



## **Effect of Forest Microhabitat and Larval Stage on Overwintering Survival, Development, and Phenology of *Spathius galinae* (Hymenoptera: Braconidae), Biological Control Agent of Emerald Ash Borer, *Agrilus planipennis* (Coleoptera: Buprestidae)**

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# Effect of forest microhabitat and larval stage on overwintering survival, development, and phenology of *Spathius galinae* (Hymenoptera: Braconidae), biological control agent of emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae)

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## Abstract

*Spathius galinae* Belokobylskij (Hymenoptera: Braconidae) is among several parasitoids introduced as classical biocontrol agents for control of the invasive emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), in the US. However, the successful establishment of this parasitoid and its efficacy in suppressing emerald ash borer vary among the release sites and regions. We hypothesize that forest microhabitats and parasitoid overwintering life stages affect parasitoid overwintering survival, development, and emergence phenology of adults in the spring. To test this, we placed logs containing 3 different larval stages of *S. galinae* (early instar larva, late instar larva, and cocoon) in ventilated jars in 2 distinct microhabitat sites, i.e., urban forest and mature natural forest. These were deployed in late fall of 2020 and remained in the field through winter. The emergence of adult parasitoids was recorded in spring through summer of 2021. Parasitoids in the warmer urban site emerged more quickly, emerging 12 d earlier than parasitoids in the cooler mature forest site. None of the *S. galinae* deployed as early instar larvae diapaused in either urban or forest sites. In both habitats, 27.1 to 32.1% of late instar larvae diapaused. Across overwintering larval stages, the median time of *S. galinae* emergence was about 24 d in the urban microhabitat, significantly shorter than that (36 d) observed in the mature forest microhabitat. Our findings suggest that environmental factors profoundly affect *S. galinae* phenology, and consequently emerald ash borer biological control.

Key Words: biological control; parasitoid; invasive species

## Resumen

*Spathius galinae* Belokobylskij (Hymenoptera: Braconidae) se encuentra entre varios parasitoides introducidos como agentes de biocontrol clásico para el control del barrenador esmeralda del fresno invasivo, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), en los EE. UU. Sin embargo, el establecimiento exitoso de este parasitoide y su eficacia para suprimir el barrenador esmeralda del fresno varían entre los sitios y regiones de liberación. Nuestra hipótesis es que los microhábitats del bosque y los estadios de vida de hibernación del parasitoide afectan la sobrevivencia, el desarrollo y la fenología de emergencia de los adultos en la primavera. Para probar esto, colocamos troncos que contenían 3 estadios larvales diferentes de *S. galinae* (larva de estadio temprano, larva de estadio tardío y capullo) en frascos ventilados en dos sitios de microhábitat distintos, un bosque urbano y un bosque natural maduro. Estos se desplegaron a fines del otoño del 2020 y permanecieron en el campo durante el invierno. Se registró la aparición de parasitoides adultos desde la primavera hasta el verano del 2021. Los parasitoides en el sitio urbano más cálido emergieron más rápidamente, emergiendo 12 días antes que los parasitoides en el sitio de bosque maduro más frío. Ninguno de los *S. galinae* se desplegó como larvas de estadio temprano en diapausa en sitios urbanos o forestales. En ambos hábitats, entre el 27,1 y el 32,1% de las larvas de estadio tardío entraron en diapausa. A lo largo de los estadios larvales de hibernación, la media del tiempo de emergencia de *S. galinae* fue de aproximadamente 24 días en el microhábitat urbano, significativamente más corto que el (36 días) observado en el microhábitat de bosque maduro. Nuestros hallazgos sugieren que los factores ambientales afectan profundamente la fenología de *S. galinae* y, en consecuencia, el control biológico del barrenador esmeralda del fresno.

Palabras Clave: control biológico; parasitoide; especies invasivas

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The emerald ash borer (*Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae) is an invasive woodboring pest in North America. Originally from Asia, this beetle has been responsible for billions of dollars in losses and injury to forestry, recreation, and ornamental industries due to its pro-

pensity to infest and kill ash trees (*Fraxinus* sp. L.; Oleaceae) (Kovacs et al. 2010; Herms & McCullough 2014). Few management and prevention options are available, and those that are, such as insecticide injections and infested tree removal, are cost prohibitive, labor intensive, and ecologi-

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cally unsustainable (McCullough 2019). As a result, there has been great interest in developing more acceptable management strategies.

One method that has been pursued as a long-term, low-cost, self-sustaining way by which to reduce ash borer populations is classical biological control (Duan et al. 2018). Several parasitoids of emerald ash borer were selected initially for testing and were subsequently released beginning in 2007; these were *Tetrastichus planipennis* Yang (Hymenoptera: Eulophidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Duan et al. 2018). While *T. planipennis* and *O. agrili* have spread and established reasonably well, *S. agrili* is detected infrequently in subsequent monitoring efforts (Abell et al. 2016; Jennings et al. 2016; Margulies et al. 2017). Additional foreign exploration in Russia and subsequent testing identified *Spathius galinae* Belokobylskij & Strazenac (Hymenoptera: Braconidae) as a potential classical biological control agent of North American emerald ash borer (Belokobylskij et al. 2012; Duan et al. 2012, 2015). *Spathius galinae* is a gregarious endoparasitoid of third and fourth instar emerald ash borer larvae (Belokobylskij et al. 2012; Duan et al. 2014), and can cause > 60% emerald ash borer larval parasitism in its native range, the Russian Far East (Duan et al. 2012). Given that it originated from coastal Russia, it was surmised that *S. galinae* phenology and cold tolerance might align well with the conditions in the northern US, leading to improved establishment (Duan et al. 2018). Since initial releases in 2016, *S. galinae* has established and spread in several states throughout the eastern and northcentral US (Duan et al. 2019). Recent studies suggest that *S. galinae* is capable of persisting and spreading at least 14 km away from release sites within 3 to 5 yr following release (Quinn et al. 2022), and has begun to exert significant suppression of emerald ash borer populations at some northeastern ash-dominated forests (Duan et al. 2022). Most recently, Aker et al. (2022) detected multiple established populations of *S. galinae* in Maryland, USA, at sites up to 90 km from the nearest release point approximately 3 yr after release, indicating rapid, long-distance spread.

Among the most important characteristics of successful classical biological control agents are synchrony with target insect phenology and climatic compatibility. To this end, laboratory and semi-field studies of *S. galinae* suggest high levels of synchrony with emerald ash borer phenology (Jones et al. 2020; Quinn et al. 2022). Overwintering *S. galinae* most frequently are observed as prepupae or cocoons (Duan et al. 2020; Ragozzino et al. 2020). Mature overwintering larvae in cocoons demonstrate high cold tolerance, with average supercooling occurring at  $-25^{\circ}\text{C}$  in the laboratory, suggesting high compatibility with much of emerald ash borer's invaded range (Chandler et al. 2020; Wittman et al. 2021). However, since *S. galinae* actively parasitizes emerald ash borer larvae into the fall (Quinn et al. 2022), it is possible that *S. galinae* may be unable to develop to the cocoon stage before temperatures become too low for continued development. Laboratory rearing studies conducted at  $25^{\circ}\text{C}$  have shown that it takes approximately 10 d from parasitization for *S. galinae* to develop to the cocoon stage, and a total of 29 d for development to adult (Duan et al. 2014). The purpose of this study was to determine the effect of *S. galinae* developmental stage on overwintering success and subsequent adult emergence in the field. We hypothesize that forest microhabitats and parasitoid overwintering life stages affect parasitoid overwintering survival, development, and emergence phenology of adults in the spring.

## Materials and Methods

### INSECTS

Third or fourth instar emerald ash borer larvae infesting green ash (*Fraxinus pensylvanica* Marshall; Oleaceae) logs that had been exposed to *S. galinae* were obtained from the USDA-APHIS facility in Brighton, Michi-

gan, USA. Each log contained 5 to 10 emerald ash borer larvae. Detailed procedures for emerald ash borer larvae rearing on green ash bolts and subsequent exposure to *S. galinae* have been well-described, including the temperature-dependent developmental rate of *S. galinae* (Duan et al. 2013, 2014). Using this information, parasitoid-exposed emerald ash borer logs were incubated at  $25^{\circ}\text{C}$  ( $\pm 1.5^{\circ}\text{C}$ ) and long d photoperiod (16:8 h L:D) in an environmental chamber for different durations to produce *S. galinae* larvae of the following stages: (1) early instar (incubated for 1 d before deployment), (2) late instar larvae (5 d), and (3) cocoons (10 d).

### STUDY SITES

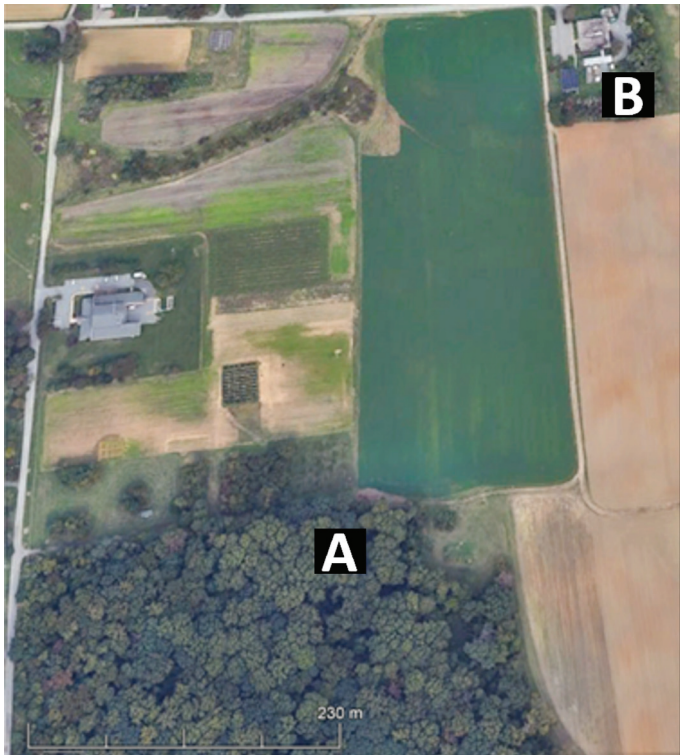
The study was conducted in 2 distinct habitats (sites) in areas adjacent to the USDA-ARS Beneficial Insects Introduction Research Unit at the Louis A. Stearns Laboratory in Newark, Delaware, USA. One site consisted of several isolated small patches (about 0.2 ha) of American birch (*Betula papyrifera* Marshall; Betulaceae), maple (*Acer* spp. L.; Sapindaceae), white or green ash (*Fraxinus* spp.) trees, which were planted about 15 yr ago nearby the Stearns Laboratory buildings (about 20–50 m away from the buildings). This site represents the typical “urban” habitat, and had a mean temperature of  $10.0 \pm 8.6^{\circ}\text{C}$ , mean RH of  $68.2 \pm 21.8\%$ , and mean light of  $2053 \pm 472.8$  lux for the study period. The second site consisted of a larger and more mature mixed hardwood lot in the southern campus of the University of Delaware, Newark, Delaware, USA, where maple (*Acer* spp.) and oak (*Quercus* spp. L.; Fagaceae) were the dominant tree species mixed with less abundant tree species including white ash (*Fraxinus americana* L.; Oleaceae), walnut (*Juglans* spp. L.; Juglandaceae), and pine (*Pinus* spp. L.; Pinaceae). This University of Delaware woodlot represents a typical natural forest and had a mean temperature  $9.1 \pm 9.6^{\circ}\text{C}$ , mean RH =  $72.9 \pm 19.6\%$ , and mean light  $581.4 \pm 242.1$  lux (Fig. 1).

### DEPLOYMENT IN THE FIELD

Logs were deployed from 10 Nov to 4 Dec 2020 and remained in the field until 26 Jul 2021. Daily ambient temperature and relative humidity were measured using HOBO data loggers (Temperature, Relative Humidity External Data Logger, model U12-012; Onset Computer Company, Bourne, Massachusetts, USA) housed inside jars, and light intensity was measured with a light meter (HD450 Light Meter/Data-logger; Extech, Nashua, New Hampshire, USA) during sunny d in early spring. Logs containing parasitized larvae were placed in 3.8 L polyethylene terephthalate jars (Uline, Pleasant Prairie, Wisconsin, USA) prepared with two 7.6 cm diam circular cutouts covered with 0.06 cm polypropylene mesh screen near the bottom (Fig. 2). A 9 cm  $\times$  10.5 cm  $\times$  1.5 cm instant standard floral foam brick (Aquafoam, Syndicate Sales, Kokomo, Indiana, USA) was placed at the bottom of each jar and soaked in water to keep the logs moist after insertion into the foam. To affix the jars onto trees in the field, two 10-gauge, 8.89 cm nails (Grip-Rite, PrimeSource Building Products, Irving, Texas, USA) were hammered immediately under the jar to hold it up. A 55 cm length of 24-gauge green floral wire (OOK, Hillman Group, Cincinnati, Ohio, USA) was stretched across the jar and was fastened to 2 nails inserted on either side of the jar. An additional 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.

A total of 21 logs were deployed in the urban forest and 21 logs were deployed in the mature forest (Table 1). Each log contained either early instar larvae, late instar larvae, or cocoon *S. galinae*. Jars were watered once per wk or as needed to keep the floral foam wet and to check for emerging *S. galinae*. Once parasitoids began emerging in the spring, the jars were checked and any emerged wasps collected every Mon, Wed, and Fri until no emergence was observed for 2 consecutive wk, suggest-





**Fig. 1.** Experimental microhabitats near the USDA-ARS Louis A. Stearns Laboratory in Newark, Delaware, USA. Letters indicate habitat type and approximate experiment locations: (A) mature forest, a larger, more mature wooded area; (B) urban forest, small, highly disturbed woodlot.



**Fig. 2.** Deployment jar for logs containing emerald ash borer larvae parasitized by *Spathius galinae*. Logs were inserted in floral foam in 3.8 L polyethylene terephthalate jar with 2 mesh cutouts for ventilation and excess water drainage. The jar was attached to the tree by resting the bottom of the jar on 2 nails hammered into the tree while a length of wire wrapped around the 2 nails on either side of the jar. Another wire looped around the neck of the jar and was fastened to the nail at the top. Water was added to the jars as needed to ensure adequate hydration of the logs and larvae.

ing that all living wasps had been collected. Logs were removed from the field 7 mo after deployment and dissected in the laboratory.

## ANALYSIS

All analyses were performed with SAS JMP Pro 16 (SAS Corporation, Cary, North Carolina, USA). *Spathius galinae* mortality and diapause by microhabitat (urban or woodlot), stage at time of deployment (early instar, late instar, or cocoon), and their interactions were analyzed with nominal logistic regression. The effect of microhabitat and *Spathius* stage at time of deployment on emergence from the logs over time was analyzed via Kaplan Meier survival analysis.

## Results

### MORTALITY AND DIAPAUSE RATES

Overall, mortality rates were low (16.9–33.5%) but varied significantly among different stages and habitats (Fig. 3A). Habitat ( $\chi^2 = 5.4$ ;  $df = 1$ ;  $P < 0.02$ ), overwintering stage ( $\chi^2 = 29.0$ ;  $df = 2$ ;  $P < 0.0001$ ), and their interaction ( $\chi^2 = 40.1$ ;  $df = 2$ ;  $P < 0.0001$ ) were significant predictors of *S. galinae* mortality. Greatest levels of mortality were observed in mid-instar compared to early instar *S. galinae* ( $P < 0.001$ ) and *S. galinae* in the mature forest habitat. The proportion of *S. galinae* that entered diapause was significantly affected by stage at time of deployment ( $\chi^2 = 51.4$ ;  $df = 2$ ;  $P < 0.0001$ ), but not significantly affected by microhabitat ( $\chi^2 = 5.7$ ;  $df = 1$ ;  $P > 0.05$ ). Notably, significantly fewer early instar *S. galinae* entered diapause compared to late instars ( $P < 0.001$ ). None of the *S. galinae* deployed as early instars diapaused in either habitat (Fig.

3B). A significantly greater proportion of larvae deployed as late instars diapaused compared to early instars ( $P < 0.02$ ) (mean proportion diapaused =  $0.49 \pm 0.12$  and 0, respectively), but there was no significant difference in the proportion of *S. galinae* cocoons diapaused compared to the other 2 stages ( $P > 0.05$ ) (mean proportion of cocoons diapaused =  $0.28 \pm 0.091$ ) (Fig. 3B).

### EMERGENCE PHENOLOGY

Of the 680 *S. galinae* deployed, 236 emerged as adults by the end of the study period (34.7%). The date of first emergence was 22 Apr 2021 in the urban forest (Fig. 4A) and 6 May 2021 in the mature forest (Fig. 4B). The median time of emergence was 24 d (95% CI = 22–27 d) in the urban microhabitat and 36 d (95% CI = 36–37 d) in the mature forest microhabitat. In the urban habitat, the median time to emergence was not significantly affected by stage ( $\chi^2 = 0.26$ ;  $df = 2$ ;  $P > 0.05$ ) (Fig. 4A). The difference in emergence timing by stage was significant in the mature forest habitat, where early instar larvae emerged 12 to 13 d later than late instars or cocoons ( $\chi^2 = 61.8$ ;  $df = 2$ ;  $P < 0.0001$ ) (Fig. 4B). When considered by deployment stage, the proportion of *S. galinae* that emerged as adults when deployed as early instars was more than double that of the late instar larvae. Surprisingly, *S. galinae* deployed

**Table 1.** Deployment numbers and timing of logs containing emerald ash borer parasitized by *Spathius galinae*. Logs were deployed from 10 Nov 2020 through 4 Dec 2020 and remained in the field until 26 Jul 2021. Each jar contained 1 log in which there were 5 to 10 parasitized emerald ash borer larvae. *Spathius galinae* in the logs were at the following developmental stages at time of deployment: (1) early instar (incubated for 1 d before deployment), (2) late instar larvae (5 d), and (3) cocoons (10 d).

Deployment date	Stage	Habitat	Number of jars deployed
10 Nov 2020	Early instar	Urban forest	4
10 Nov 2020	Early instar	Mature forest	4
16 Nov 2020	Late instar	Urban forest	3
16 Nov 2020	Late instar	Mature forest	3
20 Nov 2020	Cocoon	Urban forest	3
20 Nov 2020	Cocoon	Mature forest	4
24 Nov 2020	Early instar	Urban forest	3
20 Nov 2020	Early instar	Mature forest	3
30 Nov 2020	Late instar	Urban forest	4
30 Nov 2020	Late instar	Mature forest	4
4 Dec 2020	Cocoon	Urban forest	4
4 Dec 2020	Cocoon	Mature forest	3

as cocoons exhibited emergence levels (72.9%) that were in between those of early and late instars (100% and 62.9% of viable larvae at each stage, respectively).

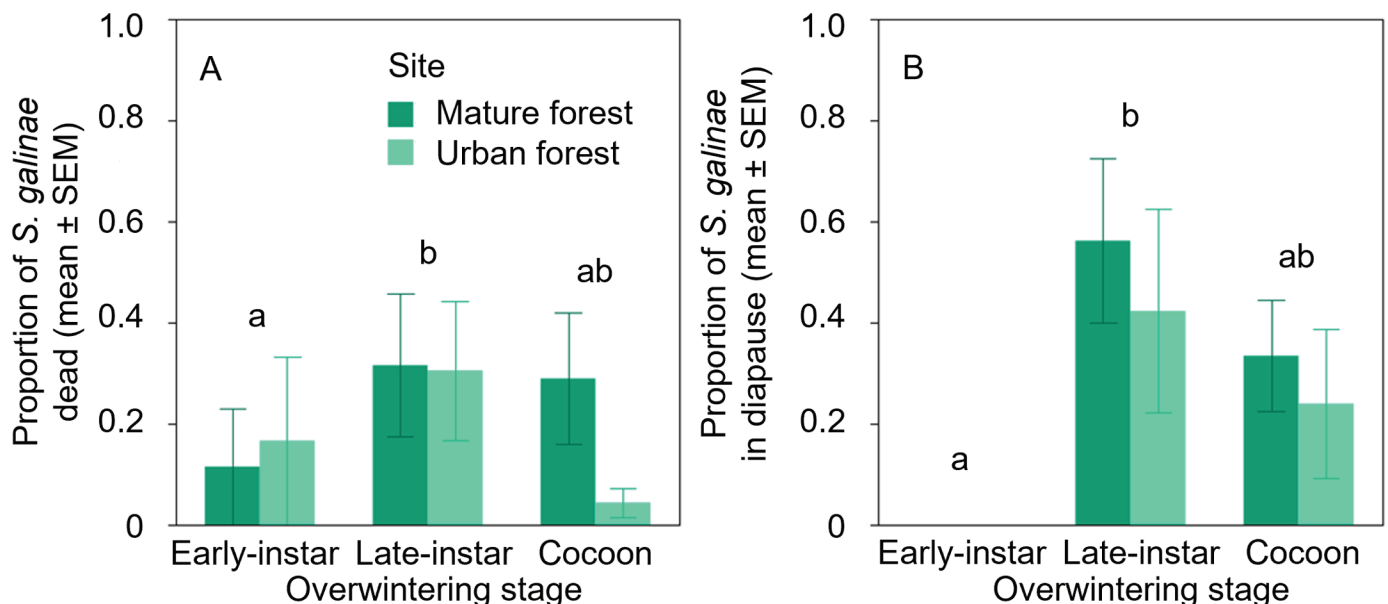
## Discussion

Our study showed that *S. galinae* larval stage at the outset of overwintering and microhabitat can significantly impact emergence timing but does not affect larval mortality. In addition, the parasitoid larval stage at the outset of overwintering also can affect significantly the rate of overwintering larvae to diapause. Early instar larvae were delayed by 2 wk compared to more advanced *S. galinae* in emergence as adults

in both habitats, and time to median emergence in the urban habitat was 10 d faster than in the mature forest. Emergence was greater in the urban habitat compared to the mature forest, and larvae deployed as early instars experienced greater levels of mortality and did not diapause. Taken together, our data suggest that (1) *S. galinae* may develop more quickly in urban woodlots because they are warmer than mature woodlots, and (2) young *S. galinae* larvae may be less likely to successfully overwinter or enter diapause. While the differences in outcomes are relatively small and the spatial scope of this study is somewhat limited, our findings indicate that habitat could impact the success of emerald ash borer classical biological control in important ways.

*Spathius galinae* development is dependent upon environmental conditions, with increased temperature decreasing time to eclosion (Duan et al. 2014). While more variable, the mean temperature in the urban habitat was about 1.0 °C greater than that of the mature forest during from the winter to spring months (Dec–May). This likely is due to increased solar, and thus heat, penetration which are typical edge effects and are less pronounced in larger, mature forest habitats (Dovčiak & Brown 2014). The relatively higher temperature likely was responsible for the decreased time to eclosion in the urban habitat. More fragmented habitats experience greater environmental extremes which may, in turn, affect overwintering success (Gurr et al. 2017). This is especially important in the context of extreme weather events, such as the 2019 polar vortex in Michigan, which killed 4.5 to 26 % of emerald ash borer larvae and 18 to 50% of *S. galinae* larvae (Duan et al. 2020). Ongoing climate change likely will increase the frequency and severity of these events, which could lead to poor parasitoid outcomes and increased risk of host outbreaks (Hance et al. 2007). Human activity generally has led to increased disturbance and habitat fragmentation (Steffen et al. 2011), meaning that fewer patches of habitat similar to our mature forest habitat will be available for emerald ash borer and *S. galinae* going forward, and those that remain may be more susceptible to environmental extremes, which may affect *S. galinae* phenology and success.

Our finding that early instar *S. galinae* larvae may be less likely to successfully overwinter or diapause is important to our understanding



**Fig. 3.** Proportion of dead (A) and diapaused (B) *Spathius galinae* by stage at time of deployment, and overwintering microhabitat. Fate was determined by dissecting all logs once emergence was complete. Letters of the same type and case within the same subfigure indicate significance when data are considered by stage alone ( $P < 0.05$ ).

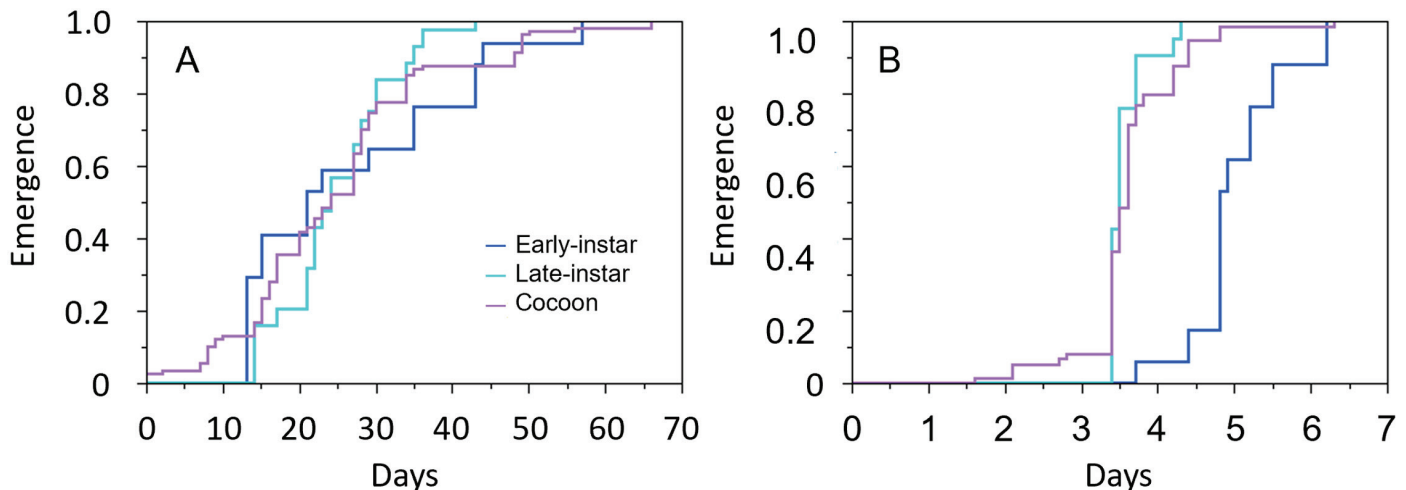


Fig. 4. Survival analysis of *Spathius galinae* emergence from urban (A) and mature forest (B) sites over time by stage at time of deployment.

of the ecology of introduced *S. galinae*. Sentinel log studies confirm that *S. galinae* is active well into the fall (Quinn et al. 2022). Since it takes approximately 10 d from parasitization for *S. galinae* to develop to the cocoon stage at 25 °C (Duan et al. 2014), it follows that development would slow and each instar would be prolonged later in the yr when temperatures are lower. This could have long-term impacts on the level to which *S. galinae* regulates emerald ash borer. Sufficient biocontrol agent numbers are necessary to achieve saturation of the environment and subsequent regulation of the target host (Yeates et al. 2012; Williams et al. 2021). If *S. galinae* emergence is reduced or delayed, this could mean that (1) the ratio of *S. galinae* to emerald ash borer may be suboptimal for full coverage, and (2) emerald ash borer that overwinter as susceptible third or fourth instars may have enough time to develop to the J-stage (mature late fourth instars), which is unsusceptible to parasitism.

This study is the first to demonstrate the possible impact of *S. galinae* larval stage at time of overwintering on diapause. Future studies should continue to explore the effects of microhabitat and stage at time of overwintering on *S. galinae*. Both factors may affect behavior, long-term fecundity, or other aspects of *S. galinae* biology and ecology. Microhabitat also may affect emerald ash borer mortality and phenology and should be investigated in the future. Additionally, release efforts of *S. galinae* should be focused earlier in the year to give *S. galinae* more opportunities to parasitize appropriate-stage emerald ash borer larvae while still providing sufficient time to develop to the late instar or cocoon stage for overwintering. This will depend upon regional temperature trends. Further research into the developmental thresholds of emerald ash borer and its parasitoids across ecoregions is needed. Continued monitoring of emerald ash borer and its biocontrol agents is essential for determining the success of this classical biocontrol program.

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