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Research

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Biological Control - Parasitoids and Predators

Differences in Early Season Emergence and Reproductive Activity Between *Spathius agrili* (Hymenoptera: Braconidae) and *Spathius galinae*, Larval Parasitoids of the Invasive Emerald Ash Borer (Coleoptera: Buprestidae)

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Abstract

Both *Spathius agrili* Yang and *Spathius galinae* Belokobylskij and Strazanac are host-specific parasitic wasps introduced for biological control of emerald ash borer in North America. *Spathius agrili* is native to northeastern China and *S. galinae* comes from a more northern, colder climate in the Russian Far East. Their origin may lead to differing abilities to adapt to climate and their host in North America. We conducted both field and laboratory experiments to determine the timing of early season emergence and synchronization of each parasitoid species to their host in the United States, and if manipulating prerelease conditions could affect emergence time. A cold acclimatization treatment prior to parasitoid emergence was assessed and compared with untreated control group reared with standard rearing protocols. Stands of naturally emerald ash borer-infested ash were sampled at two locations in Virginia throughout the experiment to determine when the parasitoid-susceptible life stage (third to fourth instar) occurred. Untreated *S. galinae* emerged approximately 2 wk earlier than any other cohort, whereas cold acclimatized *S. galinae* emerged later than any other cohort. Emergence time of *S. agrili* was unaffected by cold acclimatization. Cold acclimatization treatment did not affect the parasitism rate of either species, nor did it have multigenerational effects. Emergence time of the subsequent generation of *S. agrili* was delayed by cold acclimatization treatment, whereas *S. galinae* experienced no multigenerational effects. At Virginia field sites, susceptible EAB larvae were present during the emergence time of all four groups of parasitoids. Untreated *S. galinae* had the least overlap with any susceptible EAB larvae.

Key words: emerald ash borer, *Spathius agrili*, *Spathius galinae*, climate

The emerald ash borer (EAB), *Agrilus planipennis* Fairemaire, a pest of ash (*Fraxinus* spp.) trees native to Asia, was accidentally introduced to North America in the mid-1990s (Cappaert et al. 2005, Bray et al. 2011, Siegert et al. 2014). Since it was first discovered in 2002 in southeastern Detroit, Michigan and Ontario, Canada (Haack et al. 2002), this invasive beetle has established populations in over 35 U.S. states and five Canadian provinces, killing millions of North American ash trees by 2019 (Emerald Ash Borer Information 2019). Currently, management of this invasive beetle in North America involves several options, including trunk injections of systemic pesticides, removal of infested trees, and biological control with specialized natural enemies (parasitoids) from the pest's native range (Bauer et al. 2008, Mercader et al. 2015). Because of the lack of efficient detection methods and difficulty of applying insecticides in natural forests, biological control is considered to be a critical long-term option for protection of ash stands in natural ecosystems (Duan et al. 2018).

Three hymenopteran parasitoids were introduced from northeast China, part of the pest's native range, in 2007 as biocontrol agents against EAB in the United States (Bauer et al. 2007, 2008; Liu et al. 2007). These included two larval parasitoids, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae; Yang et al. 2006) and *Spathius agrili* Yang (Yang et al. 2005), and the egg parasitoid *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae; Zhang et al. 2005). To date, these three Chinese parasitoids have been released in 29 U.S. states as well as three of the five Canadian provinces invaded by EAB (Duan et al. 2018, Canadian Food Inspection Agency 2019, MapBiocontrol 2019). Releases of *S. agrili* and *T. planipennisi* occur either as mature adults, larvae, or pupae in ash bolts to be hung on infested ash trees depending on availability (USDA-APHIS/ARS/

FS 2019). Recent field studies conducted in Midwest, mid-Atlantic, and northeastern United States have shown that *T. planipennisi* and *O. agrili* have established self-sustaining populations in many of the released areas in these regions (Duan et al. 2013, 2015; Davidson et al. 2016; Jennings et al. 2016). However, none of the published studies have confirmed the establishment of *S. agrili* from these regions (Hooie et al. 2015, MapBiocontrol 2019). Reasons for the failure of *S. agrili* to establish are not exactly known. Some hypotheses include a combination of biotic and abiotic factors, such as the asynchronization of adult parasitoid emergence with EAB larvae, or climatic conditions at release sites. Cold storage of mass reared adults has been attempted, which showed that a cold storage treatment at 10°C for 3 mo or longer significantly decreased emergence, longevity, and fecundity of *S. agrili* (Gould et al. 2011).

Recently, another larval parasitoid, *Spathius galinae* Belokobylskij and Strazanac was collected in the Russian Far East, north of areas where *S. agrili* was collected in Tianjin province, China (Belokobylskij et. al. 2012, Duan et al. 2012). It was approved for release in the United States in 2015 (Duan et al. 2015, Federal Register 2015, USDA-APHIS/ARS/FS 2019). Pre-release climate matching indicated that *S. galinae* is well suited to the climate of the northcentral and northeastern United States, where ash is abundant (Duan et al. 2018, USDA-APHIS/ARS/FS 2019). Releases of *S. galinae* also occur by two methods; adult release or larvae/pupae in ash bolts hung on infested ash trees (USDA-APHIS/ARS/FS 2019). In 2016, releases of *S. galinae* were made in several northeastern U.S. states, followed later by releases in the northcentral and Great Lake states (MapBiocontrol 2019). A recent field study conducted in the northeastern United States showed that this newly introduced EAB larval parasitoid successfully established self-sustaining populations with significantly increased abundance or density two years after the major field releases in six hardwood forests in Connecticut, Massachusetts, and New York (Duan et al. 2019). However, little information is available on this parasitoid's ability to reproduce and establish in the mid-Atlantic and southern regions of the United States, where EAB has expanded its range.

The two congener species, *S. agrili* and *S. galinae*, share many life history traits that are promising for biological control. Both species are idiobiont larval parasitoids, paralyzing their host upon attack, have increased clutch size with increased host larval size, and show preference for third- and fourth-stage EAB larvae (Yang et al. 2006, 2010; Belokobylskij et al. 2012; Duan et al. 2014; Watt and Duan 2014). Differences between species arise in longevity of adults, fecundity, and sex ratio. *Spathius agrili* adults lived for an average of 8 wk with males living slightly longer (Gould et al. 2011), whereas *S. galinae* adults lived for a median 7 wk with females living slightly longer (Duan et al. 2014). Fecundity and sex ratios between these congener species differ as well. *Spathius agrili* has an average clutch size of 5.4 ± 0.2 eggs and is capable of laying 51.2 eggs over her life with approximately three quarters female sex ratio (Gould et al. 2011). *Spathius galinae* has an between eight and 16 offspring per host and is capable of producing up to an average 47 progeny over her life with a sex ratio (f:m) of 1.9:1 (Belokobylskij et al. 2012, Duan et al. 2014, Watt et al. 2016).

In the present study, we first determine the early-season adult emergence phenology of both these two congener species *S. agrili* and *S. galinae* and assess a 7-d cold acclimatization as a method of delaying their emergence. Secondly, we evaluate if a 7-d cold acclimatization treatment affects the longevity, parasitism rate, sex ratio, and/or fecundity of cold acclimatized wasps. Finally, we evaluate the subsequent generation, to ensure no multigenerational effects occurred. We hypothesized that a cold acclimatization treatment would alter the emergence time of early season adult parasitoids and affect the seasonal synchronization between parasitoid adults and suitable EAB larvae. We also hypothesized that the cold acclimatization treatment would decrease longevity, parasitism rate, fecundity, and would have a multigenerational effect on reproduction.

Materials and Methods

Host Larvae for Fecundity and Reproduction Experiment

All EAB larvae used in the fecundity and reproduction experiments were third or fourth instars reared at the USDA Beneficial Insect Introduction Unit (BIIRU) in Newark, DE. EAB larvae were reared in tropical ash, *F. uhdei* Wenz., bolts (1–2 cm diam, 20–25 cm length), using the methods described in Duan et al. (2012). Between three and seven larvae were reared per bolt, depending on bolt diameter and length. Larval-inoculated bolts were shipped to the Price's Fork Research Station (PFRC), in Blacksburg, VA in an insulated cooler weekly from May through September. The base of each bolt was wrapped in a paper towel soaked in distilled water, and all bolts were placed in a black plastic bag to prevent desiccation prior to use.

Early Season Emergence

All *S. agrili* and *S. galinae* cocoons used in this experiment were reared by the USDA APHIS PPQ Biological Control Production Facility, in Brighton, MI (afterward referred to as the "APHIS Brighton Facility"). Green ash, *F. pennsylvanica* Marshall, bolts (12–15 cm, 2–4 cm dia.) were infested with EAB that were allowed to mature until the third to fourth instars. Infested bolts were then subjected to gravid females of either *S. agrili* or *S. galinae* for 7 d. Two treatment groups were established: The control group reared to late-instar larvae using the standard rearing condition of 28°C used by the APHIS Brighton Facility, and a group reared at 28°C then exposed to a cold acclimatization treatment of 10°C for 1 wk as late-instar larvae. Each treatment group was established for both *S. agrili* and *S. galinae*, for a total of four cohorts of *Spathius* spp.: cold acclimatized *S. agrili*, cold acclimatized *S. galinae*, untreated *S. agrili*, and untreated *S. galinae*. Forty bolts of each cohort were produced and then shipped to Virginia Tech.

Each bolt was placed individually in a mesh-ventilated 3.78-liter square plastic container (Plastic Grip Jug 1 Gallon S-15711 ULine Plastics) in a brick of water-soaked floral foam (Oasis). Containers were placed outdoor in the shade under a stand of 20-yr old conifer trees at PFRC (37.212273, −80.489594) on 8 March 2017, and a tarp was hung above them to prevent rain water accumulation in the containers. Temperature was measured constantly using a HOBO Pro v2 temperature monitor (Fig. 1). Containers were observed twice per week until any emergence was first observed, at which point they were observed daily. All observed wasps were sexed, and a subset were used for further experiments. After emergence ended and no parasitoids emerged for 7 d, bolts were observed twice weekly for any delayed emergence until 1 October 2017.

Early Season Longevity, Parasitism Rate, Sex Ratio, and Fecundity

After emergence, pairs of male and female wasps were taken from each cohort and placed in a new mesh ventilated container (as described above), containing one to two bolts of tropical ash infested with a total of three to seven third- to fourth-instar EAB. The bottom 1–2 cm of each bolt were placed in saturated floral foam. Clover honey was streaked on top of the mesh ventilated lid to serve as a

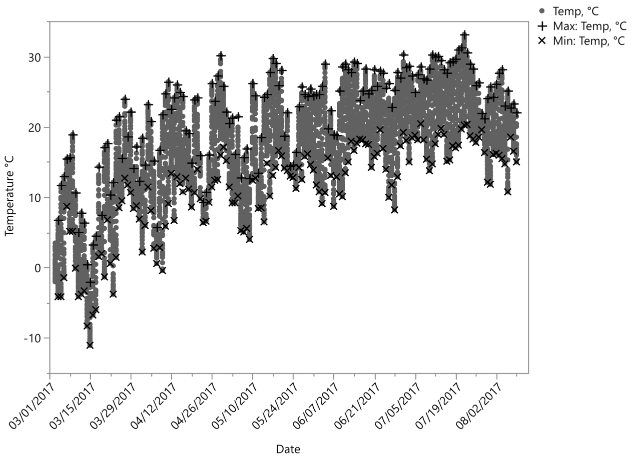

Fig. 1. Daily temperature data from the hemlock stand where bolts were placed at Prices Fork Research Station, Blacksburg, VA. Temperature was recorded every 30 min. Daily high and low temperatures are indicated by + and x, respectively.

food source for the adult *Spathius*. Containers were placed in an incubation growth chamber (I-41LL, Percival Scientific) set to the average weekly day and night temperature of Blacksburg, VA (Fig. 1), matching photoperiod, and 60 ± 10% relative humidity in order to mimic field conditions. Containers were observed daily. After 1 wk, the mated pair was removed from the container and was placed in a new container with new bolts of EAB-infested tropical ash. This was repeated weekly until the female *Spathius* died. In the event the male died, the female was moved alone. Longevity of adult parasitoids was determined by observing these mating pairs date of death.

After paired adults were removed from a container, the container was observed daily for progeny emergence, until seven consecutive days of no emergence. Once emergence had completed, bolts were dissected using a sharp utility knife to remove only the outermost layer of bark, revealing the EAB galleries and parasitoid cocoons. The fate of each EAB larvae was scored as healthy, parasitized (with presence of parasitoid cocoons or larvae), or killed by other (unknown) factors. To determine fecundity, any unemerged parasitoid adults, pupae, and larvae were counted and added to the count of emerged parasitoids.

Subsequent Generation Emergence

Newly emerged adults were removed from the container on the day they were observed, and sexed. Emergence time was calculated as days since EAB larvae were exposed to paired adults until emergence of the new adult *Spathius* by individual. This information was tabulated by cohort to determine parasitism rate and time to emergence by cohort.

Subsequent Generation Parasitism Rate

After adults emerged, they were paired within cohort and placed in a mesh ventilated plastic container containing one to two bolts of EAB-infested tropical ash (as described above). As with the previous generation, after 1 wk the mated pair was removed and placed in a new container with new bolts of EAB-infested tropical ash. This was repeated weekly until the female *Spathius* died. Once emergence had completed, bolts were dissected, the fate of each EAB larvae was scored, and any unemerged parasitoids were counted (as described above).

Field Sampling of EAB Larvae

To determine EAB larval life stage in the field locally, two sites were located in Virginia, at Douthat State Park, Millboro, VA (37.89409, −79.8015) and Mid-County Park, Christiansburg, VA (37.17123, −80.4119; MCP). Each site was visited once per month during the summer of 2017. One to two symptomatic trees were selected, felled, and debarked in 1-m sections using methods described in USDA–APHIS/ARS/FS (2019). All EAB larvae present were collected and their lifestage was assessed. All larvae were brought back to the PFRC and observed for 3 wk for any endoparasitoid emergence.

Statistical Analyses

The nonparametric Kaplan–Meier survival platform (Kaplan and Meier 1958) was used to calculate the median emergence time and 95% confidence intervals for all four cohorts. Days since exposure to Virginia field conditions was used as the time to event, and the count of daily emergence used as the frequency of the event. This was performed following the protocol outlined in Duan et al. (2011). A log-rank and Cox proportional hazard test (based on the Kaplan-Meier survival platform) were performed to determine significance in differences between pairs of treatments (*S. agrili* cold-treated and untreated *S. agrili*, *S. galinae* cold-treated and untreated *S. galinae*, *S. agrili* cold-treated and *S. galinae* cold-treated, untreated *S. agrili* and untreated *S. galinae*). If no difference was found between cold acclimatization treatment and untreated, then treatment groups were pooled for comparisons between *S. agrili* and *S. galinae*. Due to multiple comparisons across all groups of cohorts, then four pairwise comparisons, the Bonferroni correction was used and the type I error rate (alpha) was set to 0.1.

Longevity of adult parasitoids was calculated in weeks using a one-way ANOVA to determine differences between treatments within each species, and between species. Due to a low number of individuals followed through their lifetime, cohorts were pooled by species, with the assumption that cold acclimatization treatment had no effect on longevity. Sex ratio was determined by calculating the proportion of female to male progeny, any replicates with only male offspring were removed from the analysis due to haplodiploidy. The weekly replicates were used to determine parasitism rate by dividing the number of parasitized larvae by the total larvae provided, and fecundity based on a count of the parasitoid progeny per individual female. Data were then analyzed in a one-way ANOVA to determine differences between cold acclimatization treatment and untreated within species.

For assessment of the subsequent generation, the Kaplan–Meier survival platform was used again to determine differences between pairs of cohort treatments, as was performed for the previous generation emergence. Median emergence time and 95% confidence intervals were calculated through the survival platform. Log-rank and Cox proportional hazard test were performed between pairs to determine significance. Multigenerational effects on parasitism rate were assessed using a count of EAB larvae fate analyzed by a one-way ANOVA. Again, due to multiple comparisons across all groups of cohorts, then four pairwise comparisons, the Bonferroni correction was used and the type I error rate (alpha) was set to 0.1 for emergence analysis.

All data were analyzed in SAS JMP Pro 14.0.0 (SAS Institute 2019).

Results

Early Season Emergence

Emergence of cold acclimatized *S. agrili* began on 2 May and completed emergence on 14 June. Untreated *S. agrili* emergence began on 5 May and completed emergence on 16 June. Emergence of both

Table 1. The summary of the Kaplan–Meier survival curve for early season emergence of *S. agrili* and *S. galinae* under cold acclimatization and untreated conditions

Group	Adults emerged	Median Time (d)	Median Time (calendar date)	Lower 95%	Upper 95%	25% Emerged	75% Emerged
Cold Acclimatized *S. agrili*	30	79.5a	May 26	76	82	70	82
Cold Acclimatized *S. galinae*	85	93c	June 9	83	95	78	98
Untreated *S. agrili*	48	82.5a	May 29	82	83	78	84.5
Untreated *S. galinae*	381	68b	May 15	68	70	58	79
Combined	544						

a. Significant differences in median emergence time are indicated by lettered grouping next to median time. Comparison between all groups indicated significant differences between groups. Pairwise analysis between *S. galinae* treatments indicated cold acclimatization significantly delayed emergence. Pairwise analysis between untreated *S. agrili* and untreated *S. galinae* indicated *S. agrili* untreated emerged significantly later than *S. galinae* untreated. Pairwise analysis between cold acclimatized *S. agrili* and cold acclimatized *S. galinae* indicated that cold acclimatized *S. agrili* emerged significantly later than cold acclimatized *S. galinae*. Pairwise analysis between *S. agrili* treatments indicated no difference.

b. Groups without a factor (species or treatment) were not compared.

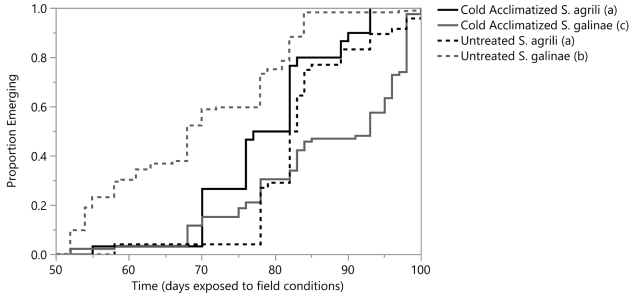

Fig. 2. Daily emergence of *S. agrili* (shown in black) and *S. galinae* (shown in gray) adults over time. Cold acclimatization and untreated control groups. Significant difference from pairwise comparisons is indicated by letter grouping in the legend (Log-Rank, *P* < 0.01).

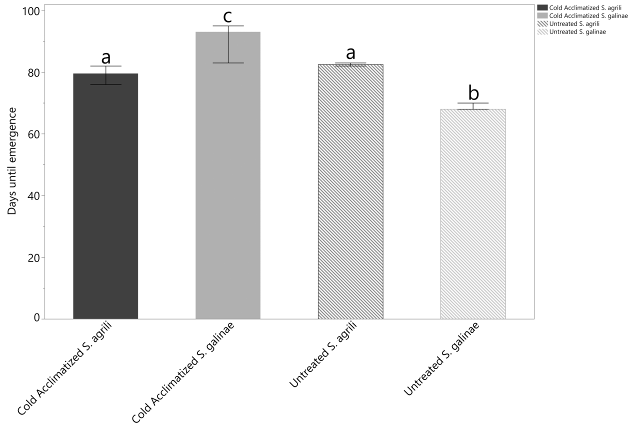

Fig. 3. Median days to emergence for both cohorts of *S. agrili* and *S. galinae* (95% CI for the Kaplan–Meier survival curve). Significant difference is indicated by letter grouping (Log-Rank, *P* < 0.01).

cohorts of *S. galinae* began on 29 April and lasted until 16 June when emergence ended (Table 1 and Fig. 2). There were significant differences in emergence times among the four cohorts (Log-Rank $\chi^2 = 128.038$, df = 3, $P < 0.0001$; Table 1 and Fig. 2). Pairwise comparisons showed that cold acclimatization treatment significantly delayed the emergence time of *S. galinae* compared with untreated *S. galinae* (Log-Rank $\chi^2 = 91.856$, df = 1, $P < 0.0001$; Table 1 and Fig. 3). Emergence of untreated *S. agrili* was significantly later than untreated *S. galinae* (Log-Rank $\chi^2 = 36.193$, df = 1, $P < 0.0001$; Table 1 and Fig. 3). In contrast, cold acclimatized *S. agrili* emerged earlier than cold acclimatized *S. galinae* (Log-Rank $\chi^2 = 20.723$, df = 1, $P < 0.0001$; Table 1 and Fig. 3). Cold acclimatization treatment did not affect emergence time of *S. agrili* compared with untreated *S. agrili* (Log-Rank $\chi^2 = 5.01$, df = 1, $P = 0.0252$; Table 1 and Fig. 3).

Early Season Longevity, Parasitism Rate, Sex Ratio, and Fecundity

Both *S. agrili* and *S. galinae* cohorts were pooled regardless of treatment due to limited replicates. In total, 21 pairs of *S. galinae* were set up, whereas a total of 10 pairs of *S. agrili* were set up. Longevity did not differ between *S. agrili* and *S. galinae* (Table 2). Cold acclimatization treatment did not affect parasitism rate for either *S. agrili* or *S. galinae* (Table 2). Due to lack of differences among treatments, all treatments were pooled by species and no difference was detected between parasitism rate of *S. agrili* and *S. galinae* (Table 2). Cold acclimatization treatment did not affect sex ratio for either *S. agrili* or *S. galinae* (Table 2). When groups were pooled regardless of treatment, sex ratio of *S. agrili* did not differ from *S. galinae*. It also did not affect fecundity for either *S. agrili* or *S. galinae* (Table 2). When groups were pooled regardless of treatment, fecundity of *S. agrili* was significantly greater than *S. galinae* (Table 2).

Subsequent Generation Emergence

There was an overall significant difference in subsequent generation emergence time among the four cohorts (Log-Rank $\chi^2 = 92.9856$, df = 3, $P < 0.0001$; Table 3 and Fig. 4). Pairwise comparisons showed that the median emergence time of cold acclimatized *S. agrili* progeny was significantly longer than that of untreated *S. agrili* progeny (Log-Rank $\chi^2 = 15.5906$, df = 1, $P < 0.0001$; Table 3 and Fig. 5). In contrast, there was no significant difference in emergence timing between cold acclimatized *S. galinae* and untreated *S. galinae* (Log-Rank $\chi^2 = 1.30$, df = 1, $P = 0.254$; Table 3 and Fig. 5).

Furthermore, there were significant differences in emergence time between the two parasitoid species. Cold acclimatized *S. agrili* had peak emergence significantly earlier than cold acclimatized *S. galinae* (Log-Rank $\chi^2 = 10.88$, df = 1, $P = 0.0010$; Table 3 and Fig. 5). Untreated *S. galinae* progeny began emergence earlier than untreated *S. agrili* progeny, although both completed emergence in similar lengths of time (Log-Rank $\chi^2 = 17.84$, df = 1, $P < 0.0001$; Table 3 and Fig. 5).

Table 2. The longevity, parasitism rate, sex ratio, and fecundity of the early season emergence of all four cohorts by treatment and pooled by species where no difference occurred

Cohort	Longevity (wk) [a]	Parasitism Rate (% attacked) [b]	Sex Ratio (% Female Progeny) [c]	Fecundity (Progeny/Adult) [d]
Cold acclimatized *S. agrili*	–	35.67 ± 7.30	59.94 ± 14.26	35.00 ± 14.97
Untreated *S. agrili*	–	28.48 ± 5.32	60.54 ± 12.75	33.00 ± 10.59
Cold acclimatized *S. galinae*	–	26.56 ± 4.64	59.51 ± 8.39	19.00 ± 3.99
Untreated *S. galinae*	–	20.86 ± 6.25	49.07 ± 12.51	14.40 ± 5.35
S. agrili (pooled)	5.22 ± 0.79	30.97 ± 4.05	60.28 ± 8.94	33.67 ± 5.87
S. galinae (pooled)	4.40 ± 0.61	24.54 ± 3.94	56.27 ± 7.04	17.35 ± 4.70

Pairwise analyses were only performed selectively based on biologically significant comparisons (ANOVA $P < 0.05$).

[a] Pairwise analysis indicated no significant difference ($F = 0.6793$; df = 1, 23; $P = 0.4187$).

[b] Pairwise analysis between Cold acclimatized *S. agrili* and untreated indicated no difference ($F = 0.6343$; df = 1, 71; $P = 0.4285$). Pairwise analysis between Cold acclimatized *S. galinae* and untreated indicated no difference ($F = 0.5362$; df = 1, 75; $P = 0.4663$). Pairwise analysis between *S. agrili* (pooled) and *S. galinae* (pooled) indicated no difference ($F = 1.2940$; df = 1, 147; $P = 0.2572$).

[c] Pairwise analysis between cold acclimatized *S. agrili* and untreated indicated no difference ($F = 0.0010$; df = 1, 17; $P = 0.9749$). Pairwise analysis between cold acclimatized *S. galinae* and untreated indicated no difference ($F = 0.4802$; df = 1, 28; $P = 0.4943$). Pairwise analysis between *S. agrili* (pooled) and *S. galinae* (pooled) indicated no difference ($F = 0.1238$; df = 1, 45; $P = 0.7266$).

[d] Pairwise analysis between cold acclimatized *S. agrili* and untreated indicated no difference ($F = 0.0119$; df = 1, 8; $P = 0.9162$). Pairwise analysis between cold acclimatized *S. galinae* and untreated indicated no difference ($F = 0.4758$; df = 1, 13; $P = 0.5034$). Pairwise analysis between *S. agrili* (pooled) and *S. galinae* (pooled) indicated significant difference ($F = 4.7071$; df = 1, 22; $P = 0.0417$).

Table 3. Subsequent generation (progeny) emergence of *Spathius agrili* and *Spathius galinae* from parents under cold acclimatization and untreated conditions from the Kaplan–Meier survival curve

Group	Adults Emerged	Median Time (d)	Lower 95%	Upper 95%	25% Emerged	75% Emerged
Cold Acclimatized *S. agrili*	68	38a	37	43	36.5	53.5
Cold Acclimatized *S. galinae*	118	45b	44	47	42	59
Untreated *S. agrili*	75	36c	36	39	35	40
Untreated *S. galinae*	31	45b	45	48	43	50
Combined	292					

a. Significant differences in median emergence time are indicated by lettered grouping next to median time (Log-Rank, $P < 0.01$).

b. Groups without a factor (species or treatment) were not compared.

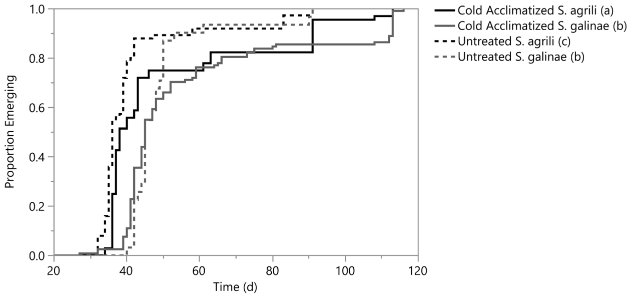

Fig. 4. Daily emergence of all subsequent generation cohorts. *Spathius agrili* (shown in black), *S. galinae* (shown in gray), cold-treated (shown in solid lines), and untreated (shown in dashed lines). Significant difference is indicated by letter grouping in the legend, (Log-Rank, $P < 0.01$).

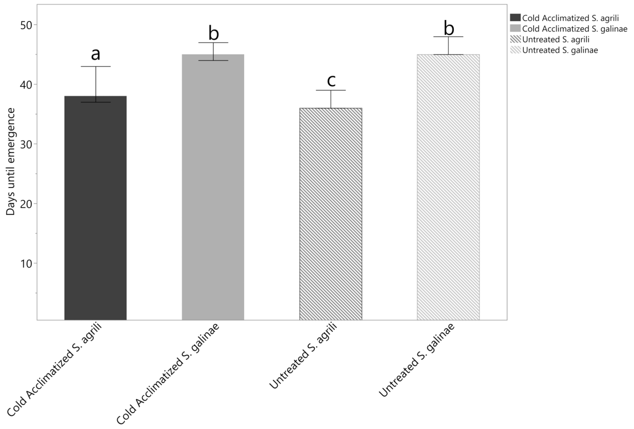

Fig. 5. Median days to emergence for the subsequent generation of both cohorts of *S. agrili* and *S. galinae* (95% CI for the Kaplan–Meier survival curve). Significant difference is indicated by letter grouping (Log-Rank, $P < 0.01$).

Subsequent Generation Parasitism Rate

There was no significant multigenerational effect of cold acclimatization treatment on the lifelong parasitism rate of the subsequent generation for either *S. agrili* ($F = 0.1935$, df = 1.74, $P = 0.6613$) or *S. galinae* ($F = 0.0063$, df = 1.56, $P = 0.9368$). When groups were pooled regardless of treatment parasitism rates did not differ between species ($F = 0.4394$, df = 1.131, $P = 0.5086$; Table 4).

EAB Larval Phenology in the Field

Very few EAB larvae were available in June, and at Douthat State Park. The majority of EAB sampled were still pupae. Total EAB larvae increased in July, with third- and fourth-instar larvae increasing to their maximum abundance. In August, the number of EAB larvae declined although some larvae were still present (Fig. 6). At MCP, a similar pattern was observed, but with much lower overall numbers.

Discussion

Early season emergence of *S. agrili* and *S. galinae* differed under Virginia field conditions. The two species displayed differential responses to cold acclimatization. Median emergence of untreated *S. galinae* occurred on May 15, approximately 2 wk (11.5–14.5 d) earlier than *S. agrili*, and 25 d earlier than cold acclimatized *S. galinae*. The emergence time of *S. agrili* was unaffected by the cold acclimatization period, median emergence occurred on May 26 (cold acclimatized) and May 29 (untreated). The last cohort to emerge was cold acclimatized *S. galinae*, median emergence occurred on June 9. With an average of 4 to 5 wk lifespan, both *S. agrili* and *S. galinae*, regardless of treatment, overlapped with third- and fourth-instar EAB at both sampled field sites. Under our experimental conditions, *S. agrili* and cold acclimatized *S. galinae* emerged later in the season when more third- and fourth-instar EAB are likely to be found in field conditions. Untreated *S. galinae* had very little overlap with available EAB larvae due to their earlier peak emergence.

Both *S. agrili* and *S. galinae* had shorter adult longevity than literature suggested. Both species of *Spathius* have similar adult longevity, 4.40–5.22 wk in this experiment, lower than their 7–8 wk longevity under laboratory rearing conditions (Gould et al. 2011, Duan et al. 2014). This could be due to differences in testing methodologies, or an effect of Virginia climate conditions.

Table 4. The parasitism rate of the subsequent generation of *S. agrili* and *S. galinae*

Cohort	Parasitism Rate (% attacked)
S. agrili (pooled)	23.52 ± 3.67
Cold acclimatized *S. agrili*	22.01 ± 4.97
Untreated *S. agrili*	25.16 ± 5.17
S. galinae (pooled)	27.23 ± 4.21
Cold acclimatized *S. galinae*	26.66 ± 8.35
Untreated *S. galinae*	27.45 ± 5.22

No groups differed when compared using pairwise ANOVA (*P* o groups

Cold acclimatization had multigenerational effects on emergence time of *S. agrili*, but not on *S. galinae*. The subsequent generation of *S. agrili* emerged 2 d earlier when the parent generation was cold acclimatized. Emergence of *S. galinae* progeny was unaffected by cold acclimatization. Cold acclimatization treatment did not affect longevity, sex ratio of progeny, or parasitism rate in either species. Differences were observed between the two species when cold acclimatized and untreated cohorts were pooled within species. The fecundity of *S. galinae* was lower than *S. agrili* which is consistent with previous literature of the both species (Gould et al. 2011, Belokobylskij et al. 2012, Duan et al. 2014, Watt et al. 2016).

Depending on the availability of EAB larvae at a given site, a cold treatment could be used to improve phenological synchronization between the phenology of the parasitoids and their host. With a better understanding of EAB phenology at individual sites, this early season release method could allow for infested bolts to be shipped to field sites earlier in the season and aid in mass rearing. In the time since this experiment was completed, further climate matching has been completed on *S. agrili* and *S. galinae*, based on EAB overwintering lifestage across its invasive range (USDA-APHIS/ARS/FS 2019). The USDA now suggests release of *S. agrili* only south of the 40th parallel due to its failure to establish in the northern United States, and *S. galinae* only north of it due to early spring larval availability (USDA-APHIS/ARS/FS 2019). As *S. galinae* establishes in the northern United States and Canada where *S. agrili* did not, each will contribute to the control of EAB as part of a complex of biological control agents in different geographical regions (Larson and Duan 2016, Duan et al. 2019). As EAB biological control guidelines are updated with new information on EAB larval development, parasitoid release timing is becoming more precise, and less likely to miss the critical period of larval availability. Therefore, this method of early season release would not be suitable for *S. galinae* without a cold treatment. *Spathius agrili* could be released this way, because

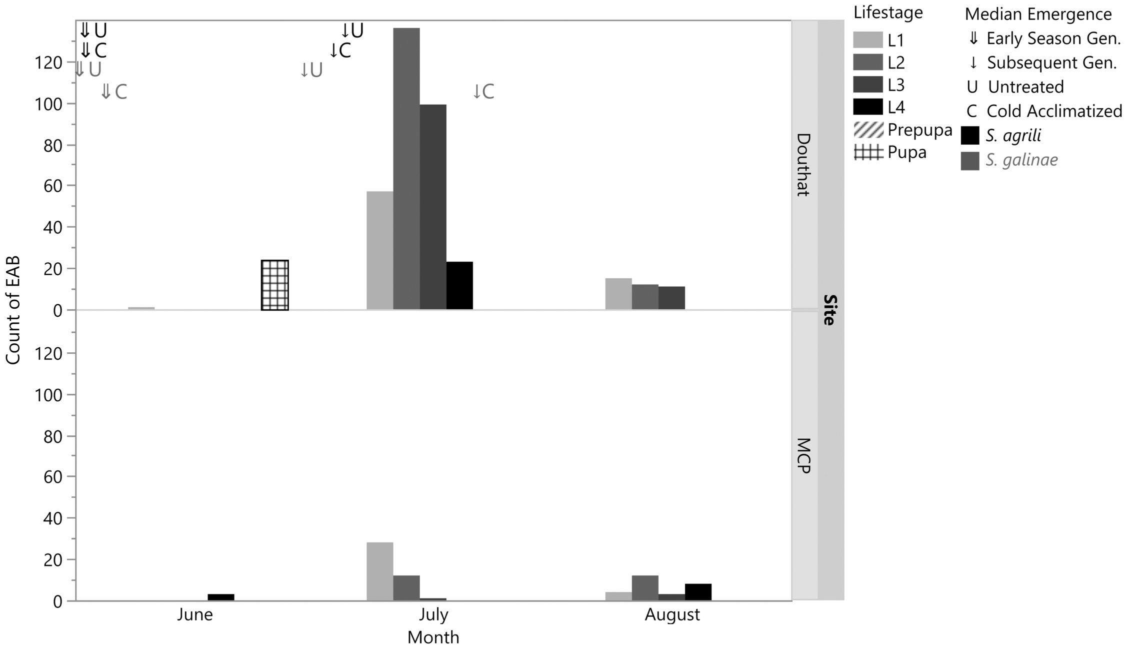

Fig. 6. Sum of all EAB larvae sorted by lifestage from monthly sampling at each field site. Sampling at Douthat took place on 15 June 2017, 13 July 2017, and 16 August 2017. Sampling at MCP took place on 2 June 2017, 11 July 2017, and 14 August 2017. Median parasitoid emergence date are indicated above EAB larval count. Subsequent generation placement is based on the assumption that the parent generation emerges at the median emergence time, and there is no preoviposition period of the parent generation. Placement is an approximation and is not to scale.

emergence occurred when third- and fourth-instar EAB were present at both sites regardless of treatment.

Further studies could examine these patterns using true overwintering conditions, or in whole tree scenarios. Whole tree sampling in field scenarios should give the true representation of the overwintering survival and early season emergence of both species, and should be conducted in the future. Additional work should focus on longer cold exposures to simulate natural winters, a more varied temperature regime to observe impacts across a range of climate change conditions, as well as geographical ranges, and larger sample sizes if possible.

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