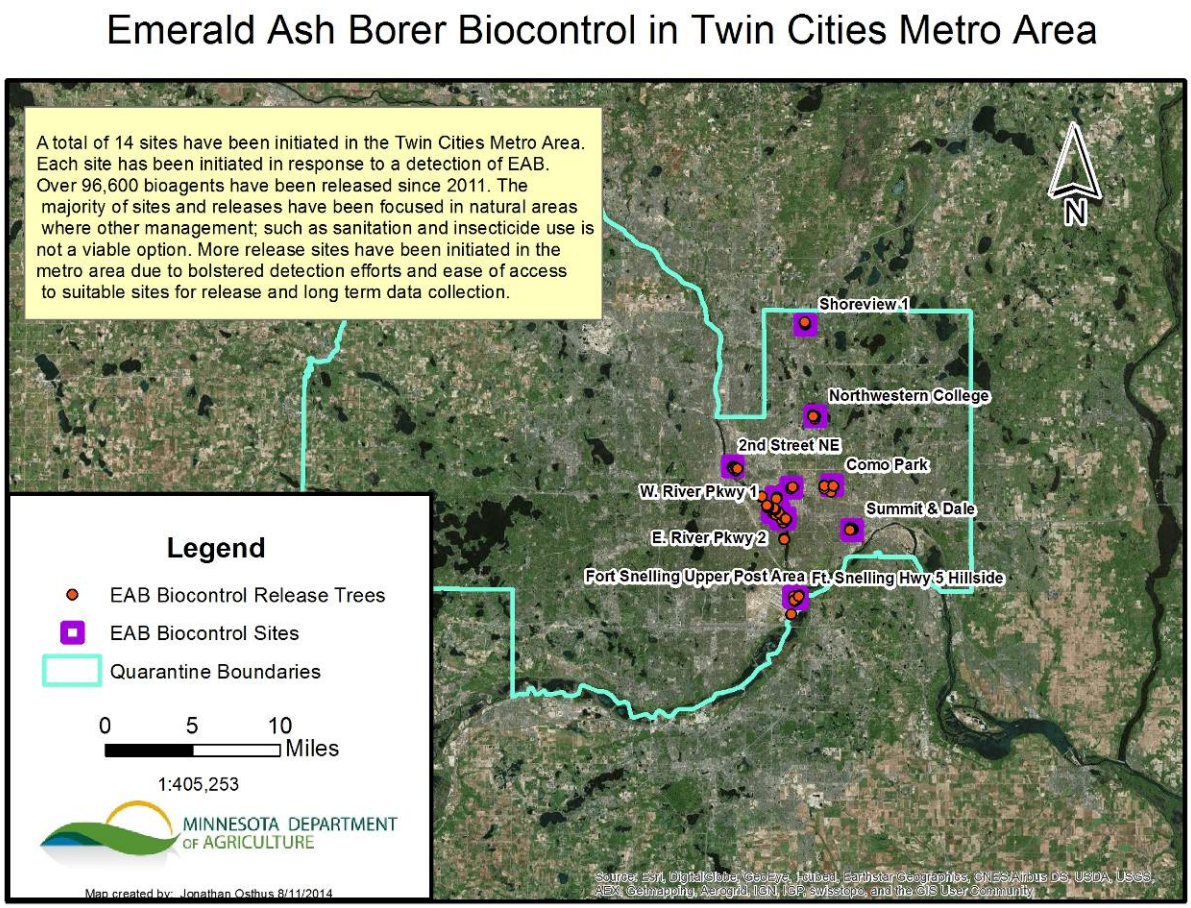
We are beginning to recover parasitoids. MDA found *Tetrastichus planipennisi* larvae that had consumed most of an EAB carcass in an EAB gallery (pictured below) on 10/23/13 at Great River Bluffs State Park. A second clutch of parasitoid larvae were found on 11/01/13. MDA emerged several adults and received a definitive species confirmation from USDA APHIS PPQ. These parasitoid finds confirm that *T. planipennisi* is attacking EAB and reproducing in the field. Also, we would know that the parasitoids are dispersing well. These larvae were found approximately 0.5 miles from the nearest release site. Releases of *T. planipennisi* were initiated in fall 2011 at the park.


EAB carcass and the developing parasitoid larvae that consumed it found within an EAB gallery.

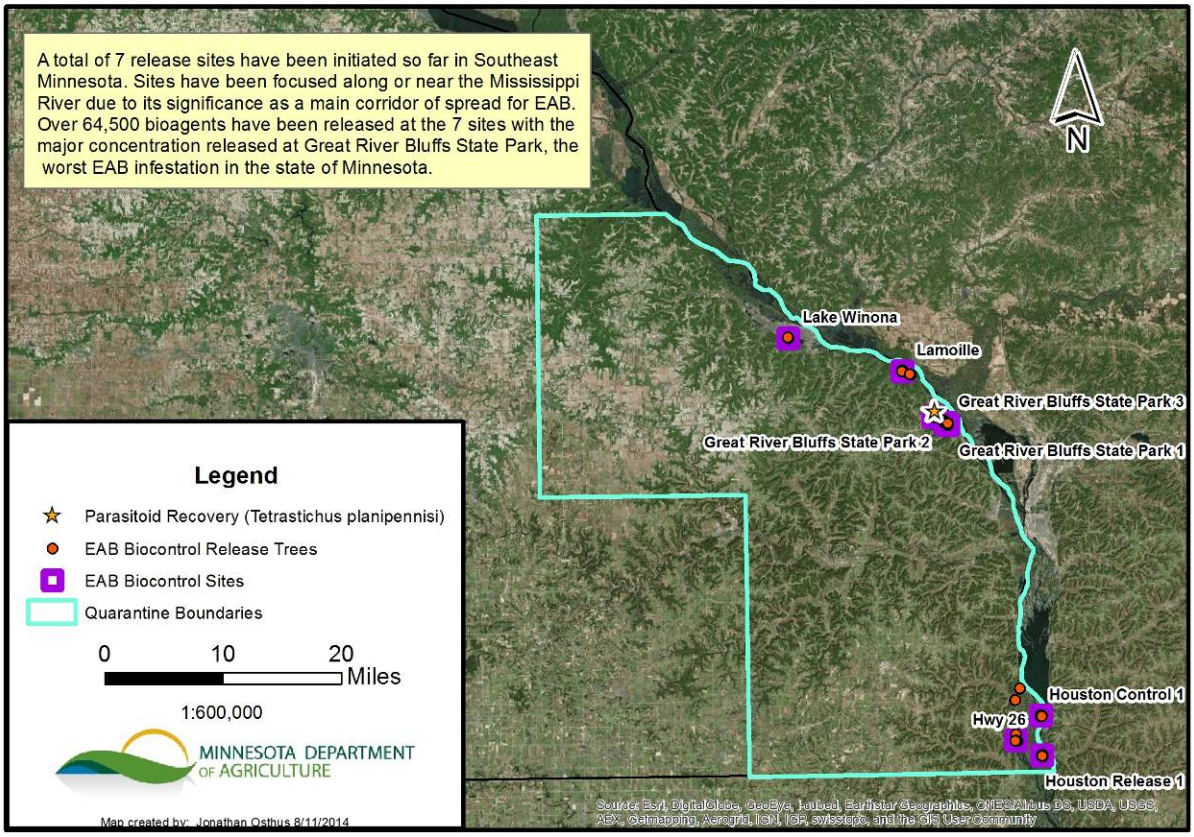
Upcoming plans include continued ash health monitoring. This will include the Duluth area due to the August 2013 EAB find in nearby Superior, WI.

Final Report Summary:
Strategies for implementing biocontrol improved over the project based upon our research findings. Research efforts demonstrated that the egg parasitoid, *Oobius agrili*, is the most cold tolerant and the larval parasitoid, *Tetrastichus planipennisi*, is the least cold tolerant. Therefore, we began releasing *T. planipennisi* earlier in the season to allow multiple generations to build a population sufficient to withstand anticipated cold induced mortality losses. We learned that *T. planipennisi* is capable of dispersing almost 5 miles within 24 hours but that most will fly ¾ miles in 24 hours. Therefore, we began releasing *T. planipennisi* over a large area at a release site rather than at a central cluster to enable faster *T. planipennisi* dispersal.

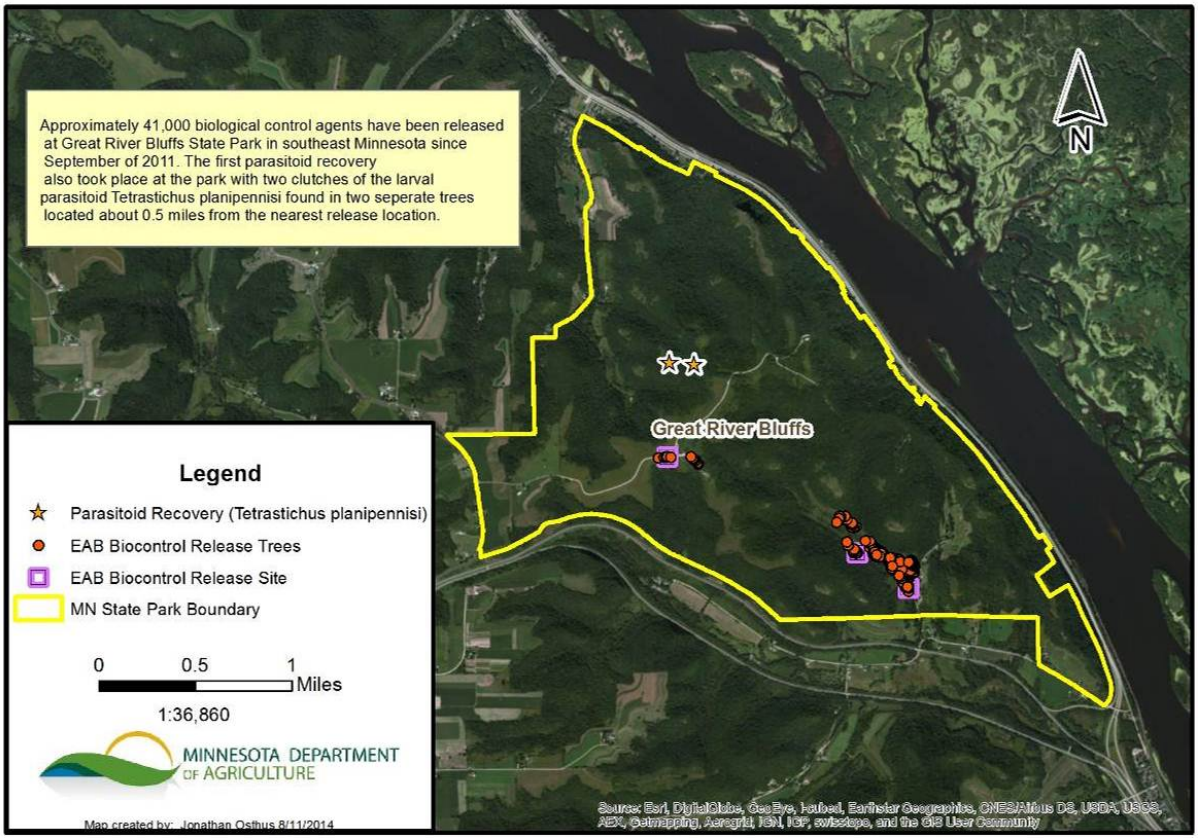
Releases of parasitoid wasps were initiated in the fall of 2010 and will continue in Phase 2 of our project. The number of release sites increased from one site in 2010 to 21 sites in 2014 in response to new EAB finds. Over 160,000 parasitoids have been released at these sites (over 127,000 during project period). Recovery of two clutches of the larval parasitoid *Tetrastichus planipennisi* demonstrate that this species is dispersing, finding EAB in the field and reproducing.



Emerald Ash Borer Biocontrol in Southeast Minnesota



Emerald Ash Borer Biocontrol at Great River Bluffs State Park, Winona County, MN



Biological control agent release totals by site and year between 07/01/11 and 06/30/14

Site	Latitude	Longitude	Year	*Spathius agrili*	*Tetrastichus planipennisi*	*Oobius agrili*	Total
2nd Street NE	44.99477	-93.26373	2013	0	1,887	0	1,887
Como Park	44.97898	-93.14738	2013	0	1,286	191	1,477
			2014	0	1,334	150	1,484
E. River Pkwy 1	44.95877	-93.21427	2011	0	702	260	962
			2012	252	934	1,346	2,532
E. River Pkwy 2	44.95643	-93.20991	2011	745	1,587	571	2,903
			2012	2,118	3,006	1,664	6,788
E. River Pkwy 3	44.95190	-93.20251	2013	0	1,549	1,324	2,873
			2014	0	625	250	875
Fort Snelling Upper Post Area	44.88626	-93.19165	2012	915	1,642	0	2,557
			2013	0	0	480	480
Ft. Snelling Hwy 5 Hillside	44.88491	-93.18779	2013	0	2,867	807	3,674
			2014	0	835	0	835
Great River Bluffs State Park 1	43.92874	-91.38466	2011	521	1,103	0	1,624
			2012	840	166	340	1,346
			2013	0	1,342	1,692	3,034
Great River Bluffs State Park 2	43.93897	-91.40949	2011	1,023	2,663	0	3,686
			2012	2,465	586	338	3,389
			2013	0	1,205	766	1,971
Great River Bluffs State Park 3	43.93150	-91.38989	2012	697	1,228	690	2,615
			2013	0	23,008	200	23,208
Houston Release 1	43.52080	-91.23666	2012	1,212	1,114	622	2,948
Hwy 26	43.53996	-91.28052	2013	0	1,499	630	2,129
			2014	0	1,015	0	1,015
Lake Winona	44.03872	-91.65294	2014	0	1,015	0	1,015
Lamoille	43.99587	-91.46054	2011	234	282	0	516
			2012	171	270	0	441
			2013	0	3,583	548	4,131
Langford Park	44.97725	-93.19489	2011	570	271	394	1,235
			2012	1,489	2,556	971	5,016
Northwestern College	45.03636	-93.16732	2013	0	2,435	616	3,051
			2014	0	500	350	850
Shoreview 1	45.11235	-93.17918	2011	888	1,536	338	2,762
			2012	809	1,653	967	3,429
			2013	0	260	0	260
Summit & Dale	44.94212	-93.12364	2011	736	1,983	0	2,719
			2012	729	2,520	974	4,223
			2013	0	260	0	260
Tower Hill Park	44.96891	-93.21265	2011	926	1,900	694	3,520
			2012	1,437	2,075	664	4,176
W. River Pkwy 1	44.95657	-93.21661	2011	1,307	1,026	926	3,259
			2012	2,124	2,072	1,665	5,861
W. River Pkwy 2	44.95102	-93.20656	2013	0	1,398	1,343	2,741
			2014	0	1,210	50	1,260
			Total	**22,208**	**81,988**	**22,821**	**127,017**

Ash health data were collected at all sites. Data fields included crown class, tree diameter, dominance in the canopy, number of epicormic shoots, EAB exit holes, bark splits and woodpecker feeding damage. These data collection will continue in Phase 2.

Crown class is an indicator ash health. After tree leaf out, tree canopies are visually rated on a scale of 1-5. A crown class of 1 is a healthy tree with a robust canopy. Ratings of 2, 3, and 4 indicate canopy decline with 4 showing the most decline. A dead tree is rated as a 5. The following visual rating images were excerpted from Appendix C of the 2013 Emerald Ash Borer Biological Control Release and Recovery Guidelines by USDA-APHIS-ARS-FS, Riverdale, Maryland.

1 **2** **3** **4** **5**

The table below shows mean canopy class rating and standard error of the mean by site and year for biological control agent release trees for 2011 - 2013. The number of trees changes at some sites by year. The standard number of release trees per site is 12. Trees are removed at some Twin Cities sites either because they are infested or as part of a structured removal. Removal of infested trees is likely to influence the mean crown class rating. The number of release trees was increased at some sites to facilitate biological control agent dispersal within the sites. Data are not available for all sites for all years because some sites were not initiated until 2012 or 2013.

Site	Year	# Trees	Mean	Std Error
2nd Street NE	2013	12	1.3	0.18
Como Park	2013	10	1.4	0.17
E. River Pkwy 1	2011	12	1.6	0.26
	2012	10	1.4	0.31
	2013	9	2.1	0.35
E. River Pkwy 2	2011	12	1.7	0.22
	2012	10	1.3	0.21
	2013	8	1.9	0.13
E. River Pkwy 3	2013	9	1.6	0.24
Fort Snelling Upper Post Area	2012	12	1.0	0.00
	2013	9	1.2	0.15
Ft. Snelling Hwy 5 Hillside	2013	12	1.2	0.11
Great River Bluffs State Park 1	2011	12	1.6	0.29
	2012	12	1.0	0.00
	2013	12	1.7	0.19
Great River Bluffs State Park 2	2011	12	1.2	0.11
	2012	12	1.2	0.11
	2013	12	1.8	0.18
Great River Bluffs State Park 3	2012	20	1.7	0.02
	2013	20	2.2	0.15
Houston Release 1	2011	12	1.4	0.26

Site	Year	# Trees	Mean	Std Error
	2012	12	1.3	0.14
	2013	11	1.6	0.29
Hwy 26	2013	12	1.4	0.19
Lamoille	2011	4	1.0	0.00
	2012	12	1.0	0.00
	2013	16	1.0	0.00
Langford Park	2011	12	1.6	0.19
	2012	12	1.2	0.11
	2013	6	1.3	0.21
Northwestern College	2013	12	1.6	0.23
Shoreview 1	2011	12	1.3	0.13
	2012	11	1.0	0.00
	2013	12	1.0	0.00
Summit & Dale	2011	12	1.0	0.00
	2012	11	1.0	0.00
	2013	8	1.5	0.19
Tower Hill Park	2011	12	2.3	0.30
	2012	6	1.2	0.17
W. River Pkwy 1	2011	12	2.5	0.19
	2012	12	2.4	0.26
W. River Pkwy 2	2013	9	1.2	0.22

The work accomplished in Phase 1 setup a strong implementation program. We initiated parasitoid releases as soon as EAB was found in an area. This gave the parasitoids the best opportunity to have an impact on EAB populations. We will continue releases and documenting changes in ash health in Phase 2. We will focus more effort on recovering parasitoids in the field and determining whether parasitoids are establishing.

V. DISSEMINATION:

Description: We will communicate about EAB biological control research and implementation with the public, land managers, and researchers. The web will be used for communication with all www.mda.state.mn.us/en/plants/pestmanagement/eab/eabbiocontrol.aspx and will be updated annually. Communication with the public will be via news media (print, television, and radio) and social media such as Facebook and Twitter. We will communicate updates with land managers at the multi-agency EAB Forum (meets 4 times/year) and in trade publications such as "The Scoop" published by the Minnesota Nursery Landscape Association. Research findings will be presented at University of Minnesota seminars, the 2012 Minnesota-Wisconsin invasive species conference, and a national Entomological Society of America meeting (LCCMR funding will not be used for meetings). After project completion, research papers will be submitted for publication.

Status as of May 31, 2012:
We conducted extensive outreach and education through many channels.

- Scientific presentations (ENRTF dollars were not used for these meetings)
    - Hanson, A.A., Venette, R.C. 2011. Thermocouple design for emerald ash borer parasitoids. Poster submitted and presented (March 14, 2011) at the 2011 Entomological Society of America – North Central Branch Meeting General Session in Minneapolis, MN.
    - Hanson, A. A., Venette, R.C., Hutchison, W.D. 2011. Cold hardiness of emerald ash borer parasitoids *Spathius agrili* and *Tetrastichus planipennisi*. Oral presentation

(November 14, 2011) at the 2011 Entomological Society of America National Meeting Student Competition in Reno, NV.

- Web
  - We updated the EAB biocontrol webpage (link above) and added a page on EAB biological control research at www.mda.state.mn.us/en/plants/pestmanagement/eab/eabbiocontrol/eabwaspresearch.aspx
  - In collaboration with MDA's general EAB program, an interactive map of confirmed EAB infestations and biological control release sites in Minnesota is now available at http://gis.mda.state.mn.us/maps/eab.htm
- Media coverage
  - There was print, television, radio, and social media coverage of biological control agent releases and our research projects. All major state media covered our stories and a few media from Wisconsin and North Dakota also covered EAB biological control stories.
    - Media events were held in Winona and at the Summit & Dale (St. Paul) infestations
    - Media ran stories on our ash health, EAB, and EAB bioagent monitoring study and the sticky traps using cadavers preliminary study
- Events
  - Open houses in the Twin Cities, Winona, and La Crescent
  - Social events are excellent opportunities to discuss EAB and EAB biocontrol with the public. We had informational tables with EAB activities and bioagent samples at:
    - Ice cream socials at Tower Hill Park and Falcon Heights
    - Cinco de Mayo in West St. Paul
  - Other events where EAB biocontrol was discussed include:
    - Shade Tree Short Course in New Brighton
    - Northern Green Expo in Minneapolis
    - Home and Landscape Expo in Minneapolis
    - Home and Garden Show in Minneapolis
    - DNR Forestry Expo in Duluth
    - Logger Training in Tower and Grand Rapids
- Training
  - Minnesota Forest Pest First Detectors receive training on EAB biocontrol. This group of skilled volunteers communicates about invasive species topics within their communities.
- Other
  - We hosted an interactive webinar to discuss plans for the upcoming field season with collaborators.
  - We provided regular updates to the EAB Forum

Status as of November 30, 2012:

Scientific presentations

- Hanson, Anthony. A.; Venette, Robert C. 2012. Cold tolerance of introduced emerald ash borer parasitoids. In: 67th annual meeting of the North Central Branch of the Entomological Society of America; 2012 June 3-6; Lincoln, NE. Abstract. Available at http://esa.confex.com/esa/2012ncb/webprogram/Paper63275.html . (Accessed September 10, 2012).
- Fahrner, S.J. 23 October, 2013. Presentation of thesis proposal and preliminary research results, Department of Entomology, University of Minnesota.
- Hanson, Anthony A.; Venette, Robert C. 2012. Will introduced emerald ash borer parasitoids overwinter in the Upper Midwest? Upper Midwest Invasive Species Conference; 2012 October 31; LaCrosse WI. Abstract available at http://www.umisc2012.org/uploads/1/0/7/5/10750703/abstracts2012final.pdf (Accessed November 29, 2012).

- Hanson, Anthony A.; Venette, Robert C. 2012. Effects of cold tolerance on potential distributions of introduced emerald ash borer parasitoids. In: 60th annual meeting of the Entomological Society of America; 2012 November 12; Knoxville, TN. Abstract available at http://esa.confex.com/esa/2012/webprogram/Paper67442.html. (Accessed November 29, 2012) **Received President's Prize**.
- Entomological Society of America Nov 11-14, Knoxville, TN
  Fahrner, S.J., Lelito, J.P., Aukema, J.E., and B.H. Aukema. Flight capacity of Tetrastichus planipennisi, an introduced parasitoid of emerald ash borer Agrilus planipennis. In: 60th annual meeting of the Entomological Society of America; 2012 November 12; Knoxville, TN.
- North Central Forest Pest Workshop Sept 24-27, Sault Ste. Marie, ON
  Fahrner, S.J., Lelito, J.P., Aukema, J.E., and B.H. Aukema. Gaining inference on the dispersal capabilities of Tetrastichus planipennisi, a classical biological control agent of emerald ash borer

Media

- There was print, television, radio, and social media coverage of our research projects. The stories received approximately 900,000 views via news outlets.

Events

- EAB biocontrol presentation for the City of Shoreview open house regarding EAB management.
- EAB biocontrol presentation for the DNR's newly formed EAB response team in Winona County.
- EAB biocontrol presentation/implementation update at the Upper Midwest Invasive Species Conference.
- Staffed a table with information on EAB biocontrol at the Heartwood Heritage festival in the Hamline-Midway neighborhood of St. Paul.
- EAB biocontrol informative display at the Minnesota State Fair located in the DNR invasive species exhibit. Display included microscopes for a close up look at samples of EAB and its natural enemies to inform the public how tiny the parasitic wasps really are.

Training

- EAB detection training at Fort Snelling Golf Course for over 50 arborists from Iowa. Included examples of EAB bioagents and their role in management.
- EAB detection training at Fort Snelling Golf Course for Minneapolis Parks and Recreation forestry division. Included examples of EAB bioagents and their role in management.
- EAB detection training at Fort Snelling Golf Course for industry and government employees that deal with management of EAB on private or public property.
- EAB informative tour at Great River Bluffs State Park for the Upper Midwest Invasive Species Conference participants. Highlighted on the tour was MDA's EAB biocontrol implementation strategy within the park.

Other

- EAB Management meetings in Shoreview and St. Paul were held to bring together current affected communities and adjacent ones in the Twin Cities to discuss what management has taken place thus far and what is planned for the future. Updates were provided on the status of EAB biocontrol for the cities of Shoreview, Minneapolis and St. Paul.
- EAB biocontrol is featured during the month of March for MISAC's annual 2013 invasive species calendar.

Status as of May 31, 2013:

Scientific papers and presentations

- Hanson, Anthony A. 2013. Cold tolerance of Chinese emerald ash borer parasitoids and implications for biological control in the Upper Midwest. Seminar. Department of Entomology, University of Minnesota. 04 April 2013. St. Paul, MN.
- Fahrner, Samuel J. 2013. A general-to-specific introduction to forest entomology: flying parasitoids, starving budworms, and some dying trees. Department of Biology - University of Iowa, Iowa City. April 5, 2013. Invited speaker.
- Hanson, Anthony A. 2013. Cold tolerance of emerald ash borer parasitoids: *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and

Tetrastichus planipennisi Yang (Hymenoptera: Eulophidae). Master's Thesis. Department of Entomology, University of Minnesota.

- Hanson, Anthony A. and Venette, Robert C. 2013. Thermocouple design for measuring temperatures of small insects. CryoLetters (Submitted).
- Hanson, A.A., Venette, Robert C., and Lelito, Jonathan P. 2013. Cold tolerance of Chinese emerald ash borer parasitoids: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). Biological Control (Submitted).

Media

- Minnesota Bound television program recorded a story on EAB and included biological control. The story will be aired at a future date to be determined.
- Media covered the EAB tours listed below.

Events and Tours

- The Senate and House Committees involving agriculture toured MDA's lab on January 16th and 23rd 2013 respectively. EAB was highlighted and legislators learned about monitoring and managing EAB infestations in Minnesota.
- MDA had a booth at the Prairie Enthusiast Conference on March 16, 2013 at Mankato State University. The booth contained examples of EAB bioagents and information on their role in EAB management.
- Demonstration of flight mill to visiting scientist Dr. Jack Gray, University of Saskatchewan, April 16, 2013
- EAB detection field tours were held on multiple dates in April and May at Langton Lake Park in Roseville and Great River Bluffs State Park near Winona. Tours were very well attended and open to everyone at no cost. They included examples of EAB bioagents and their role in management.

Status as of November 29, 2013:

Scientific papers and presentations

- North Central Forest Pest Workshop, Sept 23-26, 2013, Frontenac, MN. Fahrner, S.J., Lelito, J.P., and B.H. Aukema. Dispersal capacity of *Tetrastichus planipennisi*, an introduced parasitoid of emerald ash borer *Agrilus planipennis.*
- International Union of Forestry Research Organizations Sections 7.03.05 & 7.03.07 on Population Dynamics of Bark and-Wood Boring Insects, Sep 15-19, 2013, Banff, Alberta, Canada (used travel scholarship money and matching funds; no LCCMR project funds), Fahrner, S.J., Lelito, J.P., and B.H. Aukema. Temperature-mediated dispersal of host and parasitoid: Improving release strategies for *T. planipennisi* in the biological control of emerald ash borer *Agrilus planipennis.*
- Ecological Society of America Aug 4-9, 2013, Minneapolis, MN; Fahrner, S.J., Lelito, J.P., Aukema, J.E., and B.H. Aukema. Flight capacity of *Tetrastichus planipennisi*, an introduced parasitoid of emerald ash borer *Agrilus planipennis.*

Media

- The Star Tribune published the story "In the Twin Cities, emerald ash borer faces war in the streets" on 07/03/13. Other media also ran the story.
- Jon Osthus was interviewed live by KUMD (103.3 FM Duluth) about EAB and biocontrol.
- The US Army Corps of Engineers interviewed Jon Osthus on 09/11/13 for a video about their ACOE's role with EAB biocontrol on Mississippi River lands that they manage on 09/11/13.
- Minnesota Public Radio aired an update on EAB biological control at Great River Bluffs State Park.

Events

- EAB biocontrol was exhibited at the Heartwood Festival (Hamline-Midway area of Twin Cities) on 06/01/13.
- National Ag in the Classroom discussed EAB and biocontrol on 06/26/13.
- EAB biocontrol was exhibited at the Slice of Shoreview event 07/26-28/2013.

- EAB biocontrol was exhibited at Farm Fest 08/06-08/13.
- EAB biocontrol was exhibited at the Minnetonka EAB Open House on 08/07/13.

Final Report Summary:

Scientific papers and presentations

- Venette, R.C., and M. Abrahamson. 2014. Cold weather impacts on emerald ash borer: state of the science. Minnesota Shade Tree Advisory Committee. Eden Prairie, MN. February 20, 2014.
- Venette, R.C., L.D.E. Christianson, and A.A. Hanson. 2014. Cold tolerance of emerald ash borer and its parasitoids: tales from the north. Invited Presentation: Forest Entomology Symposium. 69th Annual Meeting of the North Central Branch of the Entomological Society of America. Des Moines, IA. March 10, 2014.
- Osthus, J.M. and M.A. Chandler. 2014. Biological control of the emerald ash borer. Minnesota Shade Tree Short Course. Roseville, MN. March 18, 2014.
- Venette, R.C., and M. Abrahamson. 2014. Cold weather impacts on emerald ash borer: state of the science. Plenary Session. Minnesota Shade Tree Short Course. Roseville, MN. March 19, 2014.

Training

- Monika Chandler trained volunteers to identify and report EAB at the Master Naturalist monthly meeting Quarry Hill Nature Center in Rochester on 02/19/14.
- Jonathan Osthus and Monika Chandler presented information on EAB and Oriental Bittersweet to Xcel Energy vegetation management contractors that cover the Upper-Midwest at their annual training on 03/12/14.

Events (add dates) and Tours

- A booth on terrestrial invasive species at the Home and Garden Show at the Minneapolis Convention Center was staffed by MDA. EAB and EAB biocontrol was a main topic of interest for those that visited the booth (02/26/14-03/02/2014).
- A booth on EAB and EAB biocontrol at the EAB Symposium held by Rainbow Tree Care in Roseville was staffed by MDA (03/05/14-03/06/14).
- Jonathan Osthus helped staff the **PlayCleanGo** booth at the Midwest Mountaineering Outdoor Adventure Expo on 04/25/14. Attendees were very receptive to the messages on ways to prevent spreading terrestrial invasive species when recreating outdoors.
- The North Dakota Forest Service traveled to Minnesota for an EAB discussion and tour at Ft Snelling State Park on 04/8/2014.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget:

Budget Category	$ Amount	Explanation
Personnel:	$ 464,724	**U of M:** One 2 year part-time faculty (1 mo/yr) mean salary $8,200/mo plus fringe benefits @ 7% for examining parasitoid establishment and dispersal (Activity 2). The total is $17,500 and is for Dr. Aukema's summer salary because he has a 9 month appointment that does not include the summer field season. **U of M:** Two 3 year full-time graduate students mean salary $28,500/yr plus fringe benefits @ 25% for bioagent cold-hardiness (Activity 1) and examining parasitoid establishment and dispersal (Activity 2). The total is $214,000. **U of M:** Two 3 year part-time school year and full-time summer field season undergraduate students mean wages $15/hr plus fringe benefits @ 7.6%, 2 students for Activity 1 and 2 for Activity 2 (40 wks @ 20 hr/wk & 12 wks in summer @ 40hrs/wk). The total is $41,200. **MDA:** One 2.7 year full-time Research Scientist 1 mean salary $42,500/yr plus fringe benefits @ 49% for EAB biocontrol implementation (Activity 3). This is a new, unclassified position within the Plant Protection Division.
Capital Equipment:	$ 14,000	**U of M:** 2 ultralow precision temperature freezers @ $7,000 each for Activity 1. The precision freezers can be set to and hold constant lower temperatures than standard freezers are necessary to test the cold-hardiness of the biological control agents. Varying levels of time at specific temperatures will be tested. Running two freezers simultaneously is necessary to perform the tests within the project timeframe. After this project is completed, the freezers will continue to be used for invasive species related activities.
Equipment/Tools/Supplies:	$ 13,800	**Equipment for MDA:** One rangefinder @ 300 for Activity 3. **Tools and Supplies for U of M (12,000) and MDA (1,500):** Activity 1 supplies include thermocouple wire ($250/yr), thermocouple connectors ($130/yr), PTFE tubing ($140/yr), 8 channel data logger ($330/yr), rearing containers ($750/yr), petri dishes ($150/yr). Activity 2 supplies include insect rearing tubes ($500/yr), field supplies such as insect collection traps and containers ($750/yr). All activity supplies include tools related to bark peeling such as draw knives and chisels ($500/yr), and miscellaneous ($1,000/yr) such as DBH tapes (for measuring tree size), spray paint, and tree tags.
Travel Expenses in MN:	$ 29,500	**Travel expenses for U of M** for Activities 1 ($3,000) and 2 ($10,900) are $13,900. **Travel expenses for MDA** are $15,600. **Vehicles:** Vehicle rental for Activities 2 and 3 during the summer field season. (Activity 2: One 3 mo. vehicle rental ($700/mo for 6 mo. for 2 yr - includes milage) and fuel ($200/mo for 6 mo/yr for 2 yr) and Activity 3: One 3 mo. vehicle rental ($700/mo for 3 mo. for 3 yr - includes milage)

		and fuel ($200/mo for 3 mo/yr for 3 yr)). MDA's vehicle pool will be used for travel vehicles during the non-field season. **Meals and lodging** for all 3 activities (Activity 1: Approx. 6 days of travel/yr each for 3 yr for 1 undergrad student, 1 grad student, and the PI; Activity 2: Approx. travel/yr for 3 yr for 1 undergrad student (3 days), 1 grad student (6 days), and the PI (3 days); Activity 3: Approx. travel/yr for 3 yr for 1 EAB biocontrol coordinator (10 days) and the PI (6 days))
TOTAL ENRTF BUDGET:	**$500,000**	

Explanation of Use of Classified Staff: N/A

Number of Full-time Equivalent (FTE) funded with this ENRTF appropriation:
One 2 year part-time faculty (1 mo/yr) = 346 hrs
Two 3 year full-time graduate students = 2080*2*3 = 12,480 hrs
Two 3 year part-time undergraduate students = 1280*2*3 = 7,680 hrs
One 2.7 year full-time Research Scientist 1 = 2080*2.7 = 5,616
Total hours: 26,122
Total FTEs = 26,122/2080 = 12.56

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
	$	$	
State (in-kind)			
Field equipment, lab equipment and lab space, computing/software, GIS and data management ($40,000 for U of M, $15,000 for MDA), graduate student advising and research management ($100,000 at U of M), project coordination and overseeing EAB biocontol implementation ($15,000 at MDA)	$ 170,000	$	
TOTAL OTHER FUNDS:	**$ 170,000**	**$**	

VII. PROJECT STRATEGY:
A. Project Partners: Receiving funds: EAB biological control research and implementation will be a joint U of M and MDA endeavor. U of M will lead research and receive funds for the research projects: Assessing bioagent cold-hardiness and method development for bioagent monitoring. MDA will lead implementation and receive funds for coordinating Minnesota's EAB biocontrol program. MDA will provide labor to support research and implementation. Both institutions will provide in-kind equipment, facilities, intellectual input, and GIS/technical support. **Not receiving funds:** We will collaborate with Dr. Luke Skinner (DNR), USDA EAB biocontrol researchers, other federal and state agencies, counties, municipalities, and private landowners. The US Forest Service will not receive funds but will provide facilities.
B. Project Impact and Long-term Strategy: All three biological control agent species were released and recovered in Michigan. We are confident that these species will establish in southern Minnesota which has a similar climate to the areas of biological control agent release and recovery. However, northern Minnesota is colder than Michigan so we are not sure that EAB and its biological control

agents will survive northern winters. Understanding their winter survival potential would inform biological control agent release decisions.

EAB biocontrol is still too new for conclusions regarding efficacy. Although EAB can spread and kill ash trees at high rates, the movement potential of parasitoids once released is less well known, especially in new environments like Minnesota. Understanding rates of establishment and spread will permit judicious use of biological control agents as new sites with EAB are detected.

Implementing EAB biological control is very time and labor intensive. Site selection, data collection, coordination with project partners, and outreach are involved. Biological control agents are in short supply due to the limitations of production and demand thereby increasing the need for strategic releases. Based upon the experience in Michigan and other states, we learned that EAB can spread and destroy ash trees very quickly. An efficient and forceful implementation strategy for Minnesota should be developed and enacted immediately. Management recommendations resulting from research should be incorporated into the strategy as they become available.

C. Spending History:

Funding Source	**M.L. 2005 or FY 2006-07**	**M.L. 2007 or FY 2008**	**M.L. 2008 or FY 2009**	**M.L. 2009 or FY 2010**	**M.L. 2010 or FY 2011**
Forest Service (supplies and salary for Activity 1)					8,000
University of Minnesota (salary to initiate Activity 2)					2,500
Minnesota Department of Agriculture (salary to initiate bioagent releases, Activity 3)					3,000

These funds were spent prior to LCCMR fund availability.

VIII. ACQUISITION/RESTORATION LIST: NA

IX. MAP(S): NA

X. RESEARCH ADDENDUM: (attached)

XI. REPORTING REQUIREMENTS:

Periodic work plan status update reports will be submitted not later than May 31, 2012, November 30, 2012, May 31, 2013 and November 29, 2013. A final report and associated products will be submitted between June 30 and August 15, 2014 as requested by the LCCMR.

Attachment A: Budget Detail for M.L. 2011 (FY 2012-13) Environment and Natural Resources Trust Fund Projects

Project Title: Emerald Ash Borer Biocontrol Research and Implementation

Legal Citation: M.L. 2011, 1st Special Session, Chapter 2, Article 3, Subd. 6b

Project Manager: Monika Chandler, Minnesota Department of Agriculture, 651-201-6537, Monika.Chandler@state.mn.us

M.L. 2011 (FY 2012-13) ENRTF Appropriation: $ 500,000

Project Length and Completion Date: 3 years, 06/30/2014

Final Report: submtited August 15, 2014 with budget amendment request submitted September 17, 2014

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Revised Activity 1 Budget 09/17/14	Amount Spent	Balance	Revised Activity 2 Budget 09/17/14	Amount Spent	Balance	Revised Activity 3 Budget 09/17/14	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Assess biological control agent winter survival potential***			***Examining parasitoid establishment and disperal***			***Coordinate biological control implementation***				
Personnel (Wages and Benefits)											
Personnel at U of M for Activities 1 ($127,600) and 2 ($145,100) total is $272,700. **Personnel at MDA** is $170,000.	136,102	136,102	0	152,937	152,937	0	175,685	175,685	0	464,724	0
U of M: One 2 year part-time faculty (1 mo./yr) mean salary $8,200/mo plus fringe benefits @ 7% for examining parasitoid establishment and dispersal (Activity 2). The total is $17,500 and is for Dr. Aukema's summer salary because he has a 9 month appointment that does not include the summer field season.					23,514						
U of M: Two 3 year full-time graduate students mean salary $28,500/yr plus fringe benefits @ 25% for bioagent cold-hardiness (Activity 1) and examining parasitoid establishment and dispersal (Activity 2). The total is $214,000.		91,074			105,418						
U of M: One 1.5 year full-time technician mean wages $15.30/hr plus fringe benefits @ 39.6% for Activities 1 and 2		35,244			15,483						
U of M: Two 3 year part-time school year and full-time summer field season undergraduate students mean wages $15/hr plus fringe benefits @ 7.6%, 2 students for Activity 1 and 2 for Activity 2 (40 wks @ 20 hr/wk & 12 wks in summer @ 40hrs/wk). The total is $41,200.		9,784			8,522						

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Revised Activity 1 Budget 09/17/14	Amount Spent	Balance	Revised Activity 2 Budget 09/17/14	Amount Spent	Balance	Revised Activity 3 Budget 09/17/14	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Assess biological control agent winter survival potential***			***Examining parasitoid establishment and disperal***			***Coordinate biological control implementation***				
MDA: One 2.7 year full-time Research Scientist 1 mean salary $42,500/yr plus fringe benefits @ 49% for EAB biocontrol implementation (Activity 3). This is a new, unclassified position within the Plant Protection Division.								135,000			
MDA: One part-time school year and full-time summer field season undergraduate students mean wages $15/hr plus fringe benefits @ 7.6% for EAB biocontrol implementation (Activity 3). This is a new, unclassified position within the Plant Protection Division.								40,685			
Equipment/Tools/Supplies											
Equipment/Tools/Supplies at U of M for Activities 1 ($20,000) and 2 ($6,000) total is $26,000. **Equipment/Tools/Supplies at MDA** is $1,800.											
Capital equipment over $3,500 Capital equipment: 2 ultralow precision temperature freezers @ $7,000 each for Activity 1. The precision freezers can be set to and hold constant lower temperatures than standard freezers are necessary to test the cold-hardiness of the biological control agents. Varying levels of time at specific temperatures will be tested. Running two freezers simultaneously is necessary to perform the tests within the project timeframe. After this project is completed, the freezers will continue to be used for invasive species related activities.	12,131	12,131	0							12,131	0
Equipment: One rangefinder @ 300 for Activity 3							0	0	0	0	0
Services (equipment repair)				1,917	1,917	0				1,917	0

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Revised Activity 1 Budget 09/17/14	Amount Spent	Balance	Revised Activity 2 Budget 09/17/14	Amount Spent	Balance	Revised Activity 3 Budget 09/17/14	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Assess biological control agent winter survival potential***			***Examining parasitoid establishment and disperal***			***Coordinate biological control implementation***				
Activity 1 supplies include thermocouple wire ($250/yr), thermocouple connectors ($130/yr), PTFE tubing ($140/yr), 8 channel data logger ($330/yr), rearing containers ($750/yr), petri dishes ($150/yr). Activity 2 supplies include insect rearing tubes ($500/yr), field supplies such as insect collection traps and containers ($750/yr). All activity supplies include tools related to bark peeling such as draw knives and chisels ($500/yr), and miscellaneous ($1,000/yr) such as DBH tapes (for measuring tree size), spray paint, and tree tags.	1,994	1,994	0	4,068	4,068	0	1,574	1,574	0	7,636	0

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Revised Activity 1 Budget 09/17/14	Amount Spent	Balance	Revised Activity 2 Budget 09/17/14	Amount Spent	Balance	Revised Activity 3 Budget 09/17/14	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Assess biological control agent winter survival potential***			***Examining parasitoid establishment and disperal***			***Coordinate biological control implementation***				
Travel expenses in Minnesota											
Travel expenses for U of M for Activities 1 ($3,000) and 2 ($10,900) are $13,900. **Travel expenses for MDA** are $15,600.											
Vehicles: Vehicle rental for Activities 2 and 3 during the summer field season. (Activity 2: One 3 mo. vehicle rental ($700/mo for 6 mo. for 2 yr - includes milage) and fuel ($200/mo for 6 mo/yr for 2 yr) and Activity 3: One 3 mo. vehicle rental ($700/mo for 3 mo. for 3 yr - includes milage) and fuel ($200/mo for 3 mo/yr for 3 yr)). MDA's vehicle pool will be used for travel vehicles during the non-field season.				650	650	0	7,858	7,858	0	8,508	0
Meals and lodging for all 3 activities: Activity 1: Approx. 6 days of travel/yr each for 3 yr for 1 undergrad student, 1 grad student, and the PI; Activity 2: Approx. travel/yr for 3 yr for 1 undergrad student (3 days), 1 grad student (6 days), and the PI (3 days); Activity 3: Approx. travel/yr for 3 yr for 1 EAB biocontrol coordinator (10 days) and the PI (6 days)	373	373	0	2,429	2,429	0	2,282	2,282	0	5,084	0
COLUMN TOTAL	**$150,600**	**$150,600**	**$0**	**$162,000**	**$162,000**	**$0**	**$187,400**	**$187,400**	**$0**	**$500,000**	**$0**

Biological Control 67 (2013) 516–529

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Cold tolerance of Chinese emerald ash borer parasitoids: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae)

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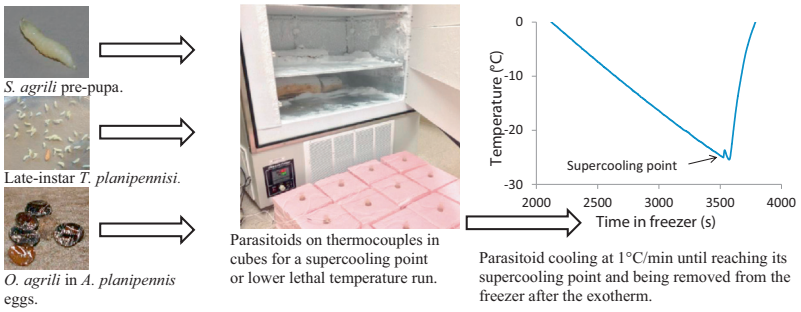
HIGHLIGHTS

- Cold acclimation decreased supercooling points of *Tetrastichus planipennisi and Spathius agrili*.
- 50% of cold acclimated *T. planipennisi* and *S. agrili* died at −19.9 and −27.3 °C.
- Mortality of *T. planipennisi* and *S. agrili* increased with cold exposure time.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 May 2013
Accepted 28 August 2013
Available online 4 September 2013

Keywords:
Agrilus planipennis
Supercooling point
Lower lethal temperature
Lower lethal time

ABSTRACT

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive insect that has caused significant ash (*Fraxinus* spp.) mortality in North America. Three Chinese parasitoids have been approved for release as part of a classical biological control program for *A. planipennis* in the United States: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). This study was designed to measure the cold tolerance of the overwintering stage for each parasitoid species in the laboratory. We exposed cold-acclimated and non-cold-acclimated individuals to temperatures from 0 to −35 °C to determine temperatures that cause body fluids to freeze, mortality after brief exposure, and mortality after long-term exposure. Cold acclimation lowered the supercooling points of *S. agrili* (median −28.8 °C) and *T. planipennisi* (median −29.4 °C). Median supercooling point for *Oobius agrili* was −30.5 °C. Cold acclimation also increased survival of diapausing *S. agrili* (50% mortality at −27.3 versus −23.7 °C for non-diapausing *S. agrili*) during brief cold exposure. *T. planipennisi* and *S. agrili* mortality increased over long term cold exposure when held at constant temperature. Half of *T. planipennisi* are predicted to fail to eclose after exposure to 0, −5, −10, and −15 °C after >84, 82, 59, and 36 days, respectively, while 50% of *S. agrili* with diapause induced in one generation would be discolored from cold injury >84 days for all exposure temperatures. Our models characterizing parasitoid mortality due to cold exposure can be used to assess the climatic suitability of a location prior to release.

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1. Introduction

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an Asian beetle that was first detected in North

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1049-9644/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.biocontrol.2013.08.015

America (Michigan, USA and Ontario, Canada) in 2002 (McCullough and Katovich, 2004), but may have been present since the mid-1990s (Siegert et al., 2009). Currently, *A. planipennis* occurs in 21 US states and two Canadian provinces but may spread to 25 North American states and provinces over the next decade (Kovacs et al., 2010). The larvae feed on the cambium, phloem, and outer sapwood of ash (*Fraxinus* spp.). Under high larval densities, galleries from the tunneling larvae girdle the tree, thereby causing crown dieback and eventually tree death. Large trees typically die three to four years after an infestation starts (Poland and McCullough, 2006). *A. planipennis* has already killed at least 50 million ash trees in Michigan alone (Smith et al., 2009). The cost of removing infested trees on developed land to slow further infestation and prevent safety hazards caused by dead trees is estimated to be $10.7 billion from 2009 to 2019 in North America (Kovacs et al., 2010). However, *A. planipennis* is not a major pest in its native range of northeastern Asia. Damage from *A. planipennis* in North America has been attributed to the lack of natural enemies and host-plant resistance (Liu et al., 2007).

The US Department of Agriculture conducted surveys in northeastern China for potential natural enemies of *A. planipennis* to initiate classical biological control in the United States (Liu et al., 2003, 2007; Gould et al., 2005; Bauer et al., 2008, in press). Three parasitoids were approved for release in North America: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Yang et al., 2005, 2006; Zhang et al., 2005). *S. agrili*, a gregarious ectoparasitoid, and *T. planipennisi*, a gregarious endoparasitoid, both oviposit through the bark where the offspring feed on and kill the host *A. planipennis* larva (Wang et al., 2010; Yang et al., 2010; Ulyshen et al., 2010a). *T. planipennisi* larvae feed within the host and rupture from the host to pupate and eclose (Duan et al., 2011). In contrast, *S. agrili* larvae feed externally until the host is consumed; afterwards larvae enter a wandering stage and move away from the consumed host, then spin cocoons, pupate, and eclose (Yang et al., 2010). In both cases, the larvae are free of their host after feeding ceases. For both larval parasitoids, development from egg to adult takes 3–4 weeks when temperatures are between 22.5 and 26.5 °C, and adults exit the gallery by chewing through the bark (Lelito, *unpublished* data; Ulyshen et al., 2010b; Yang et al., 2010). Both *T. planipennisi* and *S. agrili* have been described as multivoltine (Yang et al., 2005; Liu et al., 2007a), but emergence of the overwintering generation of *S. agrili* may instead occur periodically throughout the spring and summer (Lelito, *unpublished data*).

Oobius agrili is a solitary egg parasitoid that is typically parthenogenetic. *O. agrili* eggs develop within the *A. planipennis* egg, and emerge as adults in approximately one month when temperatures are between 22.5 and 26.5 °C (Lelito, *unpublished data*; Bauer and Liu, 2006; Liu et al., 2007b).

Each species of emerald ash borer parasitoid overwinters in a different stage or location. *A. planipennis* overwinters inside the gallery or pupal cell, often as pre-pupae, but occasionally as first to third instars (Poland and McCullough, 2006; Crosthwaite et al., 2011). The two larval parasitoids typically are not in direct contact with the host while overwintering. *S. agrili* overwinter as diapausing pre-pupae within the host gallery (Yang et al., 2010). Diapause in *S. agrili* can be induced in one or two generations; individuals with diapause induced over two generations have greater survival rates during storage at 4 °C for 120 days, which is required to break diapause, than individuals that entered diapause in one generation (Lelito, *unpublished data*; Gould et al., 2011). *T. planipennisi* has been described overwintering as late-instar larvae or pupae in the host gallery (Bauer et al., 2008; Ulyshen et al., 2011). However, it has recently been found overwintering as young larvae inside the host (L. Bauer, *personal comm.*). *T. planipennisi* is not known to enter diapause in any stage (Ulyshen et al., 2011; Duan et al., 2011, 2013). *O. agrili* overwinter as diapausing pre-pupae within the host egg (Liu et al., 2007).

The parasitoids may reduce *A. planipennis* densities in North America because the parasitoids have high parasitism rates in China, especially on North American ash trees planted in Asia (Yang et al., 2005; Liu et al., 2007; Duan et al., 2012b). Average parasitism rates for *T. planipennisi* in the laboratory typically range from 60% to 80% with 4–172 offspring per host (Lelito, *pers. obs.*; Ulyshen et al., 2010b). Likewise, parasitism rates for *S. agrili* typically range from 30% to 90% with 1–18 offspring produced per host (Yang et al., 2005, 2010). *O. agrili* parasitize up to 82% of eggs in the lab; parasitized eggs turn black (Liu et al., 2007b). However, cold may limit the northern distribution of the parasitoids in North America. The Chinese parasitoids have overwintered in initial release sites in Michigan, Maryland, Ohio, Indiana, and Illinois (Bauer et al., 2012; Duan et al., 2013). However, states such as Michigan and Maryland also have relatively mild winters compared to other areas at similar latitudes (USDA, 2012). Additional time may be required to confidently assess establishment in states where the parasitoids have only been recently released (Ulyshen et al., 2011; Duan et al., 2012a).

For insects, three measures are commonly used to assess cold tolerance at the population level: supercooling points, lower lethal temperature, and lower lethal time (e.g., Eaton and Kells, 2011; Morey et al., 2012). The supercooling point is the temperature at which insect body fluids begin to change from a liquid to solid state and is the lowest temperature an insect reaches prior to an exotherm, an increase of temperature due to the heat released as water crystallizes, as reviewed in Denlinger and Lee (2010). Brief exposure to freezing temperatures is lethal for freeze-intolerant species, and most insects are freeze-intolerant, as reviewed in Sømme (1982). Two other mortality responses can also be classified relative to the supercooling point. An insect is chill-intolerant if it dies after brief exposure to cold but before the supercooling point is reached, and is freeze-tolerant if it survives exposure to temperatures below its supercooling point. Lower lethal temperature is a measure of mortality in a population after brief exposure to a given low temperature. However, both supercooling point and lower lethal temperature measurements are limited to characterizing the consequences of brief temperature exposures that may occur during an overnight low, but do not account for cold stress that accrues with prolonged cold exposure throughout winter (Renault et al., 2002). Lower lethal time is similar to lower lethal temperature, but measures mortality at multiple lengths of exposure to a constant low temperature to simulate long-term cold exposure.

In this study, our primary objective to measure supercooling points, lower lethal temperature and lower lethal time for *O. agrili*, *T. planipennisi*, and *S. agrili*. Several insect species become more cold tolerant in response to environmental cues such as decreasing temperature or photoperiod (e.g., Kim and Song, 2000). We hypothesized that parasitoids reared under short photoperiods or cool temperatures would be more cold tolerant than parasitoids reared under long photoperiods and warm temperatures. We also hypothesized that *O. agrili* may be the most cold-tolerant species because it overwinters near the surface of the tree where it can experience lower temperatures than larval parasitoids overwintering in the host gallery.

2. Materials and methods

2.1. Rearing

O. agrili, *T. planipennisi*, and *S. agrili* were reared at the US Department of Agriculture–Animal and Plant Health Inspection

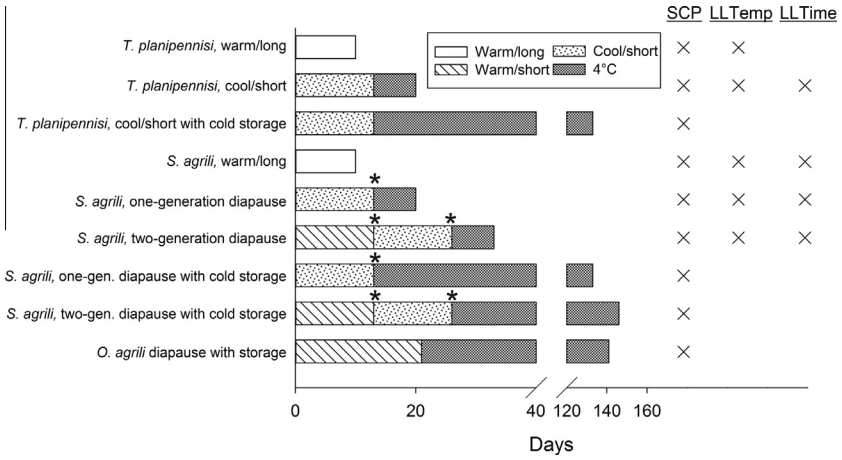

Fig. 1. Rearing conditions for *O. agrili*, *T. planipennisi*, and *S. agrili*. Species and rearing treatment combinations used during cold tolerance testing are shown on right: supercooling point (SCP), lower lethal temperature (LLTemp), and lower lethal time (LLTime). Each rearing condition occurs in a single generation excluding *S. agrili* diapause treatments where a new generation is marked with an asterisk.

Service, Emerald Ash Borer Biological Control Facility in Brighton, MI as part of a mass rearing program. Adults were allowed to parasitize the target life stage of *A. planipennis* under temperature and photoperiod conditions that would mimic summer-like conditions and potential cold-acclimating or diapause-inducing conditions in the fall and winter. Warm (26.5:22.5 °C for 16:8 h) or cool (20:15 °C for 8:16 h) rearing temperature regimes, and long (16:8 h L:D) or short (8:16 h L:D) photoperiod conditions were used in different combinations for each species (Fig. 1). In all cases, relative humidity was maintained at approximately 60–70%. Specific rearing conditions applied to each species are described below.

O. agrili were allowed to parasitize *A. planipennis* eggs under diapause-inducing conditions. *A. planipennis* eggs on coffee filter paper were presented to *O. agrili* under warm/short-photoperiod conditions to induce diapause (L. Bauer, *personal comm.*; Lelito *unpublished data*). Approximately 14 d after parasitism, *O. agrili* pupae within *A. planipennis* eggs were held at 4 °C for 4 months to simulate potential long term cold acclimation. Because the number of *O. agrili* was limited, only individuals from diapause conditions were tested.

Mated *T. planipennisi* females were allowed to parasitize *A. planipennis* larvae manually placed under the phloem of ash bolts (*Fraxinus* spp.) that were approximately 5 cm diameter × 10 cm in length (Liu and Bauer, 2007; Yang et al., 2008). Three rearing treatments were tested for late-instar *T. planipennisi*: warm/long-photoperiod conditions to produce non-cold acclimated individuals, cool/short-photoperiod conditions to produce cold acclimated individuals, and cool/short-photoperiod conditions followed by 4 °C for 4 months for long term cold acclimation (Fig. 1).

Five rearing treatments were examined for *S. agrili* pre-pupae: warm/long-photoperiod summer conditions, one-generation diapause, two-generation diapause, and one- and two-generation diapause groups acclimated at for 4 °C for 4 months. Diapause was induced in *S. agrili* in one generation by allowing adults to mate under cool/short-photoperiod conditions for 3–4 d before presenting them with a piece of ash artificially infested with *A. planipennis* larvae (as described above for *T. planipennisi*). The resulting offspring entered diapause as pre-pupae. Diapause was induced over two generations by allowing adults to mate under warm/short-photoperiod conditions for 3–4 d and allowing them to oviposit (F1 generation) under those conditions. The F1 adults were then reared under cool/short-photoperiod conditions for 3–4 d before allowing them to oviposit. The resulting offspring (F2 generation) entered diapause as pre-pupae.

Approximately one week after being parasitized, *A. planipennis* with *T. planipennisi* or *S. agrili* larvae were removed from ash bolts. Late-instar parasitoids in or on hosts were shipped in Petri dishes without ice via overnight courier to St. Paul, MN for cold-tolerance testing, all in accordance with the terms and conditions of USDA APHIS Permit P526P-11-00136. When *T. planipennisi* and *S. agrili* larvae were received, they had developed to wandering-stage larvae and were free from their host. Individual immature parasitoids of each species were placed into 1.5 ml microcentrifuge tubes using a fine-tipped paintbrush. *O. agrili* pre-pupae were kept in the remnants of the host egg to avoid potential handling stress from dissection. Each parasitoid was inspected for damage or disease, and such individuals were discarded. The remaining individuals were assigned arbitrarily to cold exposure treatments. Individuals from warm/long-photoperiod conditions were placed in a reach-in growth chamber with warm temperature and long-photoperiod conditions until they could be tested. *S. agrili* and *O. agrili* in diapause, and *T. planipennisi* from cool/short-photoperiod conditions were placed in a reach-in refrigerator with no lighting at 10 °C for 2 d and then into a refrigerator at 4 °C for 7 or 120 days before cold tolerance testing.

After the specified acclimation periods, *O. agrili* pre-pupae, *S. agrili* pre-pupae, and late-instar *T. planipennisi* were used in all cold tolerance tests. All *O. agrili* pre-pupae were kept in the egg remnant because it was dry and did not interfere with supercooling point measurements (i.e., only one exotherm from the pre-pupae and not from the egg remnant). For the larval parasitoids, we used a free-living stage to reduce potential confounding factors that might affect parasitoid cold tolerance, particularly the potential for parasitoids still in association with the living host to experience inoculative freezing within the host should the host freeze. Occasionally, *S. agrili* larvae would construct cocoons, and cold tolerance testing was performed with the cocoon intact if one was constructed.

2.2. Cold tolerance testing

For supercooling point determination and lower lethal temperature studies, "cradle" thermocouples were constructed from 0.127 mm diameter copper and constantan wires that are able to detect exotherms >0.05 °C (Hanson and Venette, 2013). Thermocouples were connected to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Temperatures (accuracy ± 0.17 °C) were recorded once per second at the first point of

contact between the copper and constantan wires (Carrillo et al., 2004).

Constraints on the availability of insects only allowed us to test supercooling points and lower lethal temperature of one species and rearing condition at a time. Supercooling points were measured for all species and rearing combinations previously described (Fig. 1). Due to limited availability of *T. planipennisi*, lower lethal temperature was only measured for individuals reared under warm/long-photoperiod and cool/short-photoperiod conditions, and lower lethal time was only measured for individuals from cool/short-photoperiod conditions. For *S. agrili*, lower lethal temperature and time were measured from warm/long-photoperiod, one-generation-, and two-generation-diapause conditions. Differences were compared among species and rearing treatments for each of the cold tolerance measures (Fig. 1). *Post hoc* comparisons among species or rearing conditions must be interpreted with caution because logistics prevented us from testing multiple species or rearing conditions at the same time. Thus, other factors which could not be controlled by our experimental design could influence apparent differences or lack thereof among treatments of interest. Lower lethal temperature and time were not measured for *O. agrili* because the number of parasitoids was limited.

2.2.1. Supercooling point

Each insect was placed in a 5 × 14 mm clear gelatin capsule (Capsuline, Pompano Beach, FL). Capsules with insects to be cooled were held on the thermocouple with a small amount of high vacuum grease (Dow Corning, Midland, MI). The thermocouples with insects were placed in polystyrene cubes constructed to cool at a constant 1 °C per minute inside a −80 °C freezer (Carrillo et al., 2004). After detection of an exotherm, the individual cube was removed from the freezer once the temperature returned to the supercooling point as per Koch et al. (2004). The thermocouple was immediately removed from the cube and allowed to warm to room temperature (ca. 25 °C). Individuals were removed from the gelatin capsules, each placed in a microcentrifuge tube, and returned to warm/long-photoperiod rearing conditions to monitor development. One to sixteen cubes containing a single species × rearing combination were used per run. When more than one cube was used in a run, we considered the run to be a block, but when only one cube was used, all runs within a day were considered to be a block. (Blocking by date only applied to *S. agrili* and *T. planipennisi* held at 4 °C for 4 months.) The number of blocks and total number of individuals tested are reported (Supplemental Table), but in general supercooling points were measured for 10–57 individuals from each rearing treatment.

Data were analyzed with SAS 9.3 (SAS Institute, 2013). Supercooling point distributions were compared within species to test the effect of rearing condition and among species for the rearing condition that appeared to give the greatest cold tolerance. Initially, supercooling point data were analyzed for normality (PROC UNIVARIATE) and if data were not normally distributed, Box–Cox transformations (Box and Cox, 1964) were attempted. Supercooling points, in general, were not normally distributed and appropriate Box–Cox transformations could not be found (data not shown). Consequently, we first tested the effect of blocking within each species × rearing combination with a Kruskal–Wallis ANOVA with a critical $\alpha = 0.005$ after a Bonferroni correction for multiple comparisons to maintain an overall $\alpha = 0.05$. No block effects were detected (Supplemental Table), so data were pooled to test for effects of rearing condition or species on supercooling points. Supercooling point distributions among rearing treatments or between species were compared using pairwise nonparametric two-sample tests for equality of probability distributions [Kuiper (1960) test in PROC NPAR1WAY]. Probability values were adjusted for multiple comparisons to maintain an overall α of 0.05 using a Bonferroni correction (critical $\alpha = 0.006$ per comparison). Supercooling points of each rearing treatment within each species were compared, and the rearing treatment with the lowest median supercooling point for each species was selected for comparisons among species. We consider tests of the complete distribution of supercooling points to be more informative than tests of central tendency (i.e., based on the median or mean) because they are able to discern changes in the tails of the distributions (i.e., effects on the most or least cold hardy individuals).

2.2.2. Lower lethal temperature

Lower lethal temperature experiments followed a randomized block design. Within each block (i.e., run of the test), parasitoids from one species × rearing-condition were assigned arbitrarily to one of four target temperature treatments. Treatment temperatures were chosen to bracket the approximate mean supercooling point for each species × rearing group based on previously collected data. Each treatment was replicated four times for a total of 16 insects per run. Insects were placed individually into gelatin capsules, onto thermocouples, and cooled as described for supercooling point measures. Insects were not removed until the target temperature was reached, even if an exotherm was detected. The duration of exposure to the target temperature was about 5 s. After removal, samples were immediately warmed to room temperature. An additional four to ten insects in separate gelatin capsules were held at room temperature for the duration of the test as a control for each block and returned to warm/long-photoperiod conditions after completion of the run. Between five and ten blocks were run for each species × rearing group (Supplemental Table). After cold exposure, individuals were removed from the gelatin capsules, each placed in a microcentrifuge tube, and returned to warm/long-photoperiod rearing conditions for further observation.

Individuals were inspected at least twice to evaluate the effects of exposure to a temperature treatment. *T. planipennisi* and *S. agrili* were examined 3 d after cold exposure for black or brown discoloration and lack of movement. Both species were also examined for eclosion 30 d after return to warm/long-photoperiod after cold exposure. Diapausing *S. agrili* were also examined at 12 and 16 weeks when eclosion was expected (Lelito, *unpublished data*). Discoloration by 16 weeks was noted to determine if non-eclosed individuals were healthy but still in diapause (creamy white) or likely dead (black or brown). Discoloration and eclosion were assessed for *T. planipennisi* and *S. agrili* reared at warm/long-photoperiod conditions by cooling individuals to −15, −20, −25, or −30 °C before being returned to warm/long-photoperiod conditions (sample sizes in Supplemental Table). *T. planipennisi* from cool/short-photoperiod conditions and *S. agrili* from one- and two-generation diapause conditions were cooled to −20, −25, −30, or −35 °C before being returned to warm/long-photoperiod conditions (sample sizes in Supplemental Table). Some adult eclosion occurred for all *T. planipennisi* rearing treatments, so discoloration measures were not included in further analyses for this species. Cohen's kappa (Cohen, 1960) was used to measure agreement between the proportion of *S. agrili* from warm/long-photoperiod conditions that were discolored by 3 d after cold exposure and the proportion that failed to eclose. This test was meant to determine if discoloration and eclosion failure agreed. The proportion of individuals where both measures indicated possible cold injury (discoloration and failure to eclose) or survival (normal color and development) was calculated as a coarse measure of overall agreement.

Logistic regression (PROC LOGISTIC; SAS Institute, 2012) was used to express cold injury as a function of brief exposure to cold temperature. Modified Abbott corrected injury measures (Appendix) were calculated for each cold exposure treatment to account for any discoloration or eclosion failure in the unexposed control group (Rosenheim and Hoy, 1989). Treatment sample size was

multiplied by the proportion of individuals surviving in the control group to give an adjusted sample size. The adjusted sample size was then multiplied by the adjusted injury percentage to calculate the number of injured individuals in the treatment group attributable to cold exposure. The adjusted number of deaths and adjusted sample size were used in logistic regression models. Initially, a logistic regression model was developed with block as the only main effect to determine if differences in cold injury occurred between runs. No block effects were detected (Supplemental Table), so final logistic regression models followed the form:

$$\text{Discoloration or eclosion failure} = \frac{1}{1 + e^{-(b_0 + b_1 t)}} \quad (1)$$

where b_0 is the intercept, which reflects the projected degree of injury at 0 °C, b_1 reflects the rate of change in injury with the change in the coldest temperature, t, to which an insect was exposed. A separate regression model was estimated for each species and rearing combination. Differences between two models were tested in PROC LOGISTIC by including a binomial categorical variable, c, with values of 0 for a reference group and 1 for a test group (Suits, 1957). The resulting model followed the form:

$$\text{Discoloration or eclosion failure} = \frac{1}{1 + e^{-(b_0 + b_1 t + b_2 c + b_3 tc)}} \quad (2)$$

where b_0, b_1, and t are as defined previously, but now for the reference group; b_2 measures the difference in injury at 0 °C (i.e., intercept) between the test and reference group; and b_3 measures the difference in rates of change in injury with change in exposure temperature between the test and reference group. To measure differences in cold tolerance between species from similar rearing conditions, *T. planipennisi* was assigned to 0 and *S. agrili* was assigned to 1. A Bonferroni correction was used to adjust probability values for the number of comparisons within species and rearing treatments to maintain an overall $\alpha = 0.05$. Exposure temperatures resulting in 50% and 90% mortality were calculated from logistic regression models for each species and rearing combination. The delta method was used to estimate 95% confidence intervals for each estimate, as reviewed in Faraggi et al. (2003).

2.2.3. Lower lethal time

Species and rearing treatments for lower lethal time measures included *T. planipennisi* from cool/short-photoperiod conditions and *S. agrili* from warm/long-photoperiod, one-generation-, and two-generation-diapause conditions (Fig. 1). Lower lethal time studies followed a factorial design with four exposure temperatures (0, −5, −10, or −15 °C) and five exposure periods (3, 14, 28, 56, or 84 d). A control group was also placed in warm/long-photoperiod rearing conditions. After receiving a shipment of parasitoids, insects were placed in microcentrifuge tubes, and individuals were randomly assigned to a temperature and time exposure. Additional blocks of the test were performed as more parasitoids became available. Before being placed in reach-in freezers (Freezer Concepts, Southbury, CT), *S. agrili* from warm/long-photoperiod conditions were held at 10 °C for 24 h to reduce the potential for cold shock, while *T. planipennisi* and diapausing *S. agrili* were moved from 4 °C coolers to their respective freezers. Parasitoids were held at constant temperature (±1 °C) and removed at the end of each assigned exposure period (sample sizes in Supplemental Table). After the specified length of exposure, treatment individuals were held at 10 °C for 24 h, followed by warm/long-photoperiod rearing conditions. Discoloration was assessed for control and exposure groups 3 d after being placed in warm/long-photoperiod conditions, and eclosion was monitored as described for lower lethal temperature studies. Due to the limited availability of *S. agrili* from one and two-generation diapause over time, individuals from the first shipment of each rearing condition were only assigned to 84-d exposures and room-temperature controls. This long exposure period had to be started first to have sufficient time for the treatment and time to break diapause (at least 192 days). After we noticed that none of the diapausing *S. agrili* were eclosing from the control or the three day exposure treatments, discoloration was noted at 16 weeks after removal from cold exposure for the remaining 14, 28, 56, and 84 d time treatments.

The proportion of *S. agrili* from warm/long-photoperiod conditions that were discolored or failed to eclose were compared with Cohen's kappa to assess the extent of agreement between the two measures over the course of the long-term cold exposure. Logistic regression models were fitted to data to describe injury over time at a constant temperature after adjusting for control injury (Appendix). Logistic regression models for lower lethal time followed the form:

$$\text{Discoloration or eclosion failure} = \frac{1}{1 + e^{-(b_0 + b_1 t + b_2 d + b_3 td)}} \quad (3)$$

where b_0, b_1, and t are as defined previously (see Eq. (1)), b_2 now measures the change in injury within increasing days of exposure, d, and b_3 measures the interaction of temperature and time ($t * d$) on changes in injury with respect to changing temperature and time exposures. The temperature by time interaction term was only included in the model for a species and rearing condition if there was a significant effect of both temperature and time, and the interaction term was also significant. A block effect was also included to determine if cold injury was different for each shipment of parasitoids received, but was excluded from the final model because no significant effect of block was detected (Supplemental Table). The effects of rearing conditions on injury were compared as for lower lethal temperature by including the categorical variable c to test for differences between reference and test groups. Exposure times resulting in 50% and 90% mortality were calculated from logistic regression models for each temperature, species, and rearing combination. The delta method was used to estimate 95% confidence intervals for each estimate.

Because few *T. planipennisi* from warm/long rearing conditions eclosed after lower lethal temperature exposures, the data from lower lethal temperature and 3 d observations from the lower lethal time studies were combined. Eclosion rates for room temperature controls from lower lethal temperature studies and lower lethal time studies were not different (data not shown). The combined data were used to generate a logistic function to describe the probability of eclosion failure as a function of brief exposure to temperatures from 0 °C to the lowest lower lethal temperature exposure.

3. Results

3.1. Supercooling point

The median supercooling point for late instar *T. planipennisi* was between −27.3 and −29.4 °C and was not the same for all rearing conditions (Table 1). *T. planipennisi* reared in cool/short-photoperiod conditions with an additional 4 mo. at 4 °C had median supercooling points that were 1.3 °C lower than those individuals that had only been exposed to cool/short-photoperiod conditions ($df = 1$; test statistic $Ka = 2.233$; $p = 0.002$; Table 1; Fig. 2A). Supercooling points for *T. planipennisi* were not different between warm/long-photoperiod and cool/short-photoperiod conditions ($df = 1$; $Ka = 1.610$, $p = 0.105$). Supercooling points were also not significantly different between runs for each rearing combination (Supplemental Table). No *T. planipennisi* adults eclosed after freezing.

Table 1
Comparison of *Tetrastichus planipennisi*, *Spathius agrili*, and *Oobius agrili* supercooling points from different rearing conditions.

Species	Rearing	Median	90th percentile	Within Sp.[a]	Among Spp.[b]
T. planipennisi	Warm/long	−27.34	−28.71	A	–
T. planipennisi	Cool/short	−28.14	−28.83	A	–
T. planipennisi	Cool/short, 4 °C acc.	−29.42	−31.11	B	ab
S. agrili	Warm/long	−25.19	−26.75	E	–
S. agrili	1 gen. diapause	−28.77	−31.02	F	a
S. agrili	1 gen. diapause, 4 °C acc.	−29.17	−31.22	F	–
S. agrili	2 gen. diapause	−29.06	−31.12	F	–
S. agrili	2 gen. diapause, 4 °C acc.	−28.09	−30.01	F	–
O. agrili	Diapause, 4 °C acc.	−30.49	−31.40	–	b

Rearing conditions are summarized in Fig. 1.
[a] Comparison of the statistical distributions of supercooling points within a species from different rearing conditions. Rearing conditions marked with the same letter are not statistically different. Comparisons of distributions were based on pairwise Kuiper's tests with a Bonferroni adjustment to maintain an overall $\alpha = 0.05$.
[b] Comparison of the statistical distributions of supercooling points among species from rearing conditions that gave the most cold-tolerant individuals. Rearing conditions marked with the same letter are not statistically different. Comparisons of distributions were based on pairwise Kuiper's tests with a Bonferroni adjustment to maintain an overall $\alpha = 0.05$.

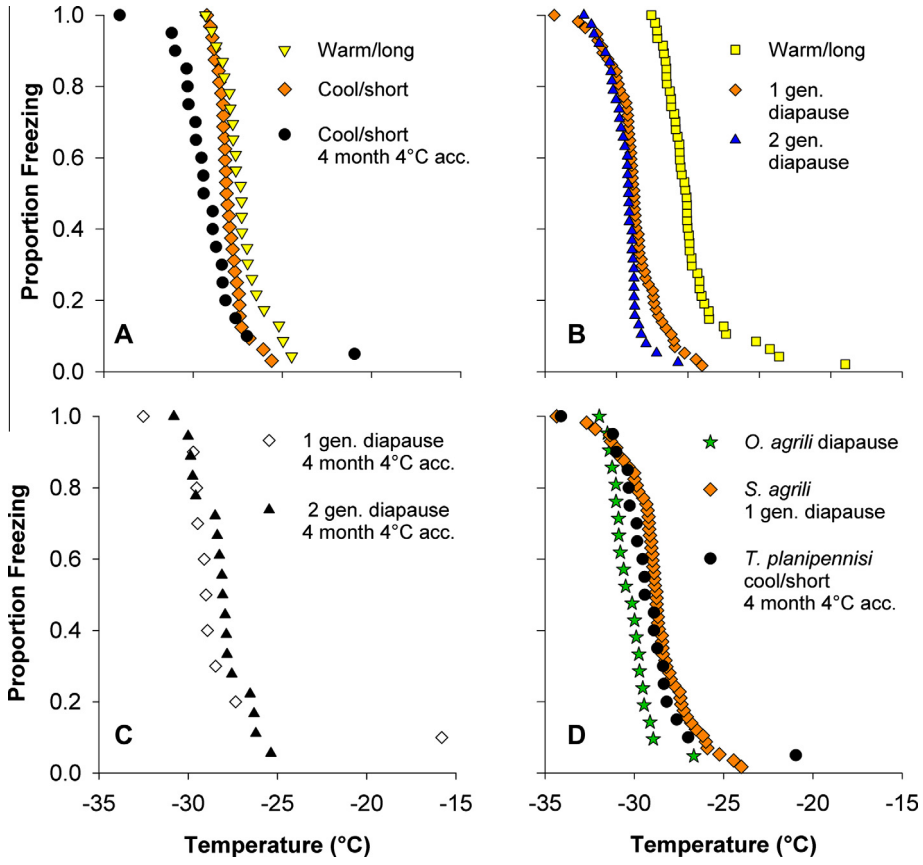

Fig. 2. Observed supercooling points for: (A) *Tetrastichus planipennisi* larvae from three rearing conditions; (B) *Spathius agrili* from three rearing conditions without an additional four months at 4 °C (C) *S. agrili* from two diapause-inducing conditions with an additional four months at 4 °C. (D) *Oobius agrili* and species from rearing combinations with the lowest supercooling points.

The median supercooling point for *S. agrili* ranged from −25.2 to −29.2 °C and was not the same for all rearing conditions (Table 1; Fig. 2B). *S. agrili* from warm/long-photoperiod conditions had supercooling points that were 3–4 °C warmer than *S. agrili* from one- or two-generation-diapause conditions without acclimation ($df = 1$; smallest $Ka = 4.077$; largest $p \leqslant 0.001$; Table 1). Supercooling points did not change when *S. agrili* from diapause conditions were acclimated at 4 °C for 4 months ($df = 1$; largest $Ka = 2.064$; smallest $p = 0.006$; Table 1; Fig. 2C). Supercooling points were also not significantly different among runs for each rearing combination (Supplemental Table). After freezing, all pre-pupae from warm/long conditions were discolored, and 91.2% ($n = 57$) from one-generation-diapause and 97.4% ($n = 38$) from two-generation-diapause were discolored.

Diapausing *O. agrili* had a median supercooling point of −30.5 (Table 1; Fig 2D). One of 21 *O. agrili* eclosed 30 d after freezing. When compared with the other parasitoid species from rearing conditions that yielded the lowest supercooling points, supercooling points of *O. agrili* were 1.3 °C lower than *S. agrili* from one-generation-diapause conditions with an additional 4 mo. at 4 °C ($df = 1$; $Ka = 2.601$, $p < 0.001$) (Table 1, Fig. 2D). Supercooling points for *T. planipennisi* reared under cool/short-photoperiod conditions

Table 2
Results of lower lethal temperature studies for *Tetrastichus planipennisi* and *Spathius agrili* from different rearing conditions: number of individuals that froze and died after freezing and estimated logistic-regression parameters to characterize discoloration or failure of adults to eclose as a function of brief exposure to low temperatures.

Species & rearing	*N* frozen	Freeze-mortality	Intercept (b_0)			Temperature (b_1)		
			Coefficient ± SE	χ^2	*p*	Coefficient ± SE	χ^2	*p*
T. planipennisi								
Eclosion:								
Warm/long	23	23	−4.871 ± 1.3762B	12.53	<0.001	−0.270 ± 0.0679B	0.07	<0.001
Cool/short	60	60	−0.534 ± 2.123	0.06	0.802	−0.102 ± 0.0821	1.53	0.216
Cool/short[a]	–	–	−7.328 ± 1.349	29.5	<0.001	−0.369 ± 0.0680	29.39	<0.001
S. agrili								
Discoloration:								
Warm/long	44	41	−15.017 ± 2.744a	29.94	<0.001	−0.633 ± 0.115a	30.46	<0.001
One gen. diapause	46	43	−26.520 ± 5.408a	24.05	<0.001	−0.970 ± 0.199a	23.87	<0.001
Two gen. diapause	62	52	−15.651 ± 2.464a	40.35	<0.001	−0.542 ± 0.085a	41.01	<0.001
Eclosion:								
Warm/long	44	44	−27.730±7.380A	14.12	<0.001	−1.186±0.304A	15.22	<0.001

Coefficients followed by the same letter are not significantly different at Bonferroni adjusted $\alpha = 0.05$; lower case letters represent comparisons among rearing treatments based on discoloration; upper case letters represent the comparison between species based on eclosion.
Coefficients are for Eq. (1) in the text.
[a] Model resulting from combined lower lethal temperature and lower lethal time data at 3 d.

with an additional 4 mo. at 4 °C were not different from *S. agrili* or *O. agrili* ($df = 1$; largest $Ka = 1.463$, smallest $p = 0.209$). Supercooling points were not significantly different among runs (Table 1).

3.2. Lower lethal temperature

For *T. planipennisi* from warm/long-photoperiod conditions, a greater proportion of adults failed to eclose as temperatures declined (Table 2; Fig. 3A). A constant, high proportion of *T. planipennisi* from cool/short-photoperiod conditions failed to eclose as adults at all treatment temperatures (Table 2; Fig. 3A). Adult eclosion was not significantly different for *T. planipennisi* from warm/long-photoperiod conditions than from cool/short-photoperiod conditions (Table 2). There was not a significant difference between runs for either rearing treatment (Supplemental Table).

There was significant agreement between the proportion of *S. agrili* that were discolored 3d after cold exposure and the proportion that failed to eclose as adults 30 d after cold exposure (Fig. 4). Discoloration increased with decreasing temperature for *S. agrili* from warm/long-photoperiod conditions and one- or two-generation diapause, but discoloration rates after cold exposure were not significantly different among rearing treatments (Table 2; Fig. 3A). Adult eclosion also decreased with decreasing temperature for *S. agrili* from warm/long conditions (Table 2; Fig. 3B). However, diapausing *S. agrili* did not eclose 16 weeks after being returned to warm/long-photoperiod conditions, so failure of diapausing *S. agrili* to eclose could not be analyzed as a function of cold exposure. Adult eclosion rates were lower for *T. planipennisi* compared with *S. agrili*, when both were reared under warm/long-photoperiod conditions (Table 2). There was not a significant difference between runs for any rearing treatment (Supplemental Table). Temperatures resulting in 50% and 90% mortality from brief cold exposures also provided for *T. planipennisi* and *S. agrili*, e.g., 50% *S. agrili* mortality at −27.4 °C for one generation diapause versus −23.7 °C for warm/long conditions (Table 3).

3.3. Lower lethal time

A smaller proportion of *T. planipennisi* reared under cool/short-photoperiod conditions eclosed as the duration of exposure to constant temperatures between 0 and −15 °C, inclusive, increased. At any length of cold exposure, fewer adults eclosed as the treatment temperature declined (Fig. 5; Table 4). There was not a significant interaction between temperature and exposure time, so a temperature by time interaction term, $t * d$, was not included in the model. No significant difference was found between shipment dates for each rearing treatment (Supplemental Table).

Some *S. agrili* from warm/long-photoperiod conditions, but not from one- or two-generation-diapause conditions, eclosed after exposure to 0 to −15 °C, inclusive, for different lengths of time. The proportion of individuals from warm/long-photoperiod conditions that failed to eclose 30 d after being removed from cold exposure was greater than the proportion that was discolored after 3 d, though there was some agreement between both measures (Fig. 6). The proportion of *S. agrili* reared under warm/long-photoperiod conditions with discoloration increased and eclosion decreased as exposure temperature decreased and exposure duration increased (Table 4; Fig. 7A and B); adult eclosion was also affected by an interaction between temperature and time ($b_3 = -0.009$; SE = 0.001; $p < 0.001$). The proportion of discolored *S. agrili* from one-generation-diapause also increased with increasing exposure duration, but decreasing temperature did not significantly affect discoloration (Table 4; Fig. 7C). Two-generation-diapause discoloration increased with both decreasing temperature and increasing exposure duration (Table 4; Fig. 7D). All *S. agrili* from 1 or 2 generation diapause conditions were discolored 16 weeks after exposure. The proportion of discolored *S. agrili* from warm/long-photoperiod conditions was significantly greater than from two-generation-diapause inducing conditions (Table 4). No significant difference was found between shipment dates for each rearing treatment (Supplemental Table). Exposure times and temperatures resulting in 50% and 90% mortality are provided (Table 5). Half of *T. planipennisi* are predicted to fail to eclose after exposure to 0, −5, −10, and −15 °C for >84, 82, 59, and 36 d, respectively. *Spathius agrili* reared in diapause inducing conditions required >84 d of exposure to 0, −5, −10, or −15 °C to achieve 50% mortality.

Our assessment of the effects of rearing treatment on lower lethal temperature for *T. planipennisi* from cool/short-photoperiod conditions was initially incomplete. The proportion of individuals that eclosed after brief exposures to temperatures between −20 and −35 °C, inclusive, did not change significantly. Eclosion failure was >80% for all temperature treatments (Fig. 3A). However, as measured in our lower lethal time studies, eclosion failure was near zero after exposure to temperatures between 0 and −15 °C, inclusive, even after 3 d (Fig. 5). The combined model suggested

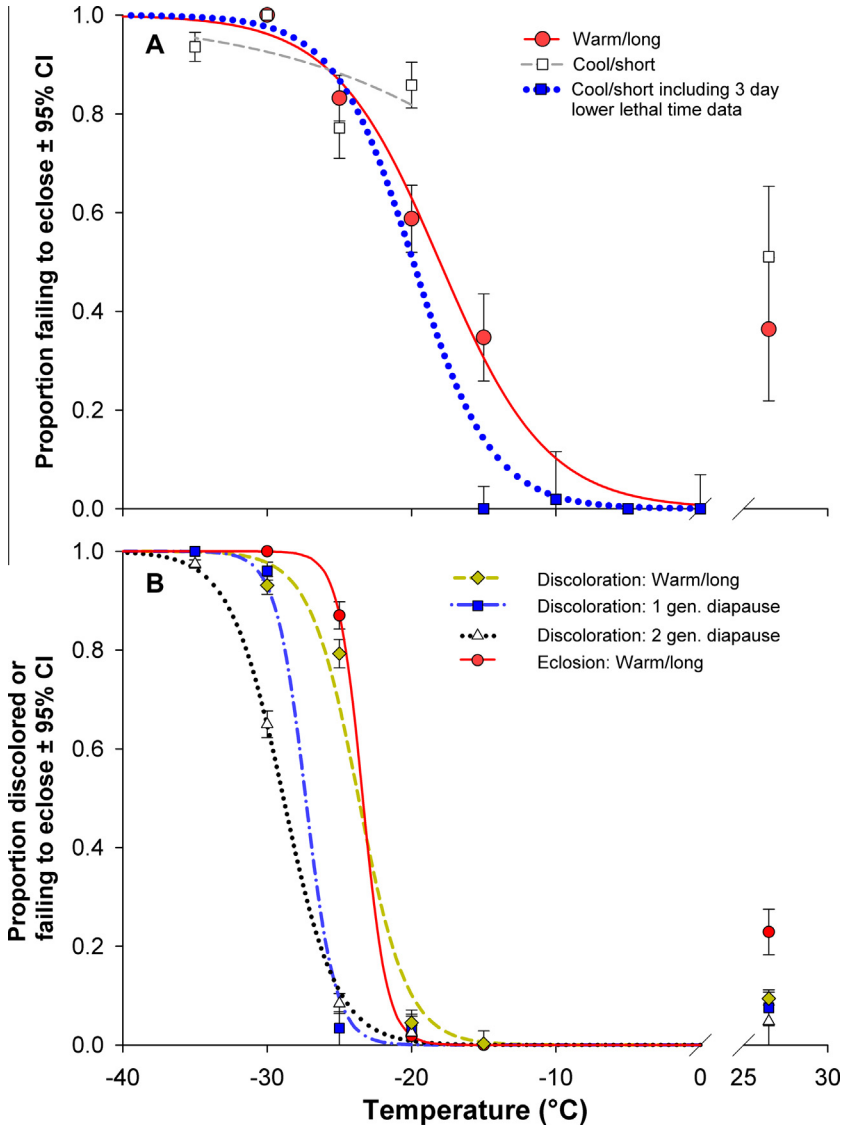

Fig. 3. Observed (symbols) and predicted (lines) proportion of emerald ash borer parasitoids that were discolored or failed to eclose after brief exposure to low temperatures: (A) *Tetrastichus planipennisi* adult eclosion. (B) *Spathius agrili* discoloration and adult eclosion. Observations at 26.5 °C were used to adjust proportions at other temperatures.

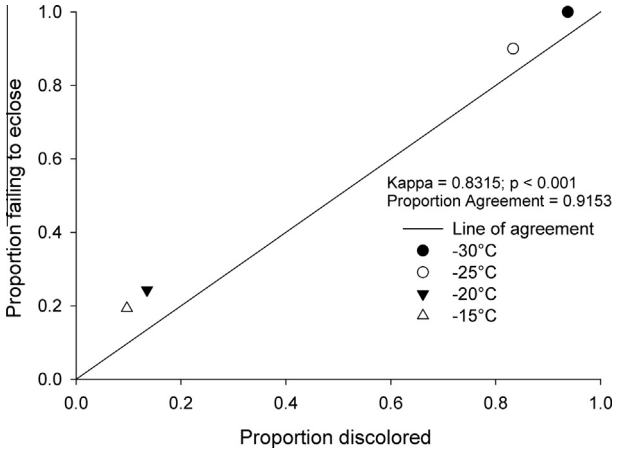

Fig. 4. Agreement between the proportion (not Abbott corrected) of *Spathius agrili* from normal rearing conditions that were discolored and those that failed to eclose in lower lethal temperature studies.

that the proportion of individuals that failed to eclose increased with brief exposure to colder temperatures, and eclosion rates were not significantly different than rates observed for *T. planipennisi* from warm/long-photoperiod conditions (Table 4; Fig. 3A).

4. Discussion

4.1. Previous research

Other researchers have reported some supercooling points and lower lethal time estimates for *T. planipennisi* and *S. agrili*, but measures of cold tolerance for *O. agrili* have not been published to date. Wu et al. (2007) reported that supercooling points of *T. planipennisi* ranged from −13.5 to −28.1 °C and *S. agrili* ranged from −19.9 to −28.4 °C. Our supercooling points of unacclimated individuals closely match these findings (Fig. 2). Lelito (*unpublished data*) also stored *T. planipennisi* larvae and *S. agrili* pre-pupae from warm/long-photoperiod rearing conditions inside ash bolts for extended periods of time at 4 °C and observed 15% *T. planipennisi* mortality at 185 d of exposure and 25% *S. agrili* mortality at 255 d of exposure. If the lower lethal time model from this work is extrapolated

Table 3
Lower lethal temperature for 50% (LT_{50}) and 90% mortality (LT_{90}) with 95% confidence intervals.

Species & rearing	LT_{50}	95% C.I.	LT_{90}	95% C.I.
T. planipennisi				
Eclosion:				
Warm/long	−18.0	−15.7 to −20.4	−26.2	−22.3 to −30.0
Cool/short	–	–	–	–
Cool/short[a]	−19.9	−18.1 to −21.7	−25.8	−22.8 to −28.8
S. agrili				
Discoloration:				
Warm/long	−23.7	−22.7 to −24.7	−27.2	−25.6 to −28.8
One gen. diapause	−27.3	−26.3 to −28.3	−29.6	−28.2 to −31.0
Two gen. diapause	−28.9	−27.8 to −29.8	−32.9	−31.1 to −34.7
Eclosion:				
Warm/long	−23.4	−22.3 to −24.5	−25.2	−24.1 to −26.4

*Values not shown since logistic regression parameters were not significant.
[a] Model resulting from combined lower lethal temperature and lower lethal time data at 3 d.

to 4 °C, 92% of *S. agrili* and >99% of *T. planipennisi* would be expected to fail to eclose. This mismatch between the projected and observed values suggests that our projections of cold injury over time should not be extrapolated outside the temperature (0 to −15 °C) and exposure time (3–84 d) ranges that were measured. Gould et al. (2011) also performed a lower lethal time study for non-diapausing *S. agrili* pupae at 10 °C and found that 41% eclosed upon return to normal rearing conditions after 3 months of exposure, but only 25% of females laid eggs.

4.2. Meaning of cold tolerance measures

Supercooling points, lower lethal temperature, and lower lethal time address different mechanisms by which low temperatures might cause injury. Supercooling points indicate the temperatures at which insects freeze. However, not all insects that freeze will die because some can survive freezing, while others die before they freeze (Sømme, 1982). If the frozen insect does not survive freezing during a supercooling point measurement, it may be either freeze-intolerant or chill-intolerant, but these alternatives cannot be distinguished until the temperature that caused mortality is known (Renault et al., 2002). As freezing is not always an indicator of mortality, supercooling point measurements alone are not fully sufficient to measure cold tolerance, but instead must be used in conjunction with measures of mortality (Renault et al., 2002). Taken collectively, our results suggest that *S. agrili* and *O. agrili* are freeze-intolerant, while *T. planipennisi* seems chill-intolerant.

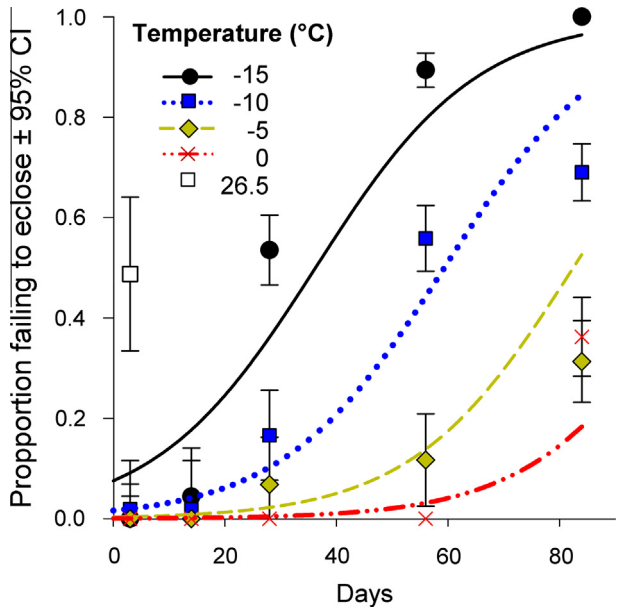

Fig. 5. Mortality of *Tetrastichus planipennisi* due to length of exposure at constant temperature. Observed (symbols) and predicted (lines) proportion of *T. planipennisi* that failed to eclose over time at constant temperatures. Observations at 26.5 °C were used to adjust proportions at other temperatures and times.

Observations of individuals after they have frozen, either during supercooling point measurements or in some lower-lethal temperature treatments, allow one to discern whether individuals have some degree of freeze tolerance. After freezing, *T. planipennisi* larvae and most *S. agrili* pre-pupae turned black or brown; none eclosed. Thus, these observations suggest *T. planipennisi* is not freeze-tolerant. However, we had difficulty breaking diapause in *S. agrili*. If eclosion is used as the measure of mortality, *S. agrili* is also not freeze-tolerant. If discoloration is the metric, a fraction (<~10%) of the diapausing *S. agrili* population might be considered freeze-tolerant. Discoloration could not be measured for *O. agrili* because the chorion of the emerald ash borer egg obscured view of the developing parasitoid, but one *O. agrili* did eclose after freezing. This lone survivor may indicate at least some *O. agrili* are freeze-tolerant. Interestingly, this individual had a supercooling point of −29.8 °C, while freeze-tolerant individuals typically have supercooling points near -10 °C (Turnock and Fields, 2005). There are exceptions to this trend among parasitoids (Salt 1959; Sømme 1964; Baust 1973; Ring 1982).

Lower lethal temperature studies showed that *T. planipennisi* and *S. agrili* were injured after brief exposures to temperatures below −20 °C. Such brief cold exposures might occur on the coldest day of the year in some locations. Median and 90th percentile supercooling points can also be compared with 50% and 90% mortality in the lower lethal temperature experiment to further assess freeze-tolerance (Fig 2; Table 3). For *S. agrili* from two-generation diapause conditions, median and 90th percentile supercooling points occurred within the 95% confidence interval for 50% and 90% mortality, respectively. This result indicates that *S. agrili* can be freeze-intolerant, but some degree of chill-intolerance also occurs among those reared under warm/long and one-generation diapause conditions. Meanwhile, *T. planipennisi* appears to be chill-intolerant as 50% mortality occurs at temperatures that are warmer than the median supercooling point. However, temperatures that result in 90% of *T. planipennisi* freezing and dying, respectively, appear to be relatively similar.

Measures of cold tolerance such as supercooling points and lower lethal temperature that use brief exposures do not account for cold stress that may have accrued from previous cold events and may underestimate injury when an insect experiences repeated low temperatures. Lower lethal time models can be used to forecast cold injury over an entire winter. However, these models often as-

Table 4
Results of lower lethal time studies for *Spathius agrili* and *Tetrastichus planipennisi* from different rearing conditions: estimated logistic-regression parameters to characterize discoloration or failure of adults to eclose as a function of exposure to low temperatures over time.

Species & rearing	Intercept (b_0)			Temperature (b_1)			Days (b_2)		
	Coefficient ± SE	χ^2	p	Coefficient ± SE	χ^2	p	Coefficient ± SE	χ^2	p
T. planipennisi									
Eclosion:									
Cool/short	−7.286 ± 0.747	95.08	<0.001	−0.319 ± 0.042	56.48	<0.001	0.069 ± 0.008	78.24	<0.001
S. agrili									
Discoloration:									
Warm/long	−5.283 ± 0.458a	133.00	<0.001	−0.126 ± 0.025a	26.15	<0.001	0.059 ± 0.005a	114.44	<0.001
One gen. diapause	−4.173 ± 0.376	102.81	<0.001	–	–	–	0.041 ± 0.006	53.36	<0.001
Two gen. diapause	−4.087 ± 0.391a	109.37	<0.001	−0.091 ± 0.026a	11.04	<0.001	0.037 ± 0.005b	28.82	<0.001
Eclosion:									
Warm/long	−4.103 ± 0.464	48.48	<0.001	0.065 ± 0.009	21.74	<0.001	−0.198 ± 0.0424	57.00	<0.001

Coefficients followed by the same letter are not significantly different at Bonferroni adjusted $\alpha = 0.05$.
Coefficients are for Eq. (3) in the text.

sume that an insect experiences a constant temperature, which is reasonable for many subterranean insects or insects that persist beneath a snowpack where temperatures changes can be buffered reviewed by Sinclair et al. (2003). Daily variations in winter temperature can affect survival rates where many freeze-intolerant and chill-intolerant individuals have lower injury under fluctuating thermal regimes when compared to constant temperatures (Colinet and Hance, 2010; Colinet et al., 2011). However, repeated freeze–thaw cycles can also increase mortality for freeze-tolerant individuals (Marshall and Sinclair, 2011, 2012). Therefore, the effect of fluctuating winter temperatures may be quite important in this system. However, properly assessing ecologically relevant temperature regimes could prove difficult given the current cost of rearing the parasitoids and the sample sizes required to assess multiple temperature and fluctuation combinations for multiple lengths of time.

4.3. Assessing mortality

Our primary interest during lower lethal temperature or lethal–time experiments was to measure the effect of cold exposure on insect mortality. Discoloration and failure to eclose represent mortality. Eclosion of adults seemed to be obvious evidence of survival and failure to eclose as evidence of death, but it was later learned that diapausing *S. agrili* require up to 26 weeks at 4 °C to break diapause (Lelito, *unpublished data*). Adult emergence was unable to be induced for individuals exposed to these conditions even after being placed in warm/long-photoperiod conditions. Individuals that failed to eclose also were discolored, so the possibility that pre-pupae were healthy but had not yet broken diapause can be ruled out. However, discoloration and adult eclosion failure rates for *S. agrili* from warm/long-photoperiod conditions were similar. Diapausing and nondiapausing individuals that froze when supercooling points were measured also had obvious discoloration. Thus, discoloration is likely to be a useful indicator of mortality when death is caused by freezing. However, discoloration after cold exposure during lower lethal time studies only weakly agreed with adult eclosion. Many individuals that had normal coloration still failed to eclose and no indication was apparent that these pre-pupae entered diapause due to the cold exposure treatment. All pre-pupae were discolored by 30 d after cold exposure if they did not eclose. These findings may indicate differences between acute and chronic cold injury in *S. agrili* (Sinclair and Roberts, 2005). Acute injury may occur from cell lysis during freezing to cause the visible discoloration of the pre-pupae. Chronic injury may be due to cold stress where the insect uses resources to survive cold, but reaches the point where it is unable to continue development.

4.4. Rearing condition effects

Rearing conditions affected the cold tolerance of *S. agrili* and *T. planipennisi*. Supercooling points were warmest for individuals from warm/long-photoperiod conditions. For *T. planipennisi*, supercooling points were unaffected by rearing under cool/short-photoperiod conditions; supercooling points were not lowered until individuals from cool/short-photoperiod were held at 4 °C for four months. For *S. agrili*, supercooling points were lowered when exposed to diapause-inducing conditions, but were not lowered further when exposed to other rearing treatments that were intended to induce cold acclimation. Based on supercooling points, it appears that cold tolerance of *S. agrili* changes more quickly than *T. planipennisi* when environmental conditions changed. Changes in supercooling points due to rearing conditions were typically small (e.g., 1.3 °C between the two cold acclimated *T. planipennisi* groups), but such small changes in cold tolerance can translate into substantial differences in potential overwintering distributions (Morey et al., 2013).

Lower lethal temperature models did not differ for *T. planipennisi* from warm/long-photoperiod conditions or cool/short-photoperiod conditions. This result suggests that the cold tolerance of *T. planipennisi* as measured by lower lethal temperature is not affected by exposure to cool/short-photoperiod conditions, a result similar to that observed with supercooling points. These two results suggest that likelihood of an individual surviv-

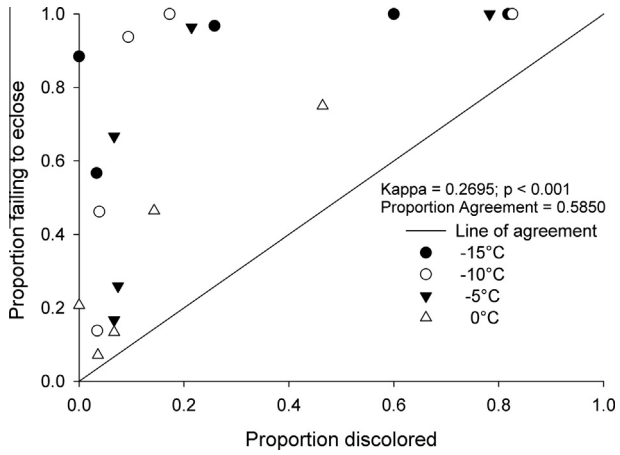

Fig. 6. Agreement between the proportion (not Abbott corrected) of *Spathius agrili* from normal rearing conditions that were discolored and those that failed to eclose in lower lethal time studies. Multiple points per temperature are independent observations through time.

ing the winter would not change if *T. planipennisi* were released in the spring or fall. However, releasing *T. planipennisi* in the spring could allow populations to build during the growing season and increase the likelihood that a fraction of the population will survive the winter.

An effect of rearing treatment on discoloration rates of *S. agrili* was not detected in lower lethal temperature studies, an interesting finding given that rearing treatments significantly affected supercooling points. This outcome suggests that while these insects lower supercooling points as rearing conditions more closely mimic fall than summer, there is not an associated increase in resilience to chilling.

In lower lethal time studies, *S. agrili* from two-generation-diapause conditions survived longer at a given temperature from 0 to −15 °C, inclusive, than *S. agrili* from warm/long-photoperiod or one-generation-diapause conditions. Because diapause induction appears to increase *S. agrili* cold tolerance, releasing *S. agrili* earlier in the year so that it has time to complete at least two generations may improve its ability to overwinter after release.

4.5. Differences in cold tolerance between species

Another objective of this study was to determine if any of the parasitoid species was more cold tolerant than the others. Lower supercooling points for *O. agrili* might be expected because the insect must survive on the surface of a tree, unlike the larval parasitoids that may be slightly buffered from extreme cold temperatures by the insulating effects of bark (Vermunt et al., 2012a). *Oobius agrili* may be the most cold tolerant of the three species based on supercooling points, but mortality measures are needed to further understand *O. agrili* cold tolerance.

Significant differences were not expected in overwintering mortality for *T. planipennisi* and *S. agrili* based on similar supercooling points. However, after brief cold exposure, a higher proportion of *S. agrili* eclosed than *T. planipennisi* when both species were reared under warm/long-photoperiod conditions. This result suggests *T. planipennisi* may have higher overwintering mortality than *S. agrili*. These findings should be interpreted with caution as late-instar *T. planipennisi* are susceptible to handling stress, which may have occurred during shipping, when parasitoids were removed from ash bolts, or when individuals were prepared for experiments (Lelito, *unpublished data*). Handling stress is common among parasitoids prior to pupation (Colinet and Boivin, 2011). Since eclosion was near 50% in the control groups it seems factors other than cold exposure were causing mortality. However, handling stress was less of an issue for *S. agrili* because discoloration of controls was typically <10% and the percentage of individuals from warm/long-photoperiod conditions that failed to eclose was only about 20%. If the larvae were already stressed, the results from this study may underestimate the level of eclosion that might occur in the field after cold exposure.

At the time these studies were conducted, *T. planipennisi* had only been described overwintering as late-instars (Bauer et al., 2008). However, recent findings suggest *T. planipennisi* can be found overwintering as early-instars in the host or as late-instars, pupae, or pharate adults outside the host, (Duan et al., 2013; L. Bauer, *personal comm.*). These additional stages may be of interest for cold tolerance research in case a different stage is more cold-

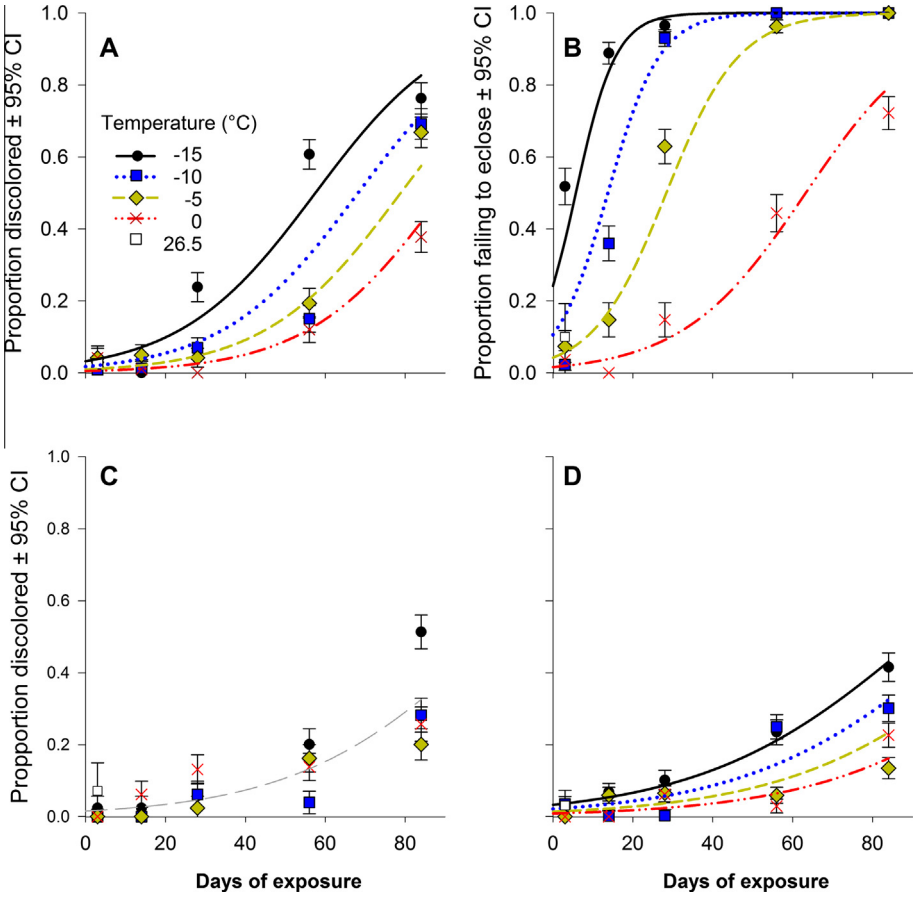

Fig. 7. Mortality of *Spathius agrili* due to length of exposure at constant temperature. Observed (symbols) and predicted (lines) mortality of *S. agrili* from different rearing conditions over time at constant temperatures: (A) warm/long-photoperiod conditions, (C) *S. agrili* one generation diapause (hashed line represents predicted mortality as a function of time; temperature did not have a significant effect on mortality), (D) *S. agrili* two generation diapause.

Table 5
Days of cold exposure resulting in 50% (LT_{50}) and 90% (LT_{90}) mortality with 95% confidence intervals for *T. planipennisi* and *S. agrili* at 0, −5, −10, and −15 °C.

Species	Rearing	Mortality measure	Exposure temperature (°C)	LT_{50}	95% C.I.	LT_{90}	95% C.I.
T. planipennisi	Cool/short	Adult eclosion	0	>84	–	>84	–
			−5	>84	–	>84	–
			−10	56	36–76	>84	–
			−15	33	19–45	51	38–63
S. agrili	Warm/long	Discoloration	0	>84	–	>84	–
			−5	70	47–92	>84	–
			−10	73	48–98	>84	–
			−15	56	39–72	>84	–
	Warm/long	Adult eclosion	0	61	41–79	>84	–
			−5	26	16–35	44	34–53
			−10	17	10–23	26	19–32
			−15	2	0–7	17	10–22

Confidence intervals not calculated for LT50s or LT90s beyond 84 days as this was the longest exposure time in the experiment.

tolerant or if the host larva affects overwintering survival of early instar parasitoids that are still within a live host (Colinet and Boivin, 2011).

4.6. Applications of models

Ultimately, the cold tolerance of these parasitoids could be considered by natural resource managers before selecting one or more parasitoids species to control emerald ash borer in an area. The suitability of the climate in areas where these species are introduced may affect both the likelihood of establishment and parasitism rates reviewed in Hajek (2004). If overwintering mortality is high for an *A. planipennis* parasitoid, the species may not be able to establish or reach population densities that would be sufficient to control *A. planipennis*. Therefore, understanding the cold tolerance of each species is an important consideration before starting a release program, particularly in northern latitudes. Forecasts for *S. agrili* from diapause-inducing conditions and *T. planipennisi* from warm/long conditions could be based initially on lower lethal temperature models and the minimum winter temperature recorded at a site. The amount of time spent at cold temperatures could be used to refine estimates of mortality at a location. Interpreting these measures of cold tolerance also requires accounting for relevant ecological conditions (Turnock and Fields, 2005). Daily minimum under-bark temperatures of *Fraxinus* spp. are typically warmer than the minimum air temperature. Using air temperature to predict mortality of *T. planipennisi* and *S. agrili* may overestimate mortality, so temperatures underneath the bark should be used to predict overwintering survival (Vermunt et al., 2012a). Large daily temperature fluctuations may also occur due to solar heating of a tree (Vermunt et al., 2012b). These warmer daytime temperatures may lead to the repair of sublethal cold injury in these parasitoids reviewed in Turnock and Fields (2005). However, assessing the effect of varying temperature on survival and sub-lethal effects is beyond the scope of this work and would an avenue for future research.

The cold tolerance of *A. planipennis* and these parasitoids can also be compared to determine how overwintering temperature may affect control of *A. planipennis* the following year. Crosthwaite et al. (2011) measured supercooling points and lower lethal temperature for *A. planipennis* and determined that freezing was lethal; and mean supercooling points of overwintering pre-pupae ranged between −28 and −30 °C. Discoloration of *S. agrili* during lower lethal temperature studies also appears to increase substantially within this temperature range. *T. planipennisi* had similar supercooling points to *A. planipennis*, but appeared to have additional pre-freeze mortality. These findings suggest *S. agrili* may have similar overwintering mortality as *A. planipennis* in a given location where both species are present, but *T. planipennisi* can experience higher overwintering mortality than *A. planipennis*. While supercooling points are similar for *O. agrili* and *A. planipennis*, *Oobius agrili* mortality needs to be measured at cold temperatures before determining the similarity in cold tolerance between the two species. Even though *S. agrili* appear likely to survive winters where *A. planipennis* is present, further research is needed to determine the minimum winter survivorship required for populations of each species to persist year-round. Nevertheless, our current results will improve efficiency in selecting parasitoids for a release site.

Acknowledgments

We would like to thank the USDA Emerald Ash Borer – Biological Control Facility in Brighton, MI for providing parasitoids. We also appreciate partial financial support provided by USDA APHIS and the Minnesota Environment and Natural Resources Trust Fund. We thank Drs. Bill Hutchison, Roger Moon, Tracy Twine, Juli Gould, Leah Bauer and anonymous reviewers for comments on a previous version of this manuscript.

Appendix A.

We adjusted estimates of mortality in response to cold exposure to account for mortality in control (i.e., room temperature) treatments, following methods developed by Rosenheim and Hoy (1989). Adjustments were based on:

$$Adj.Mortality = \frac{(1 - M_{treatment})}{\frac{(1-M_{control})}{(1-g)}}$$

where

$$g = \frac{Var(M_{control}) * t_{dist}^2}{(1 - M_{control})^2 * n_{control}}$$

Mortality in a treatment group of interest is adjusted using the observed mortality in that treatment ($M_{treatment}$) and control group ($M_{control}$). $M_{treatment}$ and $M_{control}$ can vary between 0 and 1, inclusive, but $M_{treatment}$ must be greater than or equal to $M_{control}$. The variance of the control group, $Var(M_{control}) = 4(M_{control} * (1 - M_{control})/n)$, and ($t_{dist}$) is the critical *t*-value chosen from the t-distribution based on the of the lesser sample size of the treatment or control group.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2013.08.015.

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CryoLetters **34** (3), 261-266 (2013)

THERMOCOUPLE DESIGN FOR MEASURING TEMPERATURES OF SMALL INSECTS

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Abstract

Contact thermocouples often are used to measure surface body temperature changes of insects during cold exposure. However, small temperature changes of minute insects can be difficult to detect, particularly during the measurement of supercooling points. We developed two thermocouple designs, which use 0.51 mm diameter or 0.127 mm diameter copper-constantan wires, to improve our ability to resolve insect exotherms. We tested the designs with adults from three parasitoid species: *Tetrastichus planipennisi*, *Spathius agrili*, and *S. floridanus*. These species are <3 mm long and <0.1 mg. Mean exotherms were greater for fine-gauge thermocouples than thick-gauge thermocouples for the smallest species tested, *T. planipennisi*. This difference was not apparent for larger species *S. agrili* and *S. floridanus*. Thermocouple design did not affect the mean supercooling point for any of the species. The "cradle" thermocouple design developed with the fine gauge wire was reusable and allowed for easy insect recovery after cold exposure.

Keywords: supercooling point, exotherm, parasitoid

INTRODUCTION

Contact thermocouple thermometry is commonly used to measure the surface body temperature of an insect during assessments of insect cold tolerance (5). Thermocouples are often attached to the bodies of insects with an adhesive, such as high vacuum grease or petroleum jelly (3, 9). As an insect is subjected to low temperatures, it will eventually reach a temperature at which body fluids begin to freeze (i.e., the supercooling point). Freezing is evident from a sudden increase in body temperature (i.e., an exotherm) as the heat of crystalization is released (3, 6, 9). The increase in temperature during an exotherm can be very apparent for larger insects as a result of their large mass and often high amount of fluids. However, the exotherms of minute insects, such as many insect parasitoids, (e.g., families: Braconidae, Eulophidae, and Encyrtidae) can often be small and difficult to discern from small sensor variations (AAH, personal observation). In addition, the adhesive used to attach the insect to the thermocouple often damages an insect (e.g., blocks spiracles), which is problematic when insect development after cold exposure is of interest.

Carrillo et al. (3) developed a method to control the cooling rate of insects placed inside a -80°C reach-in freezer. The insects were first placed inside polystyrene cubes with

dimensions designed to give desired and relatively constant cooling rates as the cooling rate can affect the supercooling point (10). For example, 20.3-cm cubes gave a theoretical cooling rate of approximately 1°C/min at the center of the cube when the interior temperature of the cubes was approximately <0°C. This cooling rate is standard when supercooling points are measured (10). However, the realized cooling rate could be slightly different, based in part on how close the insect was to the center of the cube. In addition, as the insect and thermocouple were lowered into the center of the cube, the thermocouple would often touch the side of the cube and dislodge the insect (4, RCV personal observation). Thus, our goal was to design a thermocouple that would help to reliably place the insect near the center of the cube, detect exotherms of small insects (2-3 mm long; <0.1 mg), recover an unadulterated insect, and would be durable for multiple uses. We developed two new thermocouple designs , which we call the coil and cradle, and used them to measure exotherms of three parasitoids: *Spathius agrili* Yang (Hymenoptera: Braconidae), *S. floridanus* Ashmead (Hymenoptera: Braconidae) and *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae). These parasitoids attack emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) an invasive insect in North America that infests ash (*Fraxinus* spp.) trees (8). Each insect was placed in a gelatin capsule, and the capsule was placed on thermocouples of differing thickness. With the insects restrained directly over the junction of the two thermocouple metals, we expected larger exotherms from thinner wire than the thicker wire, especially for smaller insects.

MATERIALS AND METHODS

Thermocouple construction: Two styles of thermocouples were constructed: a coil and cradle design. The coil design was constructed with 0.51 mm diameter (24 AWG) copper-constantan wire (accuracy = ±0.5°C). Wire was threaded through a 35-ml plastic syringe barrel (Fig. 1A). Approximately 7 cm of the plastic insulation was stripped from the end of the wire. The copper and constantan wires were wound tightly around each other for the entire length of the exposed wire. The wound wire was then formed into a spiral inside interior of the syringe tube (Fig. 1B).

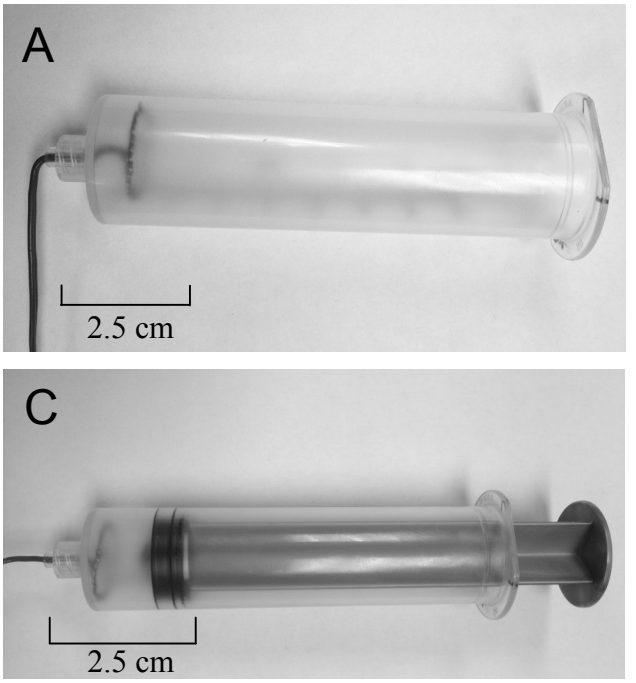

Figure 1. (A) Syringe barrel with a coil thermocouple inside. (B) Coil thermocouple with an attached *S. agrili* in a gelatin capsule. The first point where two bare wires touch after insulation ends is where temperature is detected. (C) Thermocouple with plunger inserted to reduce direct contact with air outside the insulated polystyrene cube.

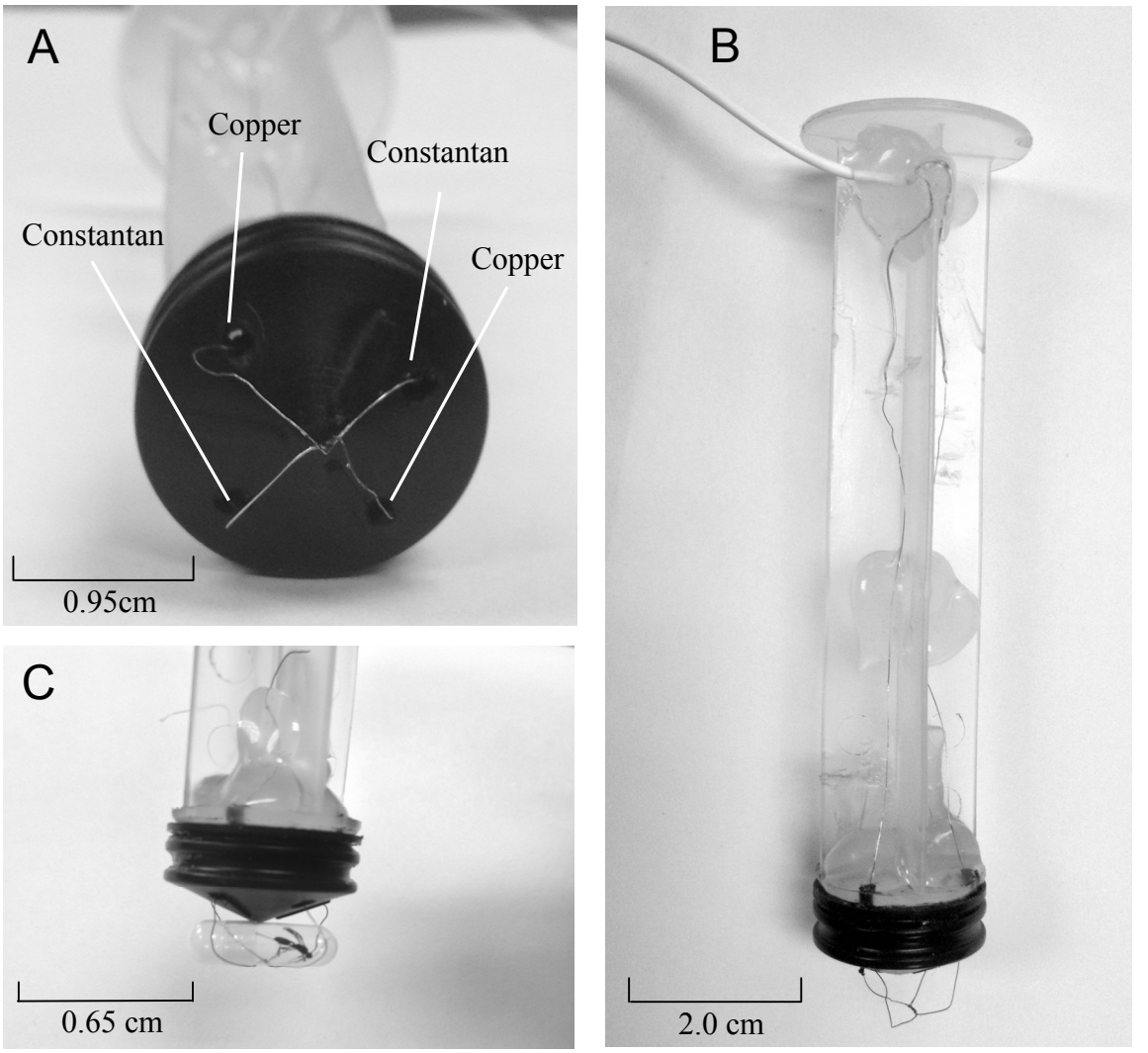

Figure 2. (A) Cradle thermocouple with holes made in the rubber plunger and the copper wire wrapped around the constantan. (B) Cradle thermocouple with glue holding bare wires in place. The split between the wires can also be glued to prevent wires from twisting. (C) Cradle thermocouple with an attached *S. agrili* in a gelatin capsule. Temperature is detected in the middle of the loops or "cradle" where the two wires are wound together.

The cradle design consisted of 0.127 mm (36 AWG) copper-constantan wire (accuracy = ±0.17°C) and a 20 ml plastic syringe barrel. Four equally-spaced holes were melted through the plastic and rubber of the syringe plunger with a hot metal probe (Fig. 2A). Approximately 10 cm of insulation was stripped from the end of the thermocouple wire. The remaining insulation was split an additional 5 mm below the stripped wire. The constantan wire was threaded through one of the holes and through a hole on the opposite side so a 1cm loop of wire protruded from the rubber plunger. The loose end of the constantan wire was secured to the side of the syringe with thermoplastic glue (Adhesive Tech, Hampton, NH). The copper wire was threaded through one of the remaining holes in the plunger, wound tightly around the constantan wire three to four times, and threaded through the remaining hole (Fig. 2A). The copper and constantan wires were glued in place to ensure bare wires did not touch before the intended connection (Fig. 2B).

Supercooling point measurements: Thermocouple wires leading out of the syringes were connected to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Supercooling points were measured for *S. agrili*, *S. floridanus*, and *T. planipennisi* adults reared from parasitized *A. planipennis* larvae at 18:6 h thermoperiod (26.5:22.5°C) and

photoperiod (L:D) at 60-70% RH. All parasitoids were reared by the US Department of Agriculture-Animal and Plant Health Inspection Service (Brighton, MI) and shipped via overnight courier to St. Paul, MN. Each insect was placed in a 5 x 14 mm clear gelatin capsule (Capsuline, Pompano Beach, FL). Capsules with insects to be cooled were held on the thermocouple with a small amount of high vacuum grease (Dow Corning, Midland, MI) at the first point of contact of the constantan and copper wires (Figs. 1C and 2C). For coil and cradle designs, the syringe plungers were pushed to the bottom of the syringe barrel leaving about 2 cm of open space, which was just enough room not to crush the insect (Fig. 1C). The thermocouples with insects were placed in polystyrene cubes calibrated to cool at a constant 1°C per minute inside a -80°C reach-in freezer and temperature was recorded every second (3). After all individuals had cooled to -30°C, the cubes were removed from the freezer and the parasitoids were placed in an 80% ethanol: water (vol:vol) solution for preservation. One run consisted of up to eight coil and eight cradle thermocouples containing a single species placed randomly in the freezer. At least two runs were performed per species. Supercooling points were determined as the lowest temperature experienced before the largest increase in temperature over 0.05°C, which was the approximate threshold where increases in temperature could be differentiated from slight background temperature variations. Exotherm height was determined as the difference between the supercooling point and the maximum temperature measured during the exotherm. In some cases we could not reliably detect an exotherm. A subset of parasitoids were later air dried for 1 wk at room temperature (~25°C) and weighed on a balance to obtain dry mass. However, individual *T. planipennisi* were <0.1 mg and could not be weighed with available equipment.

Data were analyzed using SAS 9.3 (11). Data were analyzed for normality (PROC UNIVARIATE) and homogeneity of variance (PROC GLM, Levene's test) to meet the assumptions for analysis of variance and linear regression. Supercooling points were normally distributed; potential effects of coil and cradle designs were tested with analysis of variance (ANOVA) blocked by run for each species (PROC GLM). Exotherm height for all three species and dry mass of *S. agrili* and *S. floridanus* were not normally distributed. A suitable transformation of exotherm height to correct for normality and unequal variance ($df = 5$; $F = 5.77$, $P > 0.01$) could not be found, so we used a Friedman nonparametric two-way ANOVA to test the effects of block and thermocouple on exotherm height. We applied a Bonferroni correction to account for family-wise error and maintain an overall α of 0.05 when comparing results from each ANOVA (7, 12). Logistic regression (PROC LOGISTIC) was used to evaluate the effects of block and thermocouple type on the proportion of tests in which an exotherm was detected for each species. The effect of block could not be evaluated for *S. agrili* due to quasi-complete separation. Linear regression (PROC REG) was used to determine if exotherm height on cradle thermocouples was correlated with dry mass (Box-Cox transformed (2) with $\lambda = -0.3$).

RESULTS

Mean supercooling points were not significantly different when measured on a coil or cradle thermocouple design across all species (Table 1; Fig 3A). Median exotherm height for *T. planipennisi* was significantly greater on the cradle thermocouples than on coil thermocouples (Table 1; Fig 3B). However, there was not a significant difference between coil and cradle exotherm heights for *S. agrili* or *S. floridanus* (Table 1). Supercooling points and exotherms were not significantly different between runs (Table 1).

Fig 3C illustrates the effect of thermocouple type on the proportion of tests in which an exotherm was detected. For *T. planipennisi*, exotherms were detected more frequently when measured with cradle thermocouples than coil thermocouples at Bonferonni adjusted α =

0.0167 (*df* = 1; Wald χ^2 = 7.24; $P < 0.01$); block had no effect (*df* = 1; $\chi^2 \leq 4.5$; $P \geq 0.03$). However, the proportion of tests in which an exotherm was detected was not affected by thermocouple type for *S. agrili* (*df* = 1; Wald $\chi^2 < 0.01$; $P = 0.94$) or *S. floridanus* (*df* = 1; Wald $\chi^2 = 1.85$; $P = 0.17$). Block had no effect for the latter species (*df* = 1; Wald $\chi^2 = 0.13$; $P = 0.72$). Dry mass was not a reliable predictor of exotherm height for *S. agrili* (*df* = 1,9; $F = 1.57$, $P = 0.24$) or *S. floridanus* (*df* = 1,7; $F = 0.94$, $P = 0.36$]. Mean masses were 0.7 mg (n = 10, SEM = 0.1) and 1.1 mg (n = 10, SEM = 0.1), for *S. agrili* and *S. floridanus*, respectively.

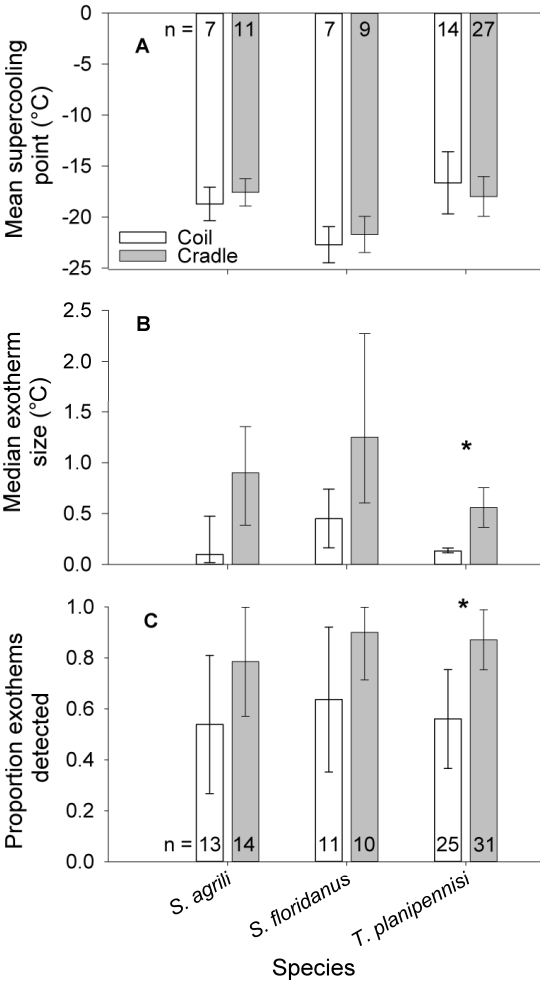

Figure 3. Mean (±95% CI) supercooling points (A), and median exotherm size (±95% CI) (B) and proportion (±95% CI) of exotherms detected (C) for adult parasitoids. Asterisk in (B & C) denotes a significant difference between values for the coil and cradle design for that species. Sample sizes were identical for supercooling point and exotherm measurement.

Table 1. Effects of block (run) and thermocouple type (coil vs. cradle) on supercooling point and exotherm height.

		Run			Thermocouple type		
Response	Species	*df*	*F*	*P*	*df*	*F*	*P*
Supercooling point	*S. agrili*	1, 15	0.01	0.93	1, 15	1.72	0.21
	S. floridanus	1, 13	6.54	0.02	1, 13	1.85	0.20
	T. planipennisi	3, 36	0.10	0.96	3, 36	0.58	0.45
Exotherm height	*S. agrili*	1, 15	6.54	0.17	1, 15	3.72	0.19
	S. floridanus	1, 13	5.22	0.04	1, 13	3.05	0.10
	T. planipennisi	3, 36	3.02	0.04	3, 36	13.57	<0.01*

* indicates significant p-value at Bonferroni adjusted α = 0.017.

DISCUSSION

Our designs worked well to measure supercooling points of small insects. Both designs gave similar supercooling points, which suggests that design type and wire thickness do not bias the supercooling point measurement. For *T. planipennisi*, the smallest insect tested, we recorded larger exotherms and detected more supercooling points with the cradle design than the coil design. Because these larger exotherms were more readily distinguishable from random fluctuations in temperature readings, supercooling points were detected that might not have been observed with thicker gauge wire. This increase in efficiency can lead to fewer samples with no recorded supercooling point and less time required processing additional individuals to reach desired sample sizes. Because of the increased sensitivity of finer gauge

wire, exotherms may also be detected if the insect is not in direct contact with the thermocouple wire, which can occur with the insect inside a gelatin capsule.

Each thermocouple design has particular advantages. The coil design uses thicker gauge wire that maintains its shape when handling. Repairs were typically only needed when plastic insulation cracked. The cradle design stabilizes finer gauge wire that tends not to form to a desired shape without support. Cradles were prone to become broken or misshapen between uses. In both cases, insects were easily recovered after exposure to cold and could be observed without interference from grease. We found these designs to be extremely useful to measure supercooling points and recommend the cradle for small insects such as small parasitoids (e.g., <3 mm long) and the coil for larger insects when exotherms are easier to detect.

Acknowledgements: We would like to thank the USDA Emerald Ash Borer - Biological Control Facility in Brighton, MI for providing parasitoids. We are grateful to Bob Koch, Mario Carrillo, Bill Hutchison, Roger Moon, Tracey Twine and anonymous reviewers for comments on previous versions of this manuscript.

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Accepted for Publication 20/01/13