REPRODUCTIVE BIOLOGY OF THE NEWLY INTRODUCED EMERALD
ASH BORER EGG PARASITOID, OOBIUS AGRILI, IN THE MID-
ATLANTICS

by

Devan A. George

A thesis submitted to the Faculty of the University of Delaware in partial
fulfillment of the requirements for the degree of Master of Science in Entomology

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IN THE MID-ATLANTIC REGION

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ABSTRACT

*Oobius agrili* (Hymenoptera: Encyrtidae) is a solitary egg parasitoid native to northeast Asia. It has been released in the United States since 2007 for biocontrol of invasive emerald ash borer (EAB) *Agrilus planipennis* (Coleoptera: Buprestidae). In order to develop a sound field release strategy that enhances the probability of its establishment in Mid-Atlantic area. We here evaluate the potential impacts of different releasing times and parasitoid diapause status on the reproductive biology of this introduced biocontrol agent under field conditions in Susquehanna State Park in northwestern Maryland.

Vials were placed on the trees either early or late in the season with parasitoids that were either previously diapaused or non-diapaused. We measured the emergence, longevity, parasitism, and diapause of the subsequent generations. Early releases of both types of *O. agrili* produced fewer progeny in diapause, allowing for greater parasitism throughout the season. Non-diapaused adults are more likely to produce diapaused eggs than diapaused adults, regardless of release time. To learn more about the *O. agrili*’s lifecycle, we studied the overwintering behavior of both diapaused and non-diapaused populations of *O. agrili* in field cages in three climatically unique locations in the mid-Atlantic states. The study showed low mortality among overwintering parasitoids, regardless of year or field site. The timeline for *O.agrili* showed varied emergence between the three sites. The results of this study will help to
determine how to better use this parasitoid for the control of *A. plannipennis* in mid-Atlantic states.
PREFACE

The increase of imports to the United States due to the growing global economy has caused the inadvertent introduction of over 4,500 species of foreign origin (Haack, 2006; Windle, 1997). Of these introduced species, approximately 15% cause serious environmental or economic damage. These include many wood-boring insect species, entering the US in wooden packaging materials, dunnage, and commodities such as nursery stock (Windle, 1997).

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is an invasive, phloem feeding insect pest of ash trees (*Fraxinus* spp.). It is able to attack all species of *Fraxinus* trees in the United States (Bauer et al., 2015; Haack et al., 2002; Herms and McCullough, 2014) as well as the white fringe tree (Oleaceae: *Chionanthus virginicus* L.) (Cipollini, 2015).

EAB was first detected in southeast Michigan and Ontario, Canada in 2002 (Haack et al., 2002). Recent dendrochronological and molecular analysis showed that this invasive beetle was probably introduced in the 1990s to the U.S. from Asia (Bray et al., 2011; Siegert et al., 2014). The emerald ash borer rapidly spread throughout the neighboring states, killing millions of ash trees (Cappaert et al., 2005; Haack et al., 2002; Herms and McCullough, 2014; Poland and McCullough, 2006) and has now spread to 35 U.S. states (Alabama, Arkansas, Colorado, Connecticut, Delaware, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maine, Maryland, Massachusetts, Michigan, Minnesota, Missouri, Nebraska, New Hampshire, New
Jersey, New York, North Carolina, South Carolina, South Dakota, Ohio, Oklahoma, Pennsylvania, Rhode Island, Tennessee, Texas, Vermont, Virginia, West Virginia, and Wisconsin) and three Canadian Provinces (EAB.info, 2018). It was first confirmed in Maryland by the USDA in 2006, in Pennsylvania in 2007, and Delaware in 2016.

There are several billion ash trees in the United States (Nowak et al., 2003) the destruction of which will have major economic impact on the diverse North American forest ecosystems. Recent analyses have shown that the economic impact of the EAB invasion in the urban forests of 25 northeastern states from 2009-2019 was estimated to be $25 billion (Aukema et al., 2011; Brockerhoff and Liebhold, 2017; Kovacs et al., 2009; Williamson and Fitter, 1996). Besides economic losses, EAB causes widespread ash mortality that cascades through ecosystems by reducing biological diversity, disrupting nutrient cycling and other essential ecological processes. There are 98 species of ash dependent specialist invertebrate herbivores that would likely be wiped out if emerald ash borer were to decimate ash forests. (Jennings et al., 2017; Ulyshen et al., 2012, 2011; Wagner and Todd, 2015)

EAB adults emerge in the spring between April and June and normally feed for 7-14 days before mating. Fertilized eggs are approximately 1 mm in diameter, ovular, and change from a milky white to amber as they mature over the few days after oviposition. EAB females produce approximately 100 eggs in their lifetime (Wang et al., 2010; Wei et al., 2007) which are laid in cracks and crevices in the bark, making human detection difficult (Abell et al., 2014). After eclosion, EAB larvae burrow through the bark and into the cambium, phloem, and xylem of the tree. There they eat
cambium, making characteristic serpentine galleries filled with frass. Larvae develop through four instars before burrowing into the hardwood to form a J-shaped overwintering chamber and undergo an obligatory diapause. Larvae feed for one (univoltine) or two (semivoltine) growing seasons before forming this J-shape (Cappaert et al., 2005). In the spring, the J-shaped larvae pupate for a month and emerge from the tree, making characteristic D-shaped exit holes. After emergence, adult EAB will begin feeding on ash leaves. Whereas the larval feeding significantly impacts the tree’s survival, adult feeding does not cause any significant damage to the tree. After two weeks of feeding, male and female EAB mate, and the females begin laying eggs. After a lethal amount of larval galleries are formed, typically in 3-4 years, the restriction of nutrients can result in tree death (Siegert, 2007). The cryptic nature of the life cycle of EAB creates serious challenges to the development of effective detection methods and viable management strategies.

Chemical control is an effective strategy in small numbers of trees, though is too expensive to treat large areas of natural ash forests (Mercader et al., 2015). Quarantines were initially established to control the emerald ash borer within the United States, restricting the movement of any Fraxinus genus firewood (USDA-APHIS, 2018). These, however, failed to contain the spread of EAB, therefore, biological control has become one of the most viable strategies for managing this invasive beetle in North America. It was hoped that woodpeckers might provide a source of native biological control of emerald ash borer and other wood boring larvae (Flower et al., 2014) but their predation typically followed severe outbreaks rather
than prevented them (Jennings et al., 2013). In contrast, traditional biological control models of introducing natural enemies from the pest’s native range can establish self-propagating populations that may effectively suppress the pest (Duan et al., 2018).

Foreign exploration for a natural enemy of the EAB began in 2003 in northeastern China, resulting in the discovery of four hymenopteran parasitoid species, including *Oobius agrili* Zhang and Huang (Encyrtidae) (Zhang et al., 2005). Using these agents, biological control of EAB was first implemented in 2007 in the United States.

*Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae), is a solitary egg parasitoid from northeast China where it is a native enemy of emerald ash borer. In its native range, *O.agrili* has been reported to cause 50-60% of EAB egg mortality (Liu et al., 2007, also Wang 2015). There have been males of *Oobius agrili* observed in northeast China (Liu et al., 2007), but only females have been observed in lab reared colonies (Duan et al., 2014; Hoban et al., 2016). Our current understanding of the lifecycle of *Oobius agrili* has been deduced from anecdotal observations made in the field in northeast China (Liu et al., 2007; Wang et al., 2010). The parasitoid’s life history in the U.S is unknown. To develop an effective arthropod biological control program, a thorough understanding of the yearly reproduction of both the host and its natural enemy is needed. To be effective, a parasitoid must have a synchronized life cycle with its host in the proposed control region to best exploit the resources provided by both the host and the environment. A synchronous life cycle will also allow the
parasitoid to maximize offspring production in the spring so as to continue the population through the following year (Tauber and Tauber, 1976).

Adults of O. agrili normally emerge in spring or early summer (May to June) from overwintering larvae inside parasitized host eggs. They can immediately attack host eggs of emerald ash borer by laying a single egg in a single host egg. Newly hatching parasitoid larvae feed on and develop inside the host eggs and can develop to adult wasps in 3 to 4 weeks at warm temperatures (Duan et al., 2014). It is suggested that O. agrili may produce two or more generations in its native range before a estivation (the process of going into diapause in the summer months) and overwintering (in diapause through the winter months) as diapausing mature larvae (Larson and Duan, 2016; Liu et al., 2007). Diapausing larvae normally resume development into pupae in the months of March-April and adults normally emerge in May and June (Liu et al., 2007).

Oobius agrili is one of the four biocontrol agents that have been introduced to the United States from the pest’s native range, Northeast Asia, since 2007. The other three biocontrol agents are EAB larval parasitoids, including Tetrastichus planipennisi Yang (Eulophidae) and Spathius agrili Yang (Braconidae) from Northeast China and Spathius galinae Belokobylskij (Braconidae) from the Russian Far East. To date, these agents have been released in 27 of 35 United States and two of three Canadian provinces invaded by EAB (MapBiocontrol 2018, also see review in Duan et al., 2018).
There have been many studies showing the impact of the larval parasitoid on ash stands infested with emerald ash borer (Duan et al., 2014, 2013, 2012) but fewer studies have investigated the establishment and effectiveness of *O. agrili* and its role in the biocontrol of EAB is largely unknown (Abell et al., 2014). The lack of field studies on *O. agrili* is largely due to the difficulty associated with observing the parasitism of EAB eggs in the field because of their small size and cryptic locations in tree bark. Bark sifting has recently been shown to be the most effective search method, yielding parasitism rates that were nearly 10 times higher than those found in visual surveys during the first year following release at a site and nearly 2 times greater than surveys conducted the following year (Abell et al., 2014).

Using the bark sifting method in Michigan, researchers showed that *O. agrili* parasitism rates in release sites 5 years after release was about 1 to 4% from 2008-2011. This percent of parasitism then increased to approximately 28% in 2014 in the release plots in Michigan (Abell et al., 2014). This is approximately half the parasitism rate (50-60% in 2005) found in China (Liu et al., 2007). This suggests that, over the next several years, the levels of parasitism in the United States could increase to parasitism rates equivalent to those in its native range (Abell et al., 2014). A rate of parasitism comparable to the 28% found in Michigan, has yet to be seen in the release plots in Maryland over a similar extended time period (up to five years after release), requiring further evaluation with an improved sampling method (Duan et al., 2011; Duan et al., 2012; Jennings et al., 2014; Larson and Duan, 2016).
Recent laboratory studies have shown that the origin of *O. agrili* adults from diapaused or non-diapaused larvae and the environmental conditions (photoperiod and temperature) affect the wasp’s reproductive biology. Adult wasps from both diapaused and non-diapaused larvae survived longer at a lower temperature (~20°C, a temperature that represents the cold fall weather in the Northeast United States), than those held in a higher temperature (~30°C, representing the warm weather in spring and summer). Regardless of temperature, both types of wasps were induced into producing diapaused progeny production by a short day (8h) photoperiod. Although, the diapaused adults exposed to a short day photoperiod had a significant increase in the amount of diapaused wasps produced over their lifetime, regardless of temperature (Hoban et al., 2016). In order to apply these results to a biological control plan, these results should be tested under field conditions.
Chapter 1

REPRODUCTIVE BIOLOGY OF *Oobius agrili*

1.1 Introduction

Using field cages, I tested the hypothesis that the release time and the origin of adult *Oobius agrili* from diapaused and non-diapaused larvae (thereafter termed as “diapause status” of adult wasps) will have significant effects on the key fitness parameters of the parasitoid reproductive biology. In order to test this parasitoid for a field release, I chose three different release times, in mid-May, mid-June, and mid-July. These release times would provide the varying environmental conditions to test the effect that photoperiod and temperature have on the longevity, fecundity, and diapause proportions of *O. agrili*. Two generationally differing adult populations were released at each of the release times, diapaused and non diapaused females. It has been shown in lab conditions that whether or not the wasp has gone through diapause has a significant effect on the rate at which adult *O. agrili* lay diapaused eggs. These conditions will subsequently affect the parasitoid establishment, abundance, and efficacy in the Mid-Atlantic United States.

1.2 Materials and Methods:

1.2.1 Parasitoids:

All *Oobius agrili* used in this study were adults reared from parasitized EAB eggs produced by the rearing facility of USDA APHIS Emerald Ash Borer Biocontrol Laboratory in Brighton, Michigan. Two generationally differing populations of adult *O. agrili* were used for the study: the diapaused and nondiapaused female adults. The
diapaused population consisted of adults that emerged from parasitized eggs that had been stored at 10°C for a week, then 4°C for 3–5 months. This process simulates the conditions overwintering *O. agrili* would experience before emerging in late spring or early summer. The non-diapaused parasitoid populations consisted of those adults that emerged from non-diapaused (i.e., not chilled) larvae, reared at 25°C for more than two generations from *O. agrili* that were originally put through 3-5 month diapause at 4°C. This was to mimic the summer conditions during which the parasitoids would not experience cold weather and continue to reproduce for at least one additional generation in mid to late summer (Duan et al., 2014).

1.2.2 Host Eggs:

Emerald ash borer eggs used in this study were freshly laid on unbleached coffee filter paper by female beetles that originated from field collected green ash trees. To produce EAB eggs for the study, EAB infested green ash (*Fraxinus pennsylvanica* Marshall) trees in Maryland, U. S. were felled from late fall (November) to mid-late winter (February). The trees were cut into meter-long pieces, and stored in a climatically controlled walk-in cooler at 3-5°C for season-long production. Tree sections were placed in emergence tubes made of cardboard Sonotube (1.2m long by 50 cm in diameter), and then held at 26-29°C with a photoperiod of 16:8 (L:D) h. The adults that emerged in these containers were collected every 1 to 2 days at the Maryland Department of Agriculture (Annapolis,
The emerged adults were sent to the USDA Agricultural Research Service (ARS) Beneficial Insects Research Unit (Newark, DE), the day of emergence or in overnight mail or hand delivered.

Once at the USDA-ARS Beneficial Insects Introduction Research Unit, the emerald ash borer adults were held in rearing containers (clear 3.5- liter jars with screen ventilated lids) at the density of approximately 15 females X 15 males per container, for 2 weeks before being moved into 1-L ventilated, clear plastic cups at 25°C, 65% RH, 16:8 (L:D) h at a density of 6-8 beetles. Adults were fed bouquets of fresh green (*F. pennsylvanica*) or tropical (*F. uhdei*) ash leaves. Mesh nylon screen was placed over the opening of the cup and an unbleached coffee filter covered the screen, secured by a rubber band. The combination of mesh and coffee filter is meant to resemble the spaces between the bark of an ash tree, so females will oviposit on the coffee paper (Duan et al. 2011, 2013). Eggs were then stored in 55°F (~12.8°C) chambers for no more than a week.

### 1.2.3 Experimental Procedure and Data Collection:

Field cages were set up in Susquehanna State Park in Havre De Grace, MD, a site located centrally in the Mid-Atlantic region. The ash stand at the study site was approximately 120 meters from a creek that drains into the Susquehanna and 100 meters away from a road with low traffic volume.
Field cages were used in this study consisting of clear plastic cups containing clear snap-cap vials (BioQuip Products, Ranchero Dominguez, CA; plastic tubes, 9 gram, 25.2 by 68 mm²) with screen-ventilated lids, made by cutting a 0.5 cm x 0.5 cm square hole and covering the hole with 790-micron nylon mesh (Supply company, Fort Meade, FL). This design allowed for the ambient temperature and humidity of the site to penetrate the vials without risking predation and hazardous weather conditions such as rain. Each vial held one diapaused or non-diapaused *O. agrili* adult and fresh EAB eggs. To provide nutrition and water to the *O. agrili*, non-diluted, clover honey was spread in thin lines on the sides of the vial using an insect pin.

The *O. agrili* in each container were provided 25 fresh eggs the first two weeks, 15 eggs for weeks three and four, 10 eggs for weeks five and six and 5 eggs each for weeks thereafter until the death of the parasitoid. This host-egg provision regime was used previously in a laboratory study (Duan and Larson, 2016), which showed no limitation of the fecundity of *O. agrili* over time.

Cages were affixed to ash trees by plastic coated wire ties strung between two 5-inch nails, driven into the tree on either side of the cup. Thirty vials consisting of 15 non-diapause adults and 15 diapaused adults were set up on June 9\textsuperscript{th} and July 14\textsuperscript{th}, 2016. The same number of vials were set up on May 26\textsuperscript{th}, June 15\textsuperscript{th}, and July 20\textsuperscript{th}, 2017. The addition of a May release in 2017 was due to new information that overwintering *O. agrili* field populations in the Mid-Atlantic begin to emerge as early as the second week of May. These set up dates were chosen to represent potential release times of *O. agrili* for use in biological control.
After a week-long exposure to host eggs, each vial was examined for *O. agrili* mortality, and parasitism of the host eggs. Surviving wasps were transferred to a new vial, containing freshly laid EAB eggs and honey. The number of eggs provided to each parasitoid and number of eggs parasitized in its previous exposure period were observed and recorded. The previously parasitized eggs were placed back on the tree to continue development to adults or mature diapaused larvae. Vials from all previous exposure periods, no longer containing an adult *O. agrili*, were monitored weekly for emergence. The number of emerged wasps was recorded, and then the emerged wasps were killed. Eight weeks after exposing the EAB eggs to the adult wasps, each egg was observed under a microscope to determine its fate, whether it was unparasitized, parasitized emerged, diapaused, or dead.

A HOBO (Temperature, Relative Humidity External Data Logger, model U12-012, Onset Computer Company, Bourne, MA) was placed in one of the field cages for the entire year. It collected both the temperature and relative humidity in the cages.

### 1.2.4 Statistical Analyses:

The survivorship of *O. agrili* in relation to release time and wasp diapause status was assessed using a Kaplan-Meier survival analysis platform. Statistical differences in the survivorship results of the adult wasps were calculated using the log-rank \( \chi^2 \) based on the survival analysis platform. The log-rank was used to determine statistical differences between the diapaused and non-diapaused adults at the May, June, and
July release times. The median survival times (±95% C.I.) were calculated for the diapaused and non-diapaused wasps using the Kaplan-Meier survival analysis.

Lifetime realized fecundity was determined using the total number of viable or live adult progeny of each wasp diapause type and release time produced over their lifetime. A two-way factorial analysis of variance (ANOVA) model was conducted to compare the main effects of wasp type (diapaused and non-diapaused), and release time (mid-May, mid-June, and mid-July) on the oviposition rate of the *O. agrili*. The progeny diapause rate from each parental wasp was calculated as proportion of diapause parasitoid progeny over the total number of parasitoid progenies produced (i.e., = total number of host eggs parasitized). Diapause rates were then transformed using arcsine square-root function. A two-way factorial analysis of variance (ANOVA) was conducted on the transformed data to detect the effect of wasp physiological status and release time on the proportion of diapaused eggs produced. JMP PRO (Version 13) statistical software was used to analyze this data.

1.3 Results:

1.3.1 Temperature and Humidity:

Temperature in 2016 consistently increased until August 23 at which point there was a large drop (Fig.1A). In 2016, the maximum weekly humidity inside of the plastic cups was 100% throughout the majority of June and July. There was a steep decrease in humidity, dropping from 84% average weekly humidity at July 12th to
54% at August 9th (Fig. 1B). In 2017, the average weekly temperature readings were all greater than 15°C (Fig. 1C). The maximum weekly humidity that year also reached 100% for most of the weeks in June and July followed by a steep decrease in humidity the weeks of July 15th and 22nd. The average weekly humidity dropped from 86% during the week of July 1st to 66% the week of July 15th. (Fig 1D).
Figure 1. Temperature and humidity readings taken at Susquehanna State Park, MD in the 2016 and 2017 field seasons.

1.3.2 *O. agrili* Adult Longevity:

In 2016, there is no significant differences in longevity of the non-diapaused and diapaused adults at any of the release times (Fig. 2&3). There are also no significant differences between the longevity of adults at each release times. In 2017, both diapaused and non-diapaused adults has a significantly greater longevity at the May release than at any other release times (log rank $\chi^2 = 20.97$, df = 5, $p = 0.0008$) (Fig. 2&3). There was no significant difference between the diapaused and non-diapaused adults at any of the release times.

Figure 2. Survivorship curves of the diapaused and non-diapaused adult *Oobius agrili* field cages at the May, June, and July release times
Figure 3. Median survival time (±95% C.I.) measured in weeks, of diapaused and non-diapaused adult *Oobius agrili* at the May, June, and July release times.

### 1.3.3 Fecundity

Neither the release time nor the parasitoid diapause type had significant effects on the realized fecundity of released *O. agrili* in 2016 (Table 1), but this was not the case in the 2017 when release time had a significant effect on the realized fecundity (the amount of eggs laid over the adult’s life time) of released *O. agrili*. In the 2017 releases, the mean fecundity was significantly greater for both diapaused and non-diapaused wasps at each successive release time (Table 2). Neither diapaused nor non-diapaused parasitoids showed a significant difference in the mean number of eggs parasitized at the June or July release in 2016 (Fig. 4). There was no significant difference in the non-diapaused and diapaused parasitoid population fecundity at any release time in either year. The decrease in fecundity in 2016 correlates with the sudden drop in temperature and significantly lower longevity in the July release.
Table 1. The 2016 effect test results from running a two-way analysis of variance on the realized fecundity of the wasps of each diapause type at each release time.

<table>
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<th>Source</th>
<th>Nparm</th>
<th>DF</th>
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<th>F Ratio</th>
<th>Prob &gt; F</th>
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<td>1</td>
<td>7.36333</td>
<td>0.0155</td>
<td>0.9015</td>
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</table>

Table 2. The 2017 effect test results from running a two-way analysis of variance on the realized fecundity of the wasps of each diapause type at each release time.

<table>
<thead>
<tr>
<th>Source</th>
<th>Nparm</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
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<td>Release Time</td>
<td>2</td>
<td>2</td>
<td>2105.1944</td>
<td>5.9363</td>
<td>0.0043*</td>
</tr>
<tr>
<td>Wasp Diapause Type *Release Time</td>
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<td>2</td>
<td>153.2500</td>
<td>0.4321</td>
<td>0.6509</td>
</tr>
</tbody>
</table>

Figure 4. Mean (+SE) lifetime fecundity of the diapaused and non-diapaused adult *Oobius agrili* at the June and July release times in 2016 (A) and the May, June, and July release times in 2017 (B). Bars with the same letters show no significant differences.
1.3.4 Diapause Rate

After running a two-way analysis of variance on the influence of the two independent variables (wasp type and release time) on the rate of diapaused egg production, in 2017 all effects were statistically significant at the 0.05 level. In the 2016 season only the wasp type yielded a significant effect on the rate of diapaused eggs produced.

In 2016, the main effect of the wasp type yielded a significant difference between diapaused adults and non-diapaused adults (Fig. 5). The interaction effect for the wasp type and release time was also significant for that year. This means that the significant effect of the release time is dependent on the wasp type being released.

The main effect of the release time on the rate of diapaused wasps produced was not significant in the 2016 season. The complete output of the effects test from the ANOVA run on the diapause rate data is listed in Table 3.

In 2016, the main effect of the wasp type yielded a significant difference between the diapaused and non-diapaused wasps (Table 3). The main effect the release time and the release time/wasp type interaction effect did not yield significant results in 2016. The interaction effect of wasp type and release time on the diapause rate in the 2017 season showed that diapause and release time have a significant effect on the rate of diapause. The complete output of the effects test from the ANOVA run on the diapause rate data is listed in Table 4.
Table 3. The 2016 effect test results of the total diapause rate of the *Oobius agrili* adults for wasp diapause type and release time, both individually and the interaction effect

<table>
<thead>
<tr>
<th>Source</th>
<th>Nparm</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wasp Diapause Type</td>
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<td>1</td>
<td>0.40427352</td>
<td>18.5798</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Release Time</td>
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<td>1</td>
<td>0.03469046</td>
<td>1.5943</td>
<td>0.2132</td>
</tr>
<tr>
<td>Wasp Diapause Type *Release Time</td>
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<td>1</td>
<td>0.03086654</td>
<td>1.4186</td>
<td>0.2399</td>
</tr>
</tbody>
</table>

Table 4. The 2017 effect test results of the total diapause rate of the *Oobius agrili* adults for wasp diapause type and release time, both individually and the interaction effect

<table>
<thead>
<tr>
<th>Source</th>
<th>Nparm</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
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<td>0.88976599</td>
<td>28.8086</td>
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<td>Wasp Diapause Type *Release Time</td>
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<td>2</td>
<td>0.59363652</td>
<td>19.2206</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

Figure 5. The average (±SE) proportion of parasitized eggs that are produced in diapause laid by the diapaused and non-diapaused adult *Oobius agrili* at the May, June, and July releases. Bars with the same letters show no significant differences.
1.4 Discussion:

In order to release *O. agrili* as an effective and efficient biological control agent for EAB, the life cycle and parasitism rates of the wasp need to be understood. *O. agrili’s* lifecycle in the Mid-Atlantic region of the United States has not previously been studied; all information has been based on observational data collected in its native range. This study was designed to determine what the best release time and wasp diapaused type for biological control release.

There was a significant difference in the longevity of adult *O. agrili* between the May, June, and July release times in 2017. Compared to the 2016 releases, the shorter life span in 2017 at all three release times correlates with the higher humidity (a nearly constant maximum humidity of 100%) recorded throughout the 2017 season. The May released adults were able to live the majority of their lifespan before the average humidity jumped after the week of June 15th. From that date onward, the minimum, average, relative humidity each week was greater than 50%. The intense humidity led to greater condensation in the vials than was present in the 2016 field season. Due to the high ambient humidity and precipitation outside of the field cages, beads of water collected along the stripes of honey that were streaked on the inside of the vials, that were used as a food source for the wasps. When the wasps would go to the honey for nourishment, they would drown in the water that had collected along the honey lines.
There was no significant difference in the fecundity of the non-diapaused and diapaused wasps at any of the release times. The release time also did not cause a significant effect on the fecundity of the adult wasps of either non-diapaused status or diapaused status. This shows that adult *Oobius agrili* released in the field to control the populations of emerald ash borer would be able to parasitize eggs at comparable rates regardless of diapause type or release time.

The release time yielded a significant effect on the rate of progeny produced in diapause. The summer solstice occurred on June 21st of both 2016 and 2017, after which the duration of daylight decreases each day. As the later releases were exposed to the shorter day length, they laid a greater proportion of diapaused eggs. There is a studied connection between the length of the day and the amount of progeny produced in diapause. Previous studies conducted on *Oobius agrili* reared in controlled growth chambers showed that the proportion of diapaused progeny produced increases as the amount of daylight is decreased (to an 8h light schedule opposed to 16h) (Hoban et al., 2016). When release times are later in the season, the days are shorter, signaling to the adult *O. agrili* that more progeny should be produced in diapause. This life-history strategy likely evolved over generations of *O. agrili*, resulting in a multivoltine behavior to increase overall fitness (Hopper, 1999).

The two diapause types of *O. agrili* also had a significant effect on the rate of diapause. The non-diapaused *O. agrili* produced an average proportion of diapaused larvae more than twice that of the diapaused *O. agrili* in the June and July releases of
both 2016 and 2017. The shorter longevity and lower diapause rates of the diapaused adult *O. agrili* are consistent with findings from lab studies (Larson and Duan, 2016).

These results suggest that a May (or early summer) release of diapaused adults into EAB infested ash stands would create a larger population of non-diapaused offspring. The diapaused *O. agrili* in this experiment simulated adults that would emerge in the spring, particularly the adults that were set up in May. Despite surviving for a shorter period of time, diapaused adult wasps would establish a population in the early to mid-summer, allowing them to continue parasitizing eggs through the peak oviposition period of EAB. According to previous studies (Brown-Rytlewski and Wilson, 2004; Cappaert et al., 2005) and anecdotal reports (Jennings et al., 2014) the peak oviposition for EAB is from June through the end of July in the United States.

A June release of diapaused adults would help sustain an actively emerging wasp population that could continue to parasitize EAB through the middle of the summer and into August and thus target the host at peak oviposition. Adults consistently laid the greatest number of eggs in the first two to three weeks after release, and laid fewer and fewer eggs each subsequent week. A release of non-diapaused wasps in July would begin to create a greater population of overwintering progeny while there was still an abundance of emerald ash borer eggs in the area.

The 2017 May release showed a low proportion of diapaused eggs produced by diapaused and non-diapaused adults. This contradicts the findings from lab reared *O. agrili* which found that non-diapaused adults laid the same proportion of diapaused
eggs regardless of the temperature at shorter day lengths (Hoban et al., 2016). The cause of the discrepancy in the May 2017 diapause rates, in which the non-diapaused eggs produced a much lower proportion of diapaused eggs than the subsequent releases, is unknown. Two possible explanations could be incorrect egg storage before the releases, or incorrect parasitoid designation prior to arrival at BIIRU. The intense humidity or the extremely varied temperature regime that the eggs experienced in the field as opposed to the controlled temperatures in lab could also have had an effect.

Finally, an August release of non-diapaused adults, producing a significantly higher rate of diapaused offspring, will produce a large population of overwintering *O. agrili*. An overwintering population is necessary to sustain *O. agrili* in the Mid-Atlantic region and defend against the semivoltine and overwintering emerald ash borer populations the following year. These release options ensure that there is an optimal parasitoid population each month of the emerald ash borer oviposition season. It also provides a plan that would promote a population of overwintering *O. agrili* for the following years.
Chapter 2

EFFECTS OF HOST HABITATS ON THE SURVIVAL AND EMERGENCE OF OVERWINTERING *OOBIUS AGRILI*

2.1 Introduction

All information about the overwintering emergence of *Oobius agrili* is based on observational studies made in Northeast China, the parasitoid’s native range. The most effective use of a biological control agent involves synchronized life cycles of the host and the parasitoid. To investigate the phenology of this egg parasitoid, diapausing *O. agrili* eggs were set up at three climatically unique sites in the Mid-Atlantic region. The three locations were monitored to determine whether the regional climate would allow a consistent emergence, or if the individual microclimates would produce varying emergence times and parasitoid mortality.

2.2 Materials and Methods

2.2.1 Production of Diapausing Parasitoid Larvae

All diapausing parasitoid larvae (inside the parasitized host eggs) used in the overwintering study were produced using field-cage rearing methods described in Chapter 1 Materials and Methods. The host (emerald ash borer) eggs were exposed to adult *O. agrili*, in field cages set up in Susquehanna State Park, Maryland, United States the from June to August of 2016, and May to August of 2017.

The parasitized host eggs, much darker in color, that did not emerge due to diapause at the end of the 2016 and 2017 field seasons were then collected, counted and used for the overwintering study.
2.2.2 Research Sites

Diapausing parasitoid larvae inside of the rearing vials for this study were placed inside the field cages (described previously) in three locations, each with a unique microclimate. I chose two sites that would have different ambient temperatures and distinctive environments, despite being within the same region: Susquehanna State Park, in Havre de Grace, Maryland, and at the USDA Beneficial Insect Research Unit in Newark, Delaware.

Susquehanna State Park is a natural forest, positioned near the mouth of the Susquehanna River, upstream from where it lets out into the Chesapeake Bay. The ash stand (39.614378, -76.152379) that contained my field cages was approximately 400 feet from a creek that drains into the Susquehanna and 350 feet away from a low traffic road. The ash stand is positioned on a steep (~27% slope) incline, at an elevation of ~100ft, with a Northeasterly aspect. The forest is old growth and field cages were hung on a tree with a DBH of 45.5cm. The site has a dense deciduous canopy cover in the summer and coniferous cover in the winter, both of which inhibit direct sunlight from reaching where the field cages posted on the ash trees.

The site chosen to be compared with Susquehanna State Park is an urban forest in Newark, DE (39.668578, -75.74193). The ash stand that held my field cages was ~35.5 km east north east of the ash stand in Susquehanna State Park. The ash stand is located at the North side of a cultivated alfalfa field, with the field cages on the south side of the trees, facing the open field. There is no slope at this location and the elevation is 31m. The urban forest is new growth forest; the tree holding the field cages for this study had a DBH of 21.6cm. There is an ~80% canopy cover for the field cages in the summer, when the ash trees are in full foliage, and then ~0% canopy cover in the winter and early spring before the trees regrow their leaves. The
surrounding terrain of the ash stand is also primarily flat, with no prominent mountains or buildings preventing sun exposure. The urban forest is directly south of the city of Newark, DE (population ~28,000).

To determine the effect of distance and environmental differences on emergence time variation between the Susquehanna State Park and the urban forest, a third site was set up to have a similar climate to the urban forest. The insectary in Newark, DE (39.668031, -75.741902) is located ~64 m from the location of field cages in the urban forest. It is a small building with ¾ walls and screen covering the space to the roof. Sunlight and wind can reach the field cages, though it is protected from precipitation and the sunlight is limited. The ambient temperature readings in the insectary are more similar to those in the urban forest than to the Susquehanna State Park readings. These conditions gave the insectary its own environment, while still having similar conditions to the urban forest.

2.2.3 Experimental Procedure

The parasitized host eggs containing diapause *O. agrili* larvae in Susquehanna State Park were counted and brought back to the laboratory, and then were redistributed in the same field cages from which they were collected to three locations described above (324 from the 2016 season, and 354 from the 2017 season). The cages at all sites were monitored twice a week from the second week of April until the first emergence. Once emergence occurred at a site, all cages at that site were monitored every three days until emergence ended. The number of emerged wasps was recorded per site and per day. The wasps that emerged were then killed so no wasps were counted more than once.
The cages were at their respective sites from November 17th of 2016 and 2017, until the following spring. Cages were taken down when the emergence of adults from the eggs ceased for two weeks. After complete emergence, the cages were brought into the lab for egg dissection. The eggs that showed no signs of emergence (lack of emergence holes) were opened with sharp forceps to find out if the larva inside was dead or was not given enough time to emerge. The number of dead *O. agrili* larvae at each site were recorded and the pieces of coffee filter paper covered in egg shells from each site were disposed of.

### 2.2.4 Temperature Recording

A HOBO (Temperature, Relative Humidity External Logger, model U12-012, Onset Computer Company, Bourne, MA) was placed in one of the plastic cup field cages that contained the eggs at each site. These temperature loggers recorded the ambient temperature and relative humidity in the cups every half an hour all day and night.

At the Susquehanna State Park site and the green ash urban forest in Newark, DE, there were bark probe temperature loggers recording the ambient temperature at the site and the temperature within the bark of the tree where the emerald ash borer eggs would be laid. These bark temperature probes were Tinytag flying lead thermistor, with dual channel internal and external (-40 to +85°C) and (-40 to 125°C). Each probe was positioned within the bark on ash trees and the wire connecting it to the logger was secured with flagging tape. The logger was then attached to the tree with a screw, positioned almost directly outside of the bark where the probe was buried. The probe and the logger recorded the temperature every hour from May 1, 2016 until August 1, 2018.
The temperatures recorded by these loggers on the outside of the tree bark and probes beneath the bark of the ash tree were analyzed to find the differences between the two microclimates. The hourly recorded temperature from the bark probe temperature logger was subtracted from the hourly recording made by the temperature logger on the outside of the same tree. The hourly difference was then coded to a unique number of the hour of day with 1:00am being 1 and 12:00am being 24. All temperatures were averaged per site per hour for the entire time the loggers were on the trees. These averages per hour were plotted on figure 12 in the results section.

2.3 Results

2.3.1 Temperature and Humidity

Temperature recordings in figure 10 are from all three sites in May and June, the months in which the *O. agrili* were emerging. There was a consistent pattern in both 2016 and 2017 in which the temperature reading in Susquehanna State Park were lower than those in the urban forest and the insectary in Newark, DE. Both graphs also show a pattern of slightly higher temperature peaks in the insectary; otherwise the insectary and the urban green ash forest experienced similar temperatures. This was expected due to the close proximity of the two locations (both within a single acre) (Fig.6).

The temperature for both years followed a similar pattern of daily averages in the first two weeks of May dropping below 14°C. There was then a steady increase in temperature resulting in the highest temperatures in the last two weeks of June. The average difference between the temperatures in the field cages in the urban forest and the Susquehanna State Park field cages was 0.32°C in 2017 and 0.64°C in 2018. The
average difference between the urban forest and the nearby insectary was .70°C in 2017 and -1.09°C in 2018.

Figure 6. Mean daily ambient temperature at each of the study sites, the urban green ash forest in Newark, DE, the Insectary in Newark, DE, and the Green Ash Stand in Susquehanna State Park, Havre de Grace, MD.

The humidity in Susquehanna State Park at the end of the summer was a major cause of parasitoid death in the 2016 and 2017 summer fecundity study. The high humidity experienced the September of 2017 in the field cages might also have played a major role in the deaths of eggs in this study.

In 2016 the weekly relative humidity declined at the first week of August, and after that the average humidity remained below 60%. Alternatively, in 2017 the weekly humidity decreased briefly (from the first week of June until the third week of June), then increased steeply again the first week of August. The maximum humidity was consistently greater than 90% and the average humidity above 80%.
Figure 7. The maximum, average, and minimum weekly average relative humidity in the field cages at Susquehanna State Park, MD for the 2016 and 2017 field seasons

2.3.2 Bark Microclimate Temperature

The difference between the ambient temperature under the bark of the ash trees and the outside air was analyzed at both Susquehanna State Park and the urban forest in Newark, DE. Figure 8 shows the average temperature difference, each hour of the day, starting with 12:00am at hour one and 11:00pm at hour 24. The average hourly temperature recorded by the bark probe temperature logger was subtracted from the hourly average temperature recorded by the logger that was placed directly outside of the bark on the same ash tree. These recordings were taken from May 1st, 2017 to July 10th, 2018, recording the temperature every hour at each site.

In Figure 8 you can see the daily pattern of the bark probe reading a higher temperature in the early morning hours, until 7:00am when the temperature difference
between the logger and the probe would become positive, meaning the logger was recording higher temperatures. In Susquehanna State Park, the average difference began to decline at approximately 12:00pm (hour 12) and then became negative at 7:00pm (hour 19). The averages for this site continued to decline from that time until 11:00pm (hour 24), with the lowest temperature holding consistent until the sun rose again.

In the urban forest, Newark, DE the temperatures for the hourly average differences between the logger and the bark probe showed a similar pattern to the Susquehanna State Park temperatures. The range in differences at the urban forest was much smaller than that at Susquehanna State Park. In Susquehanna State Park the greatest temperature difference between the logger and the bark probe was 0.813072 and the lowest difference was -0.693937, yielding a range of 1.507009. The urban forest greatest temperature is 0.454763 and the lowest temperature difference was -0.30305, yielding an average temperature range of 0.757813. This shows that the range of average difference in temperatures between Susquehanna State Park (1.507009) and the urban forest in Newark, DE (0.757813) is 0.749196.
Figure 8. The mean difference between the temperature logger and the attached bark probe, each hour of the day, in Susquehanna State Park, MD and an urban ash forest in Newark, DE.

2.3.3 Emergence Time

*O. agrili* emerged from the overwintering stage entirely in the months of May and June of both 2017 and 2018 at all three sites (Fig. 9). In both years, peak emergence occurred first in the green ash forest in Newark, DE. In 2016, the urban forest emerges 12 days before the insectary peak emergence and 18 days before Susquehanna State Park and in 2017, same day as the insectary and 14 days before Susquehanna State Park. Peak emergence for the green ash forest site was on May 26th in the 2017 spring season and May 30th in the 2018 spring season. The insectary was the second site in which peak emergence occurred in both years; the 2017 emergence peak was on June 7th and the 2018 emergence came earlier on May 30th. *O. agrili* at the natural ash stands in Susquehanna State Park emerged last, reaching its 2017 peak emergence on June 12th and the 2018 peak emergence date on June 14th.

Both years showed emergence began no earlier than May 15th in all three sites, and no emergence occurred any later than June 26th. Emergence in two out of the three sites in 2017 and 2018 was completed in two weeks. The peak Susquehanna State Park emergence in 2017 came 16 days after the peak emergence at the urban green ash forest in Newark, DE. The following year the emergence at Susquehanna State Park came 15 days after the peak emergence at the urban ash forest in Newark.

The first adult emerged from each site in both years and followed the same pattern as the peak emergence. The overwintering adults in Newark emerged first (May 20th, 2018 and May 15th, 2018), then the insectary (May 23rd, 2017 and May 21st, 2018), and then the natural ash stand in Susquehanna State Park (June 7th, 2017...
and June 5\textsuperscript{th}, 2018). The Susquehanna State Park first emergence came 17 days after the urban ash stand in Newark in the 2017 season. In the following year Newark urban forest emerged 22 days before the Susquehanna State Park site.

2.3.4 Death Rate

Throughout both years of the study, overwintering mortality rates of diapausing \textit{O. agrili} larvae were less than 7\%. Individually, each site in 2017 had less than 5\% mortality (Susquehanna state park: 4.6\%, green ash forest: 2.7\%, insectary: 0.9\%). Of all the eggs in the 2017 season, there was a total death rate of 2.7\%. The death rate in each of the sites in the 2018 emergence, although greater than those in 2017, was less than 7\% each (Susquehanna state park: 6.8\%, green ash forest: 5.9\%, insectary: 5.1\%).

Figure 9. Total number of emerged wasps per week of all overwintered \textit{Oobius agrili} kept in Susquehanna State Park, Havre de Grace, MD, the green ash forest in Newark, DE, and the insectary in Newark, DE.

### Figure 9. Total number of emerged wasps per week of all overwintered Oobius agrili kept in Susquehanna State Park, Havre de Grace, MD, the green ash forest in Newark, DE, and the insectary in Newark, DE.
2.4 Discussion

Temperature records showed that the urban forest located in Newark was warmer (average daily mean temperature difference of approximately 1°C) than the Susquehanna State Park. The urban forest is in the middle of a highly populated area which is warmed by human activities, surrounding buildings, and impervious surfaces. Alternatively, the site in Susquehanna State Park is close to large bodies of water, the Susquehanna River and the Chesapeake Bay. There is little human activity in Susquehanna state park, only a few houses, park visitors, and fishermen. The temperature in the insectary peaked several times, either matching the top temperatures recorded in the urban forest or exceeding them. These temperature spikes were most likely due to the lack of wind, precipitation, and additional insulation provided by the walls of the building.

The pattern of hourly average temperature differences observed in the bark probe data are similar to those found in other studies of the ash tree bark temperatures (Vermunt et al., 2012). There is a 2°C maximum range of temperature differences between the underbark temperature and the ambient field site temperature. This difference should be considered when consulting regional ambient temperatures to predict the timing of overwintering emergence of the egg parasitoid.

The positive values in temperature difference in the middle of the day, between the hours of 8:00am and 5:00pm, are due to the fact that the sun is up and directly warming the temperature logger located on the outside of the tree. In the eastern United States, regardless of the time of year, the process of the sun rising (from first light to the time of full sun) is always complete by 7:30am according to the National Weather Service. Before the sun rises and after the sun has set, the temperature differences become negative, indicating that the temperature beneath the bark is higher.
than the temperature outside. This could be due to the insulating effect the bark on the temperature probe or protection from weather factors such as wind, and chilling precipitation. There is a sudden drop in the average hourly temperature difference in the urban green ash forest between 12:00pm and 1:00pm. This is possibly due to the sun’s arch allowing the warming light to hit the bark covering the probe more than the temperature logger. Due to the layout of the trees in the woods, there is a brief amount of time the logger is in shadow.

The emergence pattern of the *Oobius agrili* in both the 2017 and 2018 spring season correlated with temperature patterns of those locations. The lower temperature patterns in Susquehanna State Park correlated with the later emergence times of the *O. agrili* in those field cages. The urban forest was less than an average of 1°C warmer than Susquehanna State Park in the daily temperature recordings in both 2017 and 2018.

The majority of parasitoids in this study emerged in the spring, giving a very low mortality rate in both years of the study. However, the mortality rate is slightly higher in the 2018 season. The greater number of deaths in the 2018 emergence season compared to the 2017 season was most likely caused by the high humidity recorded in the field cages at the end of the 2017 season (Fig.7). After dissection, the eggs appeared to have never developed into *O. agrili* pupae after killing the emerald ash borer egg. There was often dry, dusty contents or gelatinous yellow substance with no form inside of the unemerged parasitized egg, which lead to the conclusion that they had died before developing to adults within the egg.

The overall death rate was very low at all three of the sites, less than 7%. This suggests that the ambient temperature of the mid-Atlantic region will not inhibit the
overwintering emergence of the *O. agrili*. This could be the result of the protection provided by the experimental containers, protecting the eggs from weather, and predators. The *O. agrili* also have a diapause behavior that provides them a high tolerance to intense cold and other adverse weather conditions.

The emergence of all of the *O. agrili* took place in the months of May and June, with all peak emergence taking place between the second week of May and the second week of June. This is at the later end of the observed emerald ash borer emergence time of May through June under natural conditions in China (Wang et al., 2010; Wei et al., 2007). Emerald ash borer spends two weeks feeding before it is able to lay eggs whereas *O. agrili* is able to start laying eggs almost immediately after emerging. This difference in the host and parasitoid emergence times will allow there to be a supply of emerald ash borer eggs for the *O. agrili* to begin parasitizing upon emerging from overwintering.

The small difference between the ambient temperatures in which the eggs were held was enough to cause an approximately two-week difference in the first emergence of the urban forest and Susquehanna State Park field cages. The differences in the urban forest and the insectary in 2017 showed greater average daily temperatures in the urban forest (less than 1°C average difference). This difference in temperature resulted in a week difference in the first emergence and an approximately two-week difference in the peak emergences. In 2018, the average daily temperature differences between the urban forest and the insectary showed greater temperatures in the insectary, most likely due to the aforementioned protection the insectary gave the field cages. This is most likely the cause of the pattern of emergence of these sites being so similar, including the synchronous peak emergence times.
There was an average temperature difference between the three sites of less than 1°C. Despite this relatively small difference, the *O. agrili*’s first and peak emergence times vary between sites by a wide margin. This implies that the proximity of release sites to one another does not equate to the equivalent emergence times. Despite being at similar latitudes and in the same geographic regions, the microclimates found in the research sites were varied in more ways than just temperature. This could have serious implications for the phenology of *O. agrili*, and therefore its use as a biological control agent. If the release of this parasitoid is planned without considering the various microclimates in the overall region of the release, the lifecycle of the parasitoid and the host will be asynchronous. Since there is no current information on the effect of Mid-Atlantic microclimates on EAB, we have no way of know how this affects the host’s emergence. However, not having emergence times that are synchronous with the lifecycle of the host insect, would make an ineffective plan for biological control.
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