Factors affecting overwintering success of *Spathius galinae*, a parasitoid of the invasive emerald ash borer (*Agrilus planipennis*)

by

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ABSTRACT

Spathius galinae Belokobylskij is one of a few parasitoid wasps used as a biocontrol agent against the invasive emerald ash borer (*Agrilus planipennis* Fairmaire), a destructive beetle that has killed tens of millions of ash trees in the U.S. *S. galinae* are known to overwinter as prepupae in silken cocoons, but little is known about the overwintering success of these parasitoids at earlier life stages. Logs containing three different life stage treatments of *S. galinae* (early instar larva, mid instar larva, and prepupa) were placed in jars and deployed in two different microhabitat sites (urban woods and mature woods). The logs were deployed in late fall, remained in the field through winter, then *S. galinae* emergence was recorded in spring. Parasitoids in the warmer urban site emerged an average of 14 days earlier than parasitoids in the cooler, shaded mature woods site. *S. galinae* from all three life stage treatments successfully emerged, although the exact effect of life stage on overwintering success is not completely clear. Researchers in the future can release *S. galinae* for biocontrol through mid and late fall, but another study should be conducted to determine if young larval *S. galinae* have any less overwintering success than prepupae.

Introduction

The Emerald Ash Borer (*Agrilus planipennis* Fairmaire) is an invasive beetle from Asia that has been consuming and killing American ash trees (*Fraxinus* spp.) since its introduction to North America in 2002 (Poland & McCullough 2006). First found in Michigan, the beetle has since spread throughout much of the eastern US. Since its arrival in Delaware in 2016, the invasive beetle has done extensive damage to the state's native ash population (Delaware Department of Agriculture). In the hopes of reducing emerald ash borer (EAB) populations and curbing the spread of the beetle, USDA researchers have been studying a series of parasitoid wasps from EAB's native home range that attack the pest's larvae and eggs. The question that remains is whether these parasitoid wasps can function as effective biocontrol measures to mitigate ash damage and spread of EAB.

Currently, four parasitoids have been cleared for release by the United States Department of Agriculture into US forests to control EAB: *Oobius agrili* Zhang and Huang, *Tetrastichus planipennisi* Yang, *Spathius agrili* Yang, and *Spathius galinae* Belokobylskij and Strazenac (Duan *et al*. 2018). These insects parasitize EAB in different ways, including egg parasitism (*O. agrili*), endoparasitism of larvae (*T. planipennisi*), and ectoparasitism of larvae (*S. agrili* and *S. galinae*) (Duan *et al*. 2018). Cleared for release in 2015, *S. galinae* is the most recently introduced EAB parasitoid and has shown promise as the most effective biocontrol agent for northern populations of EAB. This parasitoid survives better in cold climates than *S. agrili*, establishes more easily after release compared to *T. planipennisi*, and parasitizes EAB hosts more efficiently than *O. agrili* (Duan *et al*. 2018, 2019). Before getting the authorization to release these parasitoids, USDA researchers had to investigate other potential hosts of the wasp (Duan *et al*. 2015). These findings revealed that *S. galinae* only attacks beetles within the genus *Agrilus*, but parasitizes *A. planipennisi* far more readily than the native *Agrilus auroguttatus*. In addition to host specificity research, experiments were carried out to determine effective methods to establish lab-reared colonies of the parasitoid (Duan *et al*. 2011). Once cleared for release, studies were performed to test how well *S. galinae* established and survived in American forests. Two years after their initial release into six study sites across three northeastern states (Connecticut, New York, and Massachusetts), Duan *et al.* 2019 successfully recovered *S. galinae* from every site. Duan *et al.* 2020 found that less than 2% of *S. galinae* recovered from Connecticut wild ash trees died from the cold temperatures; however, polar vortices and colder climates caused a higher mortality rate among parasitoids and EAB hosts in southern Michigan that same winter. Before winter arrives, *S. galinae* that parasitize EAB hosts in late summer and autumn develop from eggs to larvae to prepupae, and then remain as prepupae in spun silken cocoons all winter long in a process known as diapause (Chandler *et al*. 2020, Watt *et al*. 2016). However, researchers have noted that the prepupal stage may not be the only stage capable of overwintering, but rather just the most common stage at which diapause is initiated (Chandler *et al* 2020). The central focus of this study is to determine how well *S. galinae* can diapause when placed in the field at both larval and prepupal life stages.

It is important to understand the overwintering phenology of *S. galinae* because if only wasps in cocoons survive the winter, then all wasps deployed in the field as larvae will fail to emerge. This would indicate that future parasitoid releases would need to occur early enough in the year to provide time for the wasps to develop into prepupae before the winter. However, if larvae survive the winter, then *S. galinae* releases can occur later in autumn to allow wasps to attack the larval EAB known to remain active as late as October and November (Poland *et al*. 2015).

This study observed the overwintering phenology of *S. galinae* by placing logs containing EAB larvae parasitized by *S. galinae* into protective jars in two different field sites. Since peeling back the bark to observe the larval stage of each *S. galinae* would lead to desiccation, the developmental rates determined by Duan et al. 2011 were used to rear each log of *S. galinae* to the desired stage. The logs were kept in room temperature conditions for different amounts of time, thus allowing the *S. galinae* larvae to develop to a later stage depending upon how long they were kept at room temperature.The logs placed in the field immediately after parasitism contained small *S. galinae* larvae; logs placed in the field 7 days after parasitism contained large larvae; and logs placed in the field after 11 days contained prepupae (Duan *et al*. 2011). The number of individual parasitoids that survived the winter and emerged as adults in the spring was observed and counted.

Methods

Spathius galinae larvae were reared with late (3rd – 4th) instars of EAB larvae infesting green ash (*Fraxinus pennsylvanica*) logs at the USDA-APHIS facility in Brighton, MI. Detailed procedures for EAB larvae rearing on green ash bolts and subsequent exposure to S. galinae have been described in Ragozzino et al. (2020). Parasitoid-exposed EAB logs were incubated for different durations to produce *S. galinae* larvae of the following stages:1) early instar (incubated for 1 day before deployment), 2) mid-instar larvae (7 days), 3) prepupae (11 days).

Logs were then placed in 3.8 L PET jars (Uline, Pleasant Prairie, WI) prepared with two 7.6 cm diameter circular cutouts covered with 0.06 cm polypropylene mesh screen near the bottom (Fig. 1a). A 9 cm X 10.5 cm X 1.5 cm instant standard floral foam brick (Aquafoam, Syndicate Sales, Kokomo, IN) was placed at the bottom of each jar and soaked in water to keep the logs moist once inserted into the foam. Jars were watered once a week to stay moist. To affix the jars onto trees in the field, two 10-gauge, 8.89 cm nails (Grip-Rite, PrimeSource Building Products, Irving, TX) were hammered immediately under the jar to hold it up. A 55 cm length of 24-gauge green floral wire (OOK, HILLMAN Group, Cincinnati, OH) stretched across the jar and was fastened to two nails inserted on either side of the jar. One final nail was hammered just above the lid of the jar, and another 55 cm length of wire was wrapped around the neck of the jar and twisted onto the nail (Fig. 1b).

Figure 1**.** a) jar with two mesh cutouts for drainage and ventilation, b) jar containing log in floral foam brick; nails support the bottom of the jar while wire is stretched across the jar to keep it still and a final nail on the top holds a wire that wraps around the top of the jar, c) and d) urban forest site with widely spaced trees, less wind cover, e) and f) mature shaded forest site with more shade and denser vegetative cover surrounding it

Jars containing logs were deployed in two different sites: an urban forest site and a heavily wooded forest site (Figure 1). The urban site was wedged between the USDA-APHIS Newark, DE Beneficial Insects Introduction Research Unit and a University of Delaware agricultural field. This site was made up mostly of maples (*Acer* spp.) that were spaced widely apart with herbaceous plants covering the ground. Because of the low tree density, this site had less shade, less wind cover, and less moisture than the densely wooded forest site. Deployment in the mature woods site occurred on the periphery of University of Delaware's Wildlife Research Woods. Just outside of the fence, yet still within the wooded area, logs were deployed within the forest mostly on American holly (*Ilex opaca*) and tulip tree (*Liriodendron tulipifera*). The high tree density of this site provided consistent protection from extreme weather but was generally cooler than the urban site because the tall and numerous trees provided more shade. HOBO (Honest Observer by Onset) data from devices kept within the sites throughout the entire study period (11/10/2020 - 6/25/2021) recorded environmental information about the two sites found in Table 1.

Table 1. Average temperature, relative humidity, and available light of both study sites. Temperature and relative humidity data were collected over the entire study period from 11/10/2020 to 6/25/2021. A t-test of temperature and relative humidity found *p* values of less than 0.001 for both variables, indicating statistically significant differences between the temperature and relative humidity of both sites. Light measurements were taken on a sunny day in June (6/17/2021) over the course of a 30 minute period.

	Urban Woods	Mature Woods
Average Temperature	50 ± 16.6° F	48.3 ± 14.8° F
Average Relative Humidity	68.2 ± 21.8 %	72.9 ± 19.6%
Average Light	2053 ± 472.8 Lux	581.4 ± 242.1 Lux

Field deployment began on November 10th, 2020 and continued until the last jars were set up on December 4th, 2020. Two deployment trials were used, meaning that two separate deployment events occurred for each of the three *S. galinae* experimental stages (Table 2). By the end of deployment, a total of 42 jars had been set up in the field.

Table 2. *S. galinae* stage, deployment date, and number of jars deployed at each site in each of the two deployments

	Stage	Deployment Date	# Jars in Urban Forest	# Jars in Mature Shaded Forest
Deployment 1	Early Instar Larva	11/10/2020	4	4
	Mid Instar Larva	11/16/2020	3	3
	Prepupa	11/20/20	3	4
Deployment 2	Early Instar Larva	11/24/2020	3	3
	Mid Instar Larva	11/30/2020	4	4
	Prepupa	12/4/2020	4	3

Once emerging adult *S. galinae* were found in the jars, the logs were checked every weekday until no more emergence was observed. To collect emerged parasitoids, I used my hand or a long, thin paintbrush to gently scoop the wasps out of the jar. In July, one month after the last *S. galinae* adult emerged from a log, the jars were taken down and the logs dissected using a box cutter/utility knife. The fate and stage of all EAB and *S. galinae* were recorded.

Analysis

The data were analyzed using R v.4.1.1 (R Core Team 2021). To analyze the emergence timing, line plots were generated showing the cumulative emergence over time for each *S. galinae* stage treatment within each site. Since the emergence data for both sites appeared to be about normally distributed, a Welch's two sample *t* test was performed to compare the mean emergence times of *S. galinae* at each site. The Welch's two sample *t* test was performed using the function "t.test()" in base R. A population proportion was calculated to determine the proportion of *S. galinae* of each fate (adult, dead, diapaused) out of total *S. galinae* used in the study. Population proportions were used to represent fates rather than the number of individuals because each log had a different number of successfully parasitized emerald ash borer larvae, and each parasitized larva had a different number of wasps feeding on it. To quantify the effect of larval stage and site on parasitoid fate, a generalized linear model with a binomial distribution was used for these data. Each fate was tested under its own GLM, so a total of three GLMs were created. The GLM was generated using the function "glm()" in base R.

Results

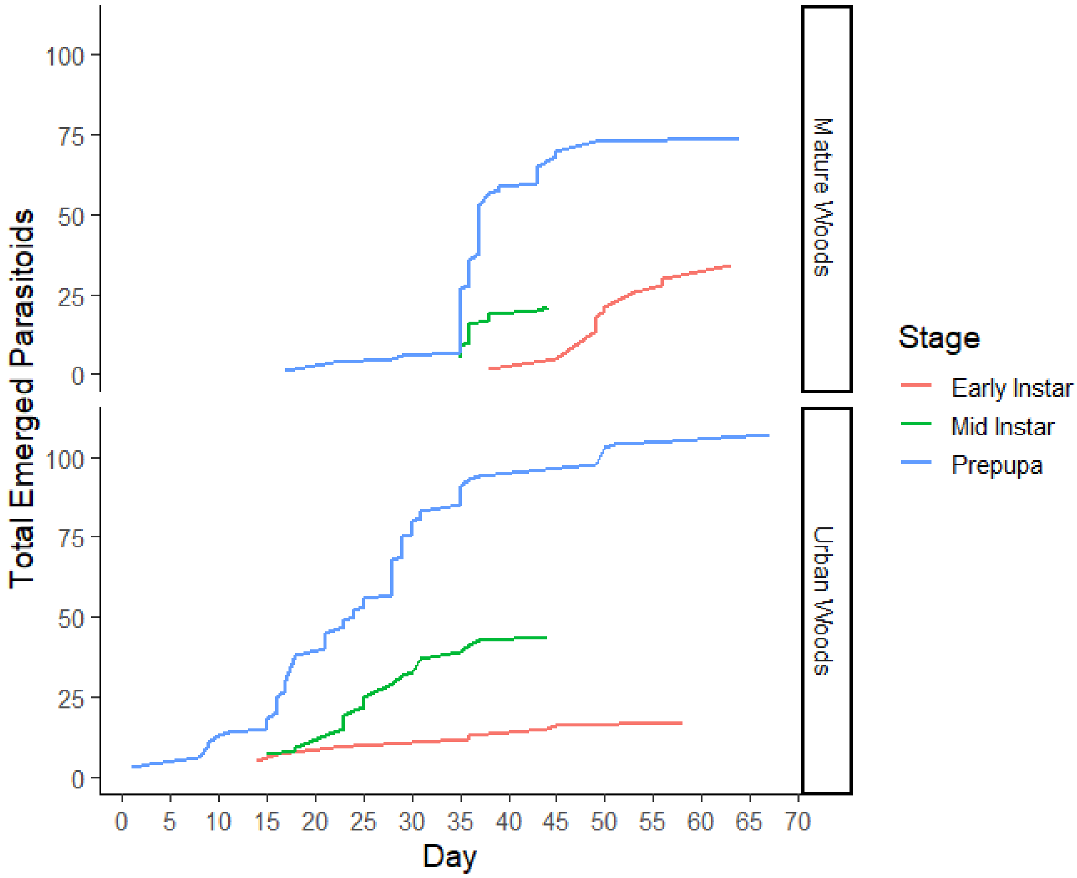

Figure 2. Cumulative emergence of adult *S. galinae* over a 67 day period, after which no future emergence was observed. The period extends from April 20th (Day 1) to June 25th, 2021 (Day 67). The graph shows the stage at which the parasitoids were deployed in the field (early instar, mid instar, prepupa), as well as the site where emergence was recorded (mature woods, urban woods).

There was a temporal difference in emergence between the urban site by the agricultural field and the mature woods in the Wildlife Research Woods (Figure 2). The mean emergence times were noticeably different with a statistically significant P value (t = -12.42, df = 293.29, P<0.0001). The mean days of emergence in the mature and urban woods sites were 40.77 and 25.94 respectively. These different means represent the noticeable delay in emergence time that the mature woods site experienced compared to the urban site.

Prior to this study, it was predicted that the early instar *S. galinae* larvae would emerge later than the mid instar and prepupa larvae. However, as the graph shows, parasitoids from all three stage treatments emerged across nearly the entire 70 day period. Prepupae *S. galinae* appear to have emerged first in both sites, but wasps from the early instar and mid instar treatments emerged at nearly the same time as one another shortly after. *S. galinae* in the early instar treatment continued emerging for a longer time interval than parasitoids in the mid instar treatment.

Another focus of this study was the overwintering success of *S. galinae* as exhibited by the fate of each individual. Figure 3 shows the population proportion of each fate of *S. galinae* out of the total parasitoids found within each treatment. Fate observations were made from a combination of daily emergence observations and from data collected once the logs were taken down and dissected.

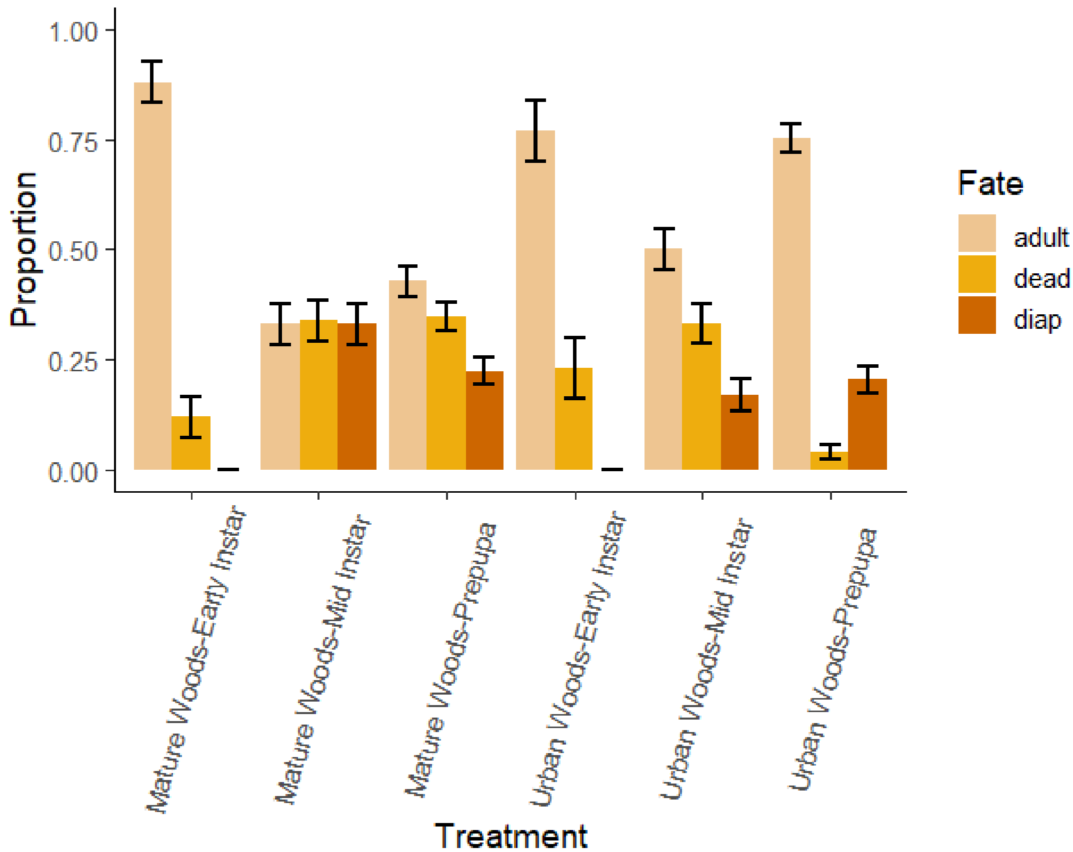

Figure 3. Population proportion of *S. galinae* of each fate (adult = developed into adults, dead = died as a larva or prepupa, diap = remained in diapause) out of total *S. galinae* observed from log dissection and emergence data.

For early instar and prepupae, the most common fate was development into adults. The mid instar *S. galinae* in the mature woods had the least success in developing into adults. All three fates were about equally as common for the mid instar larvae in the mature woods. Another striking feature is that zero individuals of the early instar stage were in the diapaused fate. The diapause fate was recorded when the *S. galinae* failed to develop into an adult, but was still alive as a prepupa or larva.

The urban woods generally showed increased proportions of successful development into adults compared to the mature woods.

The results in Figure 3 complement the generalized linear models created to show the relationships between each fate and each site and stage (Table 3). Each fate represents a separate generalized linear model.

Table 3. Generalized linear model results showing by what factor each variable change can alter the odds of that fate occurring in an early instar, urban woods larva (for adult or dead fate) or a mid instar, urban woods larva (for diapause fate). For example, the odds ratio of an adult developing from an early instar, mature woods larva is 3.46:1, but if you want to see the odds of a *mid instar*, mature woods larva, you must multiply by a factor of 0.12, which gives you an odds ratio of 0.42:1.

	Adult			Dead			Diapause		
	Odds Ratio	97.5% CI	*p*	Odds Ratio	97.5% CI	*p*	Odds Ratio	97.5% CI	*p*
(Intercept)	3.46	2.01 to 6.35	**<0.001**	0.28	0.15 to 0.49	**<0.001**	0.4	0.28 to 0.57	**<0.001**
Urban Woods	2.68	1.94 to 3.74	**<0.001**	0.4	0.27 to 0.57	**<0.001**	0.67	0.45 to 0.99	**0.043**
Mid Instar	0.12	0.06 to 0.22	**<0.001**	2.77	1.50 to 5.39	**0.002**			
Prepupa	0.26	0.14 to 0.46	**<0.001**	1.31	0.72 to 2.51	0.39	0.82	0.55 to 1.22	0.327

Discussion

The findings shown in Figure 2 reveal that *S. galinae* is fully capable of overwintering at life stages earlier than prepupa. This figure also shows how both life stage and habitat type affect emergence timing. Larval *S. galinae* emerge later in the spring than parasitoids that overwinter as prepupa, which is to be expected since larvae need to use the first few days of spring to develop into prepupae. Figure 2 also shows the delayed emergence time in the mature woods compared to the urban woods site, which is corroborated by the statistically significant results of the Welch's two sample t test. The different emergence times by site can be explained by the noticeable temperature difference between the two sites. The warmer, sunnier urban woods site allows for quicker parasitoid development than the cooler, shaded mature woods site.

Figure 3 shows that microhabitat and life stage also have an effect on developmental behavior of *S. galinae*. Parasitoids deployed in the mature woods site were less likely to develop into adults, and more likely to either die as larvae or remain in diapause. Both the results of the generalized linear models (Table 3) and Figure 3 show that there is less development into adults, which is indicative of overwintering success, among individuals in the mature woods site. Since this site is cooler, it is expected that the lower winter temperature depleted larval fat stores faster and gave rise to higher winter mortality. However, Table 1 shows that the relative humidity was higher in the mature woods than the urban woods. This increased humidity may have allowed for mold growth within the jars containing logs. Since moldy logs have been known to kill the *S. galinae* within, it is possible that higher moisture in the mature woods site made the jars containing logs more likely to mold, thus possibly increasing

S. galinae mortality within the logs. The most effective way to account for this potential mold effect in future studies is to cut more vents out of the jars to ensure as much air flow as possible in each jar.

Table 3 shows that the likelihood of the diapause fate was higher among individuals in the mature woods than for individuals in the urban woods. This is indicated by the fact that, in the Diapause GLM, the odds ratio is multiplied by 0.67 when the individual is in the urban woods, meaning that the odds ratio of the parasitoid staying in diapause will be *smaller* in the urban woods. The fate referred to as "diapause" was an unexpected fate when this study was initially planned. However, while performing dissections, I noticed that some of the *S. galinae* within the logs were neither desiccated nor emerged (an emerged parasitoid left behind an empty cocoon). These larvae were actually still alive, even after an entire warm season had passed. The fact that the parasitoid was alive, but not developing, meant that the larva somehow never broke diapause even after spring weather brought sufficient degree days for diapause to end. What is stranger about this diapause phenomenon is that not one of the 89 total *S. galinae* deployed as early instar larvae (distributed throughout 14 logs) stayed in diapause. All of the early instar parasitoids either died as larvae or successfully overwintered and emerged as adults. The GLM for the diapause fate had to be altered to account for the fact that zero individuals in this treatment went to the diapause fate. The early instar stage variable had to be removed from this GLM to account for the error it caused in the GLM.

An unexpected result highlighted by Table 3 is how the *S. galinae* deployed as early instar larvae were *the most likely* to develop into adults and *the least likely* to die as larvae over the winter. This result is demonstrated by the factors that the odds ratios are multiplied by when the individual was given the mid instar or prepupa treatment. The mid instar larvae were the least likely to develop into adults, which may have been caused by some unclear benefit that younger life stages offered for overwintering survival. It is worth noting that the *p* value for the prepupa stage in the Dead GLM is greater than 0.05, which means that the effect of prepupa stage was too inconsistent for the model to accurately measure its effect. The early instar larvae were expected to be the least successful, rather than the most successful, in developing into adults since it was believed that these larvae would have the lowest hardiness and easily die over the winter. One cause for this trend may not be the data, but rather a chance error that had significant effects on the study. Through random chance, it happened to be that the total number of *S. galinae* individuals from each stage was wildly different. There were a total of 89 early instar individuals, 215 mid instar, and 376 prepupa. Because there were comparatively so few early instar *S. galinae*, which happened by chance, it is possible that the small sample of early instar parasitoids were more susceptible to stochastic effects that caused more individuals to successfully overwinter than normally would. Alternatively, early instar larvae may have some unknown advantage for overwinter survival over older larval *S. galinae*. I would recommend that another study be conducted to better identify the relationship between *S. galinae* stage and overwintering success.

The emergence timing and overwintering success findings from this study have useful implications for biocontrol releases of the emerald ash borer parasitoid *S. galinae*. Because more shaded, cooler environments delay *S. galinae* development compared to suburban habitats, researchers should perform releases a few weeks later in spring in densely wooded sites than in more open and sunny sites. Also, while the exact effect of parasitoid stage on emergence success was not elucidated by this study, it is clear that *S. galinae* can successfully overwinter and emerge before the prepupal stage. Because of this finding, researchers should now be able to release *S. galinae* well into mid and late autumn. These autumn releases will help to ease the pressure on research labs that would otherwise be rushing to release as many parasitoids over the summer as possible. A stronger understanding of the life cycle and capabilities of this parasitoid allows researchers to use this biocontrol tool to more efficiently manage the spread and damage of emerald ash borers across the U.S. However, more research should be performed to understand exactly how the stage of *S. galinae* affects the initiation and success of diapause.

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