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Focus on spotted lanternfly

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Focus on spotted lanternfly

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Editorial: Focus on spotted lanternfly

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Editorial on the Research Topic
Focus on spotted lanternfly

The spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is a univoltine generalist pest of tree of heaven (TOH) (*Ailanthus altissima* (Mill.) Swingle [Sapindales: Simaroubaceae]) from China (1). As an invasive species, it was found in South Korea in 2004 (2, 3) and most recently Berks County, Pennsylvania in 2014 (4). The current distribution of this pest in the United States includes 14 states from Massachusetts to Indiana and New York to North Carolina (5). In addition to TOH, it also feeds on grapevines and >100 other plant and tree species (6). Large amounts of honeydew excreted during the feeding process promote sooty mold on trunk and leaf surfaces of host trees and understory plants, hindering photosynthesis and contaminating agricultural and forest crops (7). Significant damage in vineyards has been recorded in South Korea (8) and the United States (9). It is a serious threat to the collective multibillion-dollar grape, fruit, nursery, landscape, and hardwood industries in North America and around the world (10, 11).

Managing SLF populations in the field is difficult since TOH is one of the most widespread invasive alien plant species in the United States (12), where more than 400,000 hectares of grapes are cultivated each year (13). Despite concerted efforts on the study of its genetics, host range, behavior, seasonal dynamics, life cycle, dispersal potential, natural enemies, and chemical control by researchers in recent years (14), questions on effective rearing methodology, host-plant interaction, damage, detection, population ecology, behavior, communication, mortality factors, and biological control still remain. The lack of rapid and accurate survey tools creates problems for quality decision-making. Host-switching during the season further complicates population monitoring as different life stages can survive on multiple hosts. Long-distance migration may render localized eradication and control attempts ineffective. Limitations in available management options make infestation containment and population control over large areas almost impossible. However, field populations continue to expand as the result of natural dispersal and human-aided spread. Factors affecting population trends require better understanding. Cutting edge research and paradigm shifting approaches are urgently needed to prevent new infestations from establishing and to mitigate economic loss in currently infested areas.

This Research Topic addresses current knowledge gaps in biology, ecology, and management of SLF through the collection of 20 outstanding articles generated by various research groups in the forefront of the struggle from the United States and China.

To meet the challenge of establishing and maintaining a productive colony for biological control programs and other studies, Nixon et al. successfully developed a rearing method for SLF from newly hatched nymphs to adults based on potted TOH trees in the laboratory. Reliable oviposition was also achieved for females feeding on saturated TOH logs under reduced daylength.

SLF life history traits on different hosts were also studied. Laveaga et al. reported faster development and better survival for nymphs fed on a mixed diet of Concord grape (*Vitis labrusca* L. [Vitales: Vitaceae]) and TOH, as compared with TOH or Concord grape alone, which resulted in significantly heavier adults and more egg masses produced by females. Kreitman et al. observed higher survivorship and shorter development time for nymphs reared on TOH or Concord grape compared with weeping willow (*Salix babylonica* L. [Malpighiales: Salicaceae]), Oriental bittersweet (*Celastrus orbiculata* Thunb. [Celastrales: Celastraceae]), and multiflora rose (*Rosa multiflora* Thunb. [Rosales: Rosaceae]). No nymphs reached adulthood when fed on red maple (*Acer rubrum* L. [Sapindales: Sapindaceae]) whereas those reared on basil (*Ocimum basilicum* L. [Lamiales: Lamiaceae]) failed to complete the second instar. On the other hand, survival and development of both nymphs and adults on specialty crops and other secondary hosts were investigated by Elsensohn et al. Results showed that young (first to second instar) nymphs survived better on Cascade hop (*Humulus lupulus* L. ([Rosales: Cannabaceae]) and adults persisted the best on kiwifruit (*Actinidia* sp. [Ericales: Actinidiaceae]) and muscadine grape (*Vitis rotundifolia* Michx. [Vitales: Vitaceae]). Furthermore, muscadine grape alone could support spotted lanternfly through adulthood, but black walnut (*Juglans nigra* L. [Fagales: Juglandaceae]) needed to pair with TOH for improved survival and development.

Despite the apparent polyphagy for most of its life history, SLF does aggregate as adults towards TOH for defense sequestration, maturation feeding, and mating (15–17). Cooperband and Murman captured this behavior in the field when they found males were generally attracted to males while females to females in their cage studies, resulting in male-biased, sex-balanced, and female-biased adult populations on TOH trees in the early, mid-, and late stages, respectively. Further investigation by Faal et al. through olfactometer trials in the laboratory revealed that honeydew volatiles such as 2-heptanone, 2-octanone, and 2-nonanone were likely responsible for this kind of behavior. Along with plant volatiles discovered before (18), they could potentially be used as lures for the development of adult traps down the road.

SLF egg masses are found on the surfaces of a wide range of substrates from trees to plants and nonliving materials (1, 16, 17). However, oviposition is not completely random but rather selected by habitats and substrates as demonstrated by Liu. Egg mass size ranged from 0-105 eggs/mass with larger egg masses found in newly infested sites with expanding populations. Egg masses laid on American beech (*Fagus grandifolia* Ehrh. [Fagales: Fagaceae]), black birch (*Betula lenta* L. [Fagales: Betulaceae]), black cherry (*Prunus serotina* Ehrh. [Rosales: Rosaceae]), black locust (*Robinia pseudoacacia* L. [Fabales: Fabaceae]), hackberry (*Celtis occidentalis* L. [Rosales: Cannabaceae]), Norway maple (*Acer platanoides* L. [Sapindales: Sapindaceae]), pawpaw (*Asimina triloba* (L.) Dunal [Magnoliales: Annonaceae]), red maple, and sweet cherry (*Prunus avium* L. [Rosales: Rosaceae]) generally hatched better than those on other substrates.

Three studies focused on the impact of SLF feeding on its hosts. Dechaine et al. reported diminished annual diameter growth on infested TOH trees based on dendrochronological evidence after data were standardized by climate variables and tree size and age. However, no such impact was observed on infested black walnut and tuliptree (*Liriodendron tulipifera* L. [Magnoliales: Magnoliaceae]). Chemical treatment of SLF with dinotefuran through basal bark application did not help TOH growth compared with untreated controls. Islam et al. discovered genome-wide transcriptional response on grapes after heavy SLF feeding, with extensive changes in gene expression in pathways associated with biosynthesis of lignin and other structural components of cell-wall matrix and antioxidant/detoxification. Lavely et al. recorded negative impact of short-term feeding by late-stage nymphs and adults on the carbon assimilation for red maple and silver maple (*Acer saccharinum* L. [Sapindales: Sapindaceae]) in a common garden study, with no significant negative impact from SLF feeding on black walnut.

Without effective prevention and management measures, SLF has the potential to establish in most New England, mid-Atlantic, Midwest, and Pacific Coast states in the United States in addition to other suitable areas around the world (19, 20). Keena et al. predicted even wider potential climate range for SLF in North America based on upper (T_{max}) and lower (T_{min}) developmental thresholds in areas previously considered too cold to be at risk, while southward expansion into warmer regions may be limited by thermal conditions as forecasted by those models.

Accurate population estimation is difficult for SLF because of the high mobility of nymphs and adults and diverse habitats of egg masses. Lewis et al. developed an effective and low-cost lamp shade trap for egg masses after tens of designs and hundreds of field deployments in five years. This design could revolutionize SLF population monitoring and egg mass collection in the field with an average of 25 (up to 111) egg masses recovered from each trap. Guidelines for trap construction and its field installation were also included as supplementary materials for this publication. On the other hand, Belouard and Behm introduced a new way to identify adults by wing spot patterns using computer-aided photo-identification technology. After validation by larger datasets, it could have broad applications in population survey and dispersal studies for SLF adults without laborious work on marking, releasing, and recapturing of the insects.

Many chemical insecticides (e.g., chlorpyrifos, thiamethoxam, bifenthrin) are effective against SLF eggs, nymphs, and adults through topical application (21, 22). In places when cover spray is not desired, systemic insecticides can be used as the alternatives. Keyzer et al. demonstrated that dinotefuran could persist on TOH bark surface for at least 100 days when applied through basal trunk spray, providing effective management for SLF adult populations in the field.

Major mortality factors for SLF in the field include predators, parasitoids, and pathogens (23–28). Multiple strains of *Beauveria bassiana* Bals. -Criv) Vuill. (Hypocreales: Cordyciptaceae) were isolated by Clifton et al. from field infected SLF in Pennsylvania, with two of the most prevalent strains showing superior pathogenicity against nymphs and adults than a standard commercial strain in laboratory bioassays.

Anastatus orientalis Yang & Choi (Hymenoptera: Eupelmidae) is a solitary egg parasitoid of spotted lanternfly in its native range (25–27). Its potential as a biological control agent for SLF in North America is being considered. By analyzing mitochondrial DNA, Wu et al. were able to recover six haplotypes of *A. orientalis* based on specimens collected from China and South Korea, with haplotypes B, C, and D widely distributed while haplotypes A, E, and F found only in certain locations. Bao et al. investigated the impact of photoperiod experience and found increased fecundity and female-based sex ratio, but decreased longevity in the next two generations when parents were placed under long daylength. The broad host range of haplotype C was confirmed after choice and no-choice testing against 36 eastern species in 18 families by Broadley et al. and 34 southwestern species in 12 families by Gómez-Marco et al, with nontarget species in the families of Coreidae, Erebidae, Fulgoridae, Lasiocampdae, Pentatomidae, Saturnidae, and Sphingidae readily attacked. For laboratory rearing purpose, Gómez-Marco and Hoddle found that exposing SLF eggs to -40 ° C for more than 1 h effectively killed SLF eggs, however, *A. orientalis* females could still utilize them to produce progenies successfully. The potential of this egg parasitoid as an SLF biocontrol agent would rest on other haplotypes if they are found to have narrower host ranges.

Successful management of SLF in North America depends on the integration of new information from sound scientific research for early detection and rapid response activities. New findings in laboratory rearing, life history characteristics, adult behavior, host damage, potential climate range, novel survey and detection tools, pathogens and parasitoids, and chemical control in this Research Topic could help decision makers to refine their management strategies. At the same time, research community could also benefit from each study in its pursuit of better outcomes in various areas. The potential damage by SLF to other specialty crops and hardwood trees is not expected to approach the level on grapes. SLF management on grapes should focus on chemical control of SLF adults feeding on vines after emergence and those aggregating on TOH trees in the surrounding areas before mating, with microbial control of high density nymphal and adult populations and biological control being parts of the solution. Conversely, homeowners and residents in urban and suburban areas could also take advantage of the new information provided by the studies to mitigate the impact of this significant nuisance and improve their quality of life.

Author contributions

HL: Writing – original draft, Writing – review & editing. XW: Writing – review & editing. MFC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of Freezing *Lycorma delicatula* Egg Masses on Nymph Emergence and Parasitization by *Anastatus orientalis*

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Lycorma delicatula (White) (Hemiptera: Fulgoridae), native to China, was first detected in Pennsylvania, U.S. in 2014. This polyphagous pest can feed on over 70 plant species including agricultural crops, like grapes, that have high economic value. *Anastatus orientalis* Yang and Choi (Hymenoptera: Eupelmidae) is an egg parasitoid associated with *L. delicatula* egg masses in China that is being evaluated for possible introduction into the U.S. for classical biological control of *L. delicatula*. In support of this program, the suitability of frozen *L. delicatula* eggs for parasitization by *A. orientalis* was evaluated in a quarantine laboratory. Host egg masses held for four different cold storage periods (5°C for <1, 4, 8 and 11 months) were frozen at -40°C for 1 hour or 24 hours and exposed to female *A. orientalis* for parasitization for seven days. Following this experimental exposure period, rates of *L. delicatula* nymph emergence and *A. orientalis* parasitism were assessed for each of the eight different cold storage treatments. Host acceptance and suitability of frozen *L. delicatula* eggs by *A. orientalis* was assessed in terms of percentage parasitism, offspring sex ratio, and hind tibia length of emerged parasitoids. Results indicated that *L. delicatula* nymphs failed to emerge from eggs that were exposed to -40°C for 1 hour and 24 hours and *A. orientalis* could successfully parasitize *L. delicatula* eggs regardless of cold storage and freezing treatment. These results add a new tool for long term maintenance of *L. delicatula* egg masses and rearing methods for egg parasitoids of this pest. Additionally, it may be possible to field deploy sentinel eggs of *L. delicatula* frozen at -40°C to survey for resident natural enemy species capable of parasitizing eggs of this pest in advance of anticipated *L. delicatula* invasions into new areas.

Keywords: biotic resistance, Eupelmidae, Fulgoridae, invasive species, native parasitoids, proactive biological control, sentinel eggs

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INTRODUCTION

Spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), native to China (1), is an invasive species in South Korea and Japan where it invaded in 2004 and 2009, respectively (2, 3). In September 2014, *L. delicatula* was detected for the first time in Berks County, Pennsylvania, U.S. By April 2022, *L. delicatula* infestations were confirmed in an additional ten states in eastern

(Connecticut, Delaware, Maryland, Massachusetts, New Jersey, New York, Virginia, and West Virginia) and mid-western (Indiana and Ohio) areas of the U.S (4). *Lycorma delicatula* is a phloem-feeding fulgorid that has a broad host range having been recorded feeding on over 70 plant species encompassing 25 families (5–7). Feeding by high density populations causes direct damage to host plants through removal of phloem fluids and indirect damage results from the excretion of high quantities of honeydew that promote sooty mold growth (5, 8). Direct feeding damage can cause mortality to highly preferred hosts *Ailanthus altissima* (Miller) (Sapindales: Simaroubaceae) and grapevines (*Vitis vinifera* L. [Vitales: Vitaceae]). *Lycorma delicatula* is also of high concern for other economically important perennial agricultural crops like fruit (e.g., apples, *Malus domestica* Borkh [Rosales: Rosaceae]) and nut (e.g., walnuts, *Juglans* spp. [Fagales: Juglandaceae]) trees (4). Additionally, *L. delicatula* has been recorded infesting forest and ornamental shade tree species (5, 9). *Lycorma delicatula* can disperse short distances through wind-assisted gliding and long-distance dispersal is almost entirely through human-assisted movement. This occurs primarily through the accidental translocation of egg masses that are often laid indiscriminately on inert substrates (e.g., wooden pallets and railcars) that undergo subsequent transportation into uninfested areas (10–12). This type of inadvertent relocation resulted in the establishment of invasion bridgeheads in the mid-west (e.g., Indiana and Ohio) and northeastern U.S. (e.g., Massachusetts) in 2021 (4). Tree of heaven, *A. altissima*, a widely distributed and common invasive plant, is a highly preferred host for *L. delicatula* and the high abundance of this tree has likely facilitated the invasion success of *L. delicatula* (5). Waki et al. (13) modeled the potential distribution of *L. delicatula* and results indicated that large areas of the west coast of the U.S., and other parts of the world (e.g., Europe), have high potential climatic suitability for *L. delicatula* establishment and proliferation. With respect to California, a western U.S. state with an agricultural economy worth ~$50 billion per year (14), *L. delicatula* is viewed as a significant invasion threat that could cause significant problems for producers of specialty crops like grapes and nuts. Consequently, *L. delicatula* is the subject of a proactive biological control research program that is being undertaken in advance of its anticipated incursion and establishment in California (15).

Anastatus orientalis Yang and Choi (Hymenoptera: Eupelmidae) is an egg parasitoid that was discovered parasitizing *L. delicatula* eggs in northern China in 2011. This parasitoid was found during foreign exploration surveys for natural enemies for potential use in a classical biological control program targeting invasive *L. delicatula* populations in South Korea (16, 17). Interest in the use of *A. orientalis* as a classical biological control agent increased significantly in 2015 following the invasion and spread of *L. delicatula* in the U.S (18).. Accordingly, the proactive biological control program underway in California targeting *L. delicatula* has focused research efforts on *A. orientalis*. Broadley et al. (18) investigated aspects of the biology and rearing of *A. orientalis*. One finding from this study was that the number and sex ratio of progeny produced and parasitism rates of *A. orientalis* per *L. delicatula* egg mass did not differ between newly collected eggs vs. eggs stored at 5°C for up to 10 months. However, this study did not examine the number of *L. delicatula* nymphs that emerged from freshly collected *L. delicatula* egg masses, those stored at 5°C, or what nymph emergence rates would be if eggs were stored at temperatures below 5°C (e.g., -40°C) for varying time periods. Previous studies have shown that parasitoid species in the genus *Anastatus* were able to parasitize frozen hemipteran and lepidopteran eggs (19, 20). However, it is unknown if fulgorid egg masses (e.g., *L. delicatula*) would be suitable for parasitism by *A. orientalis* after freezing.

There are two objectives for this study, first, to test if exposure to -40°C for ≤ 3 days can kill *L. delicatula* eggs. The USDA-APHIS approved protocol for killing *L. delicatula* eggs (and nymphs and adults) at the Insectary and Quarantine Facility at the University of California Riverside, is to hold life stages at -40°C for 72 hours before properly removing them from the quarantine facility (USDA-APHIS Permit to Move Live Plants Pests, Noxious Weeds, and Soil number P526P-19-02058). A lethal period shorter than 72 hours for *L. delicatula* eggs may be possible and warrants determination. Second, when this study was undertaken, it was unknown if *L. delicatula* eggs killed by freezing would be suitable for parasitism by *A. orientalis*. Answering this question could increase cold storage options for *L. delicatula* eggs for laboratory rearing of *A. orientalis*. Additionally, if eggs are successfully killed at -40°C and are acceptable for parasitization by egg parasitoids it may be possible to proactively deploy unviable sentinel egg masses to survey for resident natural enemy species capable of parasitizing *L. delicatula* egg masses in areas identified as being at risk of invasion. Surveys of this nature could provide a potential measure of naturally occurring levels of biotic resistance in advance of an anticipated incursion.

MATERIAL AND METHODS

Source of *Lycorma Delicatula* Eggs for Experiments

A total of 1,554 *L. delicatula* egg masses were field collected in February and December of 2020 of which 263 egg masses with 45.4 ± 1.26 eggs/egg mass (plus 53 egg masses for pre-oviposition purposes [see below]) were randomly selected and used for experiments reported here. Collections were made in seven different locations in Pennsylvania, U.S. from *A. altissima* (**Table 1**). Entire egg masses attached to underlying bark were removed using chisels and shipped to the University of California Riverside Insectary and Quarantine Facility (UCR-IQF) under USDA-APHIS permit P526P-19-02058 and California Department of Food and Agriculture (CDFA) Permit 3458. In quarantine, all field collected egg masses were stored at 5°C and 60-75% R.H. for < 1 month, 4, 8, or 11 months until used for experiments (see below for details).

The *A. orientalis* colony was established in UCR-IQF from parasitized *L. delicatula* egg masses shipped under USDA-APHIS permit P526P-19-02066 from USDA-APHIS-PPQ Science and Technology, Buzzard Bay, Massachusetts U.S. in

October of 2019. The USDA-APHIS colony was initiated with parasitoids originally reared from *L. delicatula* eggs collected in Beijing, China, the source country of the invasive *L. delicatula* population in the U.S (18). Upon receipt at UCR-IQF, parasitized egg masses were held at 25°C and R.H. 65% for ~40 days for *A. orientalis* to complete emergence. Emerged parasitoids were used to initiate colonies [see Broadley et al. (18) for *A. orientalis* rearing protocols] that were maintained on field collected *L. delicatula* egg masses (**Table 1**) in UCR-IQF.

TABLE 1 | *Lycorma delicatula* egg mass collection sites and dates in Pennsylvania, U.S.

County	Location	Number of egg masses	Collection date	GPS
Berks	Reading	58	5-Feb-20	40°21'18.275''N-75°55'38424''W
Dauphin	Harrisburg	137	24-Feb-20	40°15'58.719''N-76°53'10.003''W
Huntingdon	Petersburg	171	20-Feb-20	40°34'19.95''N-78°2'51.633''W
Lancaster	Lancaster	50	1-Dec-20	40°2'17.268''N-76°18'20.406''W
Lebanon	Lebanon	837	26-Feb-20	40°22'32.567''N-76°27'45.402''W
	Myerstown	191	4-Feb-20	40°22'27.157''N-76°18'13.167''W
	Palmyra	110	18-Dec-20	40°18'18.111''N-76°35'30.468''W
Total		1,554		

Lycorma delicatula Egg Storage Periods and Freezing Treatments

In UCR-IQF, field collected *L. delicatula* egg masses were stored for four different periods: <1 month, 4, 8, and 11 months at 5°C before use in experiments. For each experimental cold storage period, <1 month, 4 months, 8 and 11 months, 57, 84, 24 and 24 egg masses, respectively, were randomly selected and subdivided to make eight experimental groups each of which was exposed to -40°C for two times intervals, 1 hour (egg masses <1 month, n = 17; 4 months, n = 39; 8 months and 11 months, n = 12 each) or 24 hours (egg masses <1 month, n = 23; 4 months, n = 45; 8 months and 11 months, n = 12 each) (**Table 2**). After both freezing treatments at -40°C, egg masses were "thawed" at room temperature (~25°C) for 30 minutes before being either exposed or not exposed to female *A. orientalis* for parasitization (**Table 2**). Additionally, four groups of *L. delicatula* egg masses (i.e., unfrozen treatment) not exposed to -40°C were set up under the same conditions as cold treated egg masses to measure the emergence rates of *L. delicatula* nymphs from eggs that had experienced one of the four experimental storage periods (i.e., egg masses stored at 5°C stored for <1 month, n = 13; 4 months, n = 19; 8 and 11 months, n = 6 each). Following the same protocol, an additional treatment (i.e., an unfrozen/parasitized treatment) was set up which exposed *A. orientalis* females to egg masses stored at 5°C for each of the four experimental cold storage periods (i.e., <1 month, n = 15 egg masses; 4 months; n = 18; 8 and 11 months, n = 6 each) to determine egg suitability for parasitism (**Table 2**).

Experimental Set Up and Completion of a Seven Day Pre-Oviposition Period of *A. orientalis*

All experiments were conducted in temperature and humidity controlled cabinets programmed to cycle through average Fall (i.e., September) temperatures for Beijing (average daily high 25°C, average daily low 14°C, lights on 6:00 AM, lights off 6:30 PM (i.e., L:D 12.5:11.5), 65% R.H. (see **Supplementary Table 1**) in the UCR-IQF. Fall temperatures in Beijing were used to simulate the natural conditions of the original collection area of *A. orientalis* in order to optimize parasitoid parasitism behavior and development time (18). Each experimental egg mass-parasitoid test arena was comprised of a clear plastic container 3 cm x 4 cm x 5cm (180mL clear RPTE hinged lid deli containers, AD16 GenPak, Charlotte, NC) with a modified lid that had a ventilated mesh window (1.5 cm x 2.5 cm) to facilitate air exchange. One *L. delicatula* egg mass from one of the egg storage period categories that eggs were exposed to were placed into each test unit after this seven day pre-oviposition period. The inclusion of a *L. delicatula* egg mass in this pre-oviposition period is necessary as it allows female parasitoids to mate, host feed, and mature eggs for oviposition (Gomez et al. manuscript in preparation). Five female and one male *A. orientalis*, ~24 hours of age, were introduced onto egg masses to mate and for females to complete their pre-oviposition period (18). Streaks of honey were applied to lids to provide a carbohydrate source for parasitoids and arenas were sealed with the ventilated lid. Following the seven day preoviposition period, egg masses and

TABLE 2 | Treatment assignments for experimental *Lycorma delicatula* egg masses and the number (n) of repetitions for each treatment.

Time at -40°C	Exposed to *A. orientalis*	Name of the treatment	Cold storage periods at 5°C (months)			
			<1	4	8	11
1 hour	no	1h.-40°C	n = 10	n = 15	n = 6	n = 6
	yes	1h.-40°C.Parasitism	n = 7	n = 24	n = 6	n = 6
24 hours	no	24h.-40°C	n = 12	n = 20	n = 6	n = 6
	yes	24h.-40°C.Parasitism	n = 11	n = 25	n = 6	n = 6
No exposure	no	Control	n = 13	n = 19	n = 6	n = 6
	yes	Parasitism	n = 15	n = 20	n = 6	n = 6

parasitoids were removed from test arenas. Male *A. orientalis* were replaced if they died. Females were not replaced because of the seven-day exposure required for new females (i.e., ~24 hours of age) to reach maximum parasitism performance.

Host Emergence and Parasitism Rates, Offspring Sex Ratio

Following the seven day pre-oviposition period, parasitoids were provided either one treated (i.e., -40°C for 1 hour or 24 hours) egg mass from one of the four egg storage periods (i.e., < 1, 4, 8 or 11 months at 5°C) or non-frozen egg masses from the same four storage period categories in the experimental units described above for an additional seven days. After this seven-day period (females now had a total of 14 days exposed to *L. delicatula* egg masses), parasitoids were removed from all test units and experimental egg masses inside test arenas were placed in a temperature cabinet programmed to simulate fluctuating temperatures that parasitoids would experience during fall in Beijing China for four weeks (see above). After this four-week exposure to fluctuating temperature cycles, egg masses were held at a constant 25°C and R.H. 75% until parasitoid emergence. Data collected from experimental units included the total number of *L. delicatula* eggs per experimental egg mass, the total number of emerged *L. delicatula* nymphs per egg mass, the number and gender of emerged parasitoids and the number of unemerged parasitoids (i.e., larvae, pupae and/or adult parasitoids that died and failed to emerge from eggs were found after dissection of unhatched eggs). Percentage parasitism was calculated by dividing the number of emerged and unemerged parasitoids by the total number of *L. delicatula* eggs that comprised an egg mass which was multiplied by 100. Parasitoid sex ratio was calculated as the number of female parasitoids divided by the total number of female and male parasitoids combined that emerged from each experimental egg mass.

Measurement of Hind Tibia Length as an Assessment of Parasitoid Fitness

To evaluate the effect of -40°C exposures for 1 hour or 24 hours treatments on *L. delicatula* egg quality for *A. orientalis* development, the fitness of female parasitoids that successfully emerged from eggs cold stored at 5°C for four months, and four month old eggs that were exposed to -40°C for 1 hour or 24 hours only were assessed by using measurements of right hind tibia lengths as a proxy for parasitoid size and subsequent fitness (i.e., parasitoids with larger hind tibia are assumed to be bigger and more fit than parasitoids with smaller tibia). The four month storage period was selected for this study since it is the average approximate length of the storage period that the *L. delicatula* egg masses would be held for prior to use in experiments. Excised right hind tibiae were placed onto glass slides and covered with a second glass slide. Hind tibia length was measured from its attachment to the femur to the attachment point with the tarsi using a Leica S8AP0 microscope. Slide mounted hind tibiae were photographed at a magnification of 25 × with an attached Leica DMC2900 camera and length was measured using the Leica Application Suite version 4.6.2. A total of 25 A. *orientalis* females from each treatment, both freezing treatments (i.e., 1 hour and 24 hours at -40°C) and the control treatment [i.e., Unfrozen/parasitized treatment (**Table 2**)] were measured for a cumulative total of 75 female tibiae.

Statistical Analyses

All statistical analyses were performed in R 4.1.3 (21) using RStudio 2022.02.0 Build 443 (22). Untransformed data met the assumptions of selected statistical tests and models used, unless specified otherwise. To test for differences in *L. delicatula* nymph emergence and parasitism rates between cold storage times (i.e. <1, 4, 8 and 11 months at 5°C) and treatments (i.e. controls, -40°C exposure for 1 hour or 24 hours) a Kruskal-Wallis test was performed. When differences were found, these analyses were followed by multiple pairwise comparisons using Wilcoxon rank sum test in each group with Bonferroni corrections at the 0.05 level of significance.

The sex ratio of emerged *A. orientalis* females across the four different cold storage categories of egg masses (i.e., <1 month, 4, 8, and 11 months at stored 5°C) that were frozen at -40°C for either 1 hour or 24 hours and exposed to parasitoids were compared to the sex ratio of female parasitoids that emerged from control egg masses used for parasitism (i.e., <1 month, 4, 8, and 11 months at 5°C and not exposed to -40°C). These comparisons were made using a quasibinomial GLM with a logit link function that included two variables; egg storage period (i.e., the four cold storage periods, <1 month, 4, 8, and 11 months) and treatment (i.e., egg masses that were either frozen or not frozen at -40°C for 1 hour or 24 hours, and either exposed or not exposed to parasitoids). To determine if significant effects from test variables existed ANOVA was conducted followed by a Tukey posthoc test at the 0.05 level of significance to identify differences between treatment categories and cold storage exposure treatment times. Differences in mean hind tibiae lengths between parasitoids emerging from egg masses stored at 5°C for four months on the three treatment groups (i.e., -40°C for 1 or 24 hours and not frozen at -40°C) was analyzed by ANOVA followed by a Tukey posthoc test at the 0.05 level of significance. All means are presented ± SE.

RESULTS

Effects of -40°C on *Lycorma delicatula* Nymph Emergence Rates

Irrespective of storage times (i.e., < 1, 4, 8, 11 months) at 5°C, when *L. delicatula* eggs were exposed to -40°C for either 1 hour or 24 hours zero nymphs emerged from a total of 7,557 eggs that comprised the 172 egg masses that were used in these two treatments (**Table 2**) (**Figure 1**).

The maximum percentage emergence of *L. delicatula* nymphs was from unfrozen egg masses not exposed to parasitoids which were stored for <1 month and four months with an average emergence of 72.6 ± 8.2% (n = 13 egg masses) and 56.1 ± 5.7%

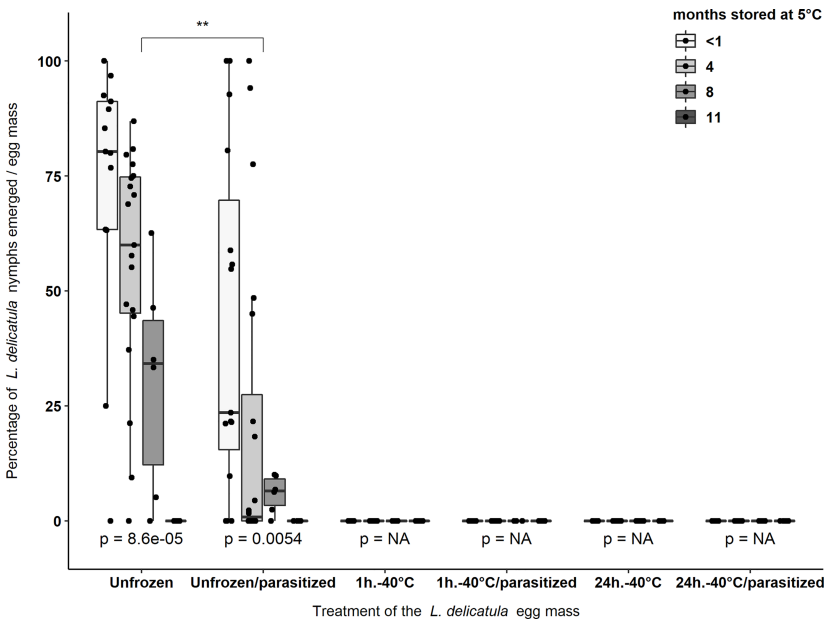

FIGURE 1 | Average percentage of nymphs that emerged from *L. delicatula* egg masses stored at 5°C and 65% R.H. for four different storage periods: <1 month, 4, 8, and 11 months. After these storage periods a subset of these egg masses were frozen at -40°C for 1 hour or 24 hours and either exposed or not exposed to *A. orientalis*. Asterisks (**) indicate significant differences ($p < 0.01$) in the percentage of *L. delicatula* nymphs that emerged from egg masses between treatment groups (i.e., Unfrozen and Unfrozen/parasitized). *p*-values < 0.05 indicate significant differences between the percentage of nymphs emerged from egg masses that were stored for different periods within the same treatment group. NA "not-applicable" indicate that no statistical analyzes were used as all data were zeros in each treatment group. Black dots represent data points.

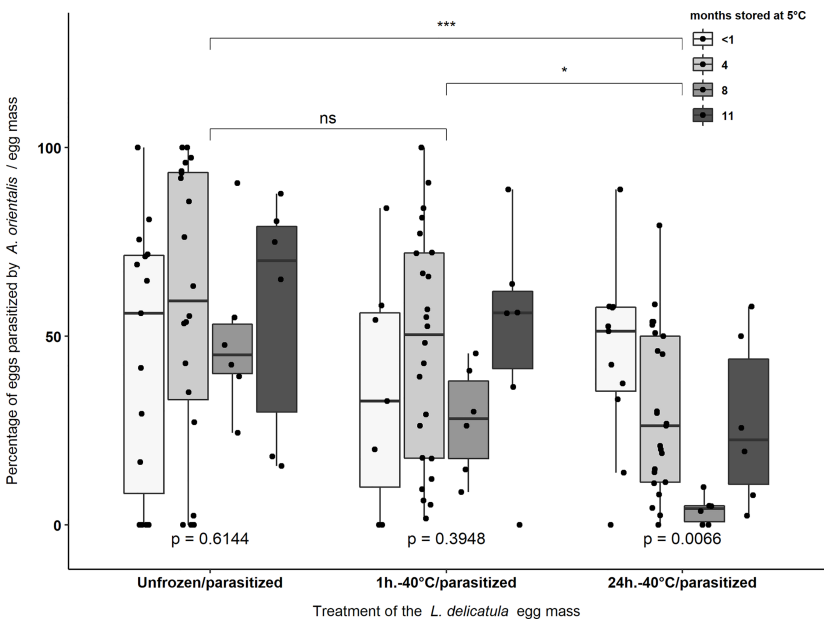

FIGURE 2 | Percentage parasitism of *L. delicatula* egg masses by *A. orientalis* that were treated at -40°C for 1 hour or 24 hours or not frozen (i.e., Unfrozen/parasitized) prior to exposure to female parasitoids. Experimental egg masses were stored at 5°C and 65% R.H. at four different periods: <1 month, 4, 8, and 11 months prior to exposure to -40°C. Asterisks indicate significant differences [(*) = $p < 0.05$; (***) = $p < 0.001$] and "ns" indicate non-significant differences in the percentage of parasitism between treatment groups. p-values < 0.05 indicate significant differences in the percentage of parasitism between egg masses that were stored for different periods within the same treatment group. Black dots represent data points.

(n = 19), respectively (**Figure 1**). Around 30.4 ± 9.8% (n = 6) nymphs emerged from egg masses stored for eight months and no nymphs emerged from unfrozen egg masses that were stored for 11 months at 5°C (n = 6). Parasitism by *A. orientalis* significantly reduced the percentage of *L. delicatula* nymphs that emerged from egg masses (χ^2 = 10.53, d.f. = 1, p = 0.001). This effect was significantly different in the egg masses stored for four months only (χ^2 = 10.04, d.f. = 1, p = 0.001) (**Figure 1**).

For control treatments not exposed to -40°C, the extent of time (i.e., <1 month, 4, 8, and 11 months) that *L. delicatula* egg masses were stored at 5°C significantly reduced percentage nymph emergence per egg mass (unfrozen egg masses not exposed to parasitoids: χ^2 = 21.43, d.f. = 3, p < 0.001; unfrozen eggs exposed to parasitoids: χ^2 = 12.69, d.f. = 3, p = 0.005) (**Figure 1**).

Parasitism of Experimental Egg Masses by *Anastatus orientalis*

A total of 6,024 eggs from 138 *L. delicatula* egg masses (mean of 43.652 ± 1.377 eggs per egg mass) stored at 5°C for the four storage periods were provided to *A. orientalis* for parasitism following three different freezing treatments (**Table 2**). *Anastatus orientalis* was able to parasitize *L. delicatula* egg masses from all storage period categories exposed to -40°C for 1 hour or 24 hours (**Figure 2**). Percentage parasitism was not affected by storage period on *L. delicatula* eggs in non-treated (i.e., the Unfrozen/parasitized treatment) (χ^2 = 1.802, d.f. = 3, p = 0.614) and frozen at -40°C for 1 hour (χ^2 = 2.979, d.f. = 3, p = 0.395). Significant differences were found in the percentage of parasitism between egg masses stored for different time periods when frozen at -40°C for 24 hours (χ^2 = 12.235, d.f. = 3, p = 0.006) with significantly lower rates of parasitism being observed for eggs that were stored for eight months prior to freezing (p = 0.017) (**Figure 2**). The average parasitism rate for each treatment was 52.9 ± 4.9% (n = 47), 43 ± 4.4% (n = 43) and 29.4 ± 3.4% (n = 4 8) for the Unfrozen/parasitized, -40°C for 1 hour, and -40°C for 24 hours treatments, respectively. The parasitism rates for egg masses treated at -40°C for 24 hours were significantly lower than the parasitism rates obtained in the other two treatments (i.e., "Unfrozen/parasitized" and "1h.-40°C/parasitized" treatments) (χ^2 = 12.221, d.f. = 2, p = 0.002) (**Figure 2**).

Sex Ratio of Emerged *Anastatus orientalis* Offspring

For the total number of emerged parasitoids (n = 2,345) from all experimental egg masses (**Table 3**), 76.4% were females and 23.6% were males. An additional 21 live parasitoid larvae, pupae, or dead adults were found when dissecting *L. delicatula* eggs. These individuals were not included in sex ratio analyses. Sex ratio of emerged parasitoids was affected by the length of the storage period at 5°C ($F_{1,3}$ = 9.72, p < 0.001) but was not affected by exposure to -40°C for 1 or 24 hours ($F_{1,2}$ = 1.47, p = 0.23) or the interaction of cold storage period and freezing treatment ($F_{1,6}$ = 0.93, p = 0.47). Egg masses stored for 8 months tended to have significantly male biased sex ratios compared to the sex ratios obtained from egg masses stored for <1, 4, or 11 months (**Figure 3**).

Hind Tibia Length of *Anastatus orientalis*

Significant differences existed between the right hind tibia length of female *A. orientalis* offspring among the three different treatments that were tested. This effect was significant for 4-month-old egg masses that were subjected to -40°C for 1 hour, and -40°C for 24 hours, and not exposed to -40°C (i.e., the Unfrozen/parasitized treatment) ($F_{2, 72}$ = 48.65, P < 0.001). The average hind tibia length of female offspring was 1.005 ± 0.009 mm, 0.98 ± 0.007 mm and 0.907 ± 0.006 mm for "1h.-40°C/parasitized", "24h.-40°C/parasitized" and the "Unfrozen/parasitized" treatments, respectively. The longest hind tibia, 1.072 mm, was measured for a female parasitoid that emerged from a 4-month-old egg mass from the "1h.-40°C/parasitized" treatment and the shortest hind tibia, 0.858 mm, was measured from a female parasitoid from the "Unfrozen/parasitized" treatment (**Figure 4**).

DISCUSSION

Exposing *L. delicatula* eggs to -40°C for either 1 or 24 hours completely prevented the development of *L. delicatula* nymphs in the 172 egg masses (i.e., 7,557 eggs) that were exposed to -40°C. This result indicates that the USDA-APHIS protocol used to kill *L. delicatula* nymphs in the UCR IQF, 72 hours at -40°C, could be reduced to a minimum of 1 hour and a maximum of 24 hours. Increased duration of storage periods at 5°C reduced the percentage of *L. delicatula* nymphs that emerged from both of the no-freeze treatments (i.e., Unfrozen and Unfrozen/parasitized treatments) from an average of ~73% of nymph emerged on egg masses stored at 5°C for <1 month (i.e., <30 days) to no nymphs emerging from egg masses stored for ~11 months (i.e., ~330 days). Previous studies have shown that storage periods up to 140 days at 5°C did not reduce the number of *L. delicatula* nymphs when compared to emergence rates from eggs that were <14 days of age at time of field collections, both with 100% of emergence (23). In this study, egg masses stored for a similar period of 120 days (i.e., 4 months) had a percentage of emergence of ~56%. The differences

TABLE 3 | Percentage of egg mass from which *Anastatus orientalis* emerged per treatment and cold storage period (n = total number of egg masses).

Time at -40°C	Name of the treatment	Cold storage periods at 5°C (months)			
		<1	4	8	11
1 hour	1h.-40°C.Parasitism	71.4% (n = 7)	100% (n = 24)	100% (n = 6)	83.3% (n = 6)
24 hours	24h.-40°C.Parasitism	90.9% (n = 11)	92% (n = 25)	66.7% (n = 6)	100% (n = 6)
No-treatment	Parasitism	66.7% (n = 15)	85% (n = 20)	100% (n = 6)	100% (n = 6)

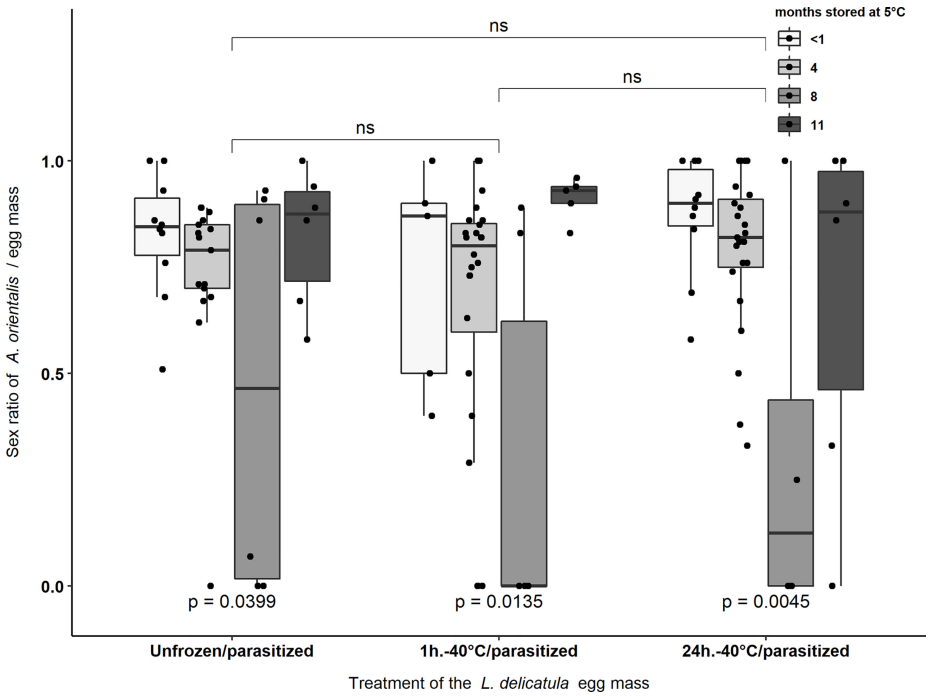

FIGURE 3 | Sex ratio of *Anastatus orientalis* offspring that emerged from *L. delicatula* egg masses stored at <1, 4, 8, or 11 months at 5°C and treated at -40°C for 1 hour or 24 hours, or not exposed to -40°C. "ns" indicate non-significant differences in the sex ratio of emerged parasitoids between egg masses of different treatments. p-values < 0.05 indicate significant differences in the sex ratio of emerged parasitoids between egg masses stored for different periods (i.e., <1, 4, 8, or 11 months) within the same treatment group. Black dots represent data points.

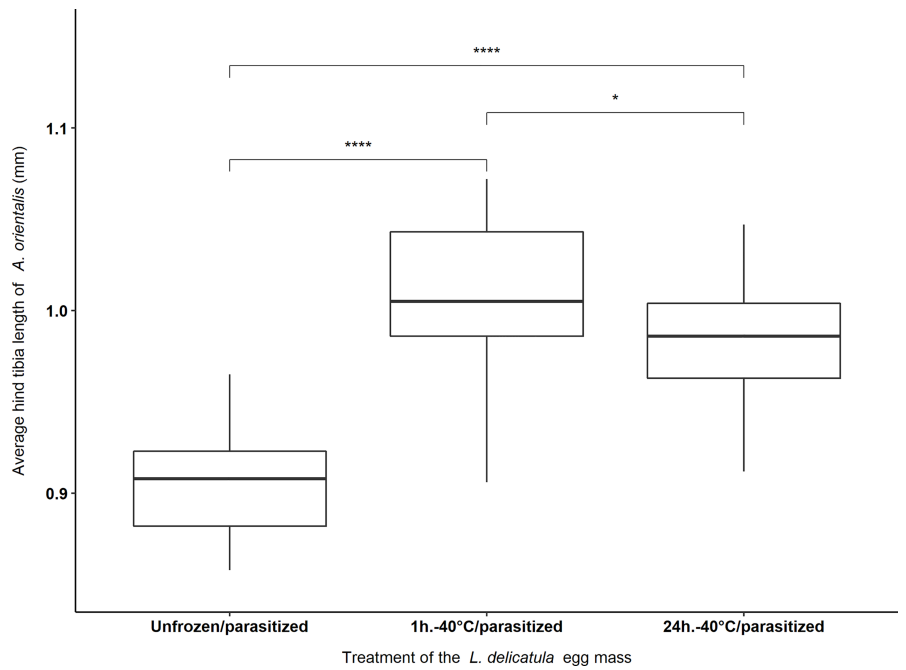

FIGURE 4 | Average hind tibia length of female *Anastatus orientalis* offspring that emerged from *L. delicatula* egg masses stored for four months at 5°C and subjected to 1 hour or 24hours at -40°C, or no freezing (i.e., Unfrozen/parasitized). Asterisks indicate significant differences between different treatments [(*) = p <0.05; (****) = p < 0.001].

in percentage emergence of *L. delicatula* nymphs from egg masses with similar storage periods used in these two different studies could be due to varying time durations in the field prior to collection, variable shipping conditions during transit, especially during movement from field collection sites on the east coast of the U.S. (i.e., Pennsylvania) to the west coast quarantine facility in Riverside California, and subsequent differences in cold storage conditions in laboratories.

Exposure studies conducted here have demonstrated that *A. orientalis* can parasitize both frozen (i.e., -40°C treatments for 1 or 24 hours) *L. delicatula* egg masses and the respective non-frozen control treatments in the same storage period category (i.e., <1, 4, 8 and 11 months at 5°C). Parasitism rates in the non-frozen egg masses averaged ~50%, which is consistent with the ~40% parasitism rate found by Broadley et al. (18). The egg masses in both treatment groups, -40°C for 1 hour and 24 hours, exhibited maximum parasitism rates of 100% and 89% for each treatment, respectively, indicating that -40°C treated eggs were suitable for *A. orientalis* parasitism. Further, -40°C treated egg masses did not affect *A. orientalis* offspring sex ratios when compared with non-frozen controls. These two results indicate that egg masses treated at -40°C did not have a significant effect on the oviposition behavior of female *A. orientalis*. Additional studies are needed to confirm if -40°C treatments can increase the long-term cold storage options for use of *L. delicatula* egg masses for colony maintenance and experiments with egg parasitoids.

Other researchers have similarly demonstrated that *Anastatus* spp. are able to parasitize frozen host eggs. For example, Haye et al. (24), showed that *Anastatus bifasciatus* (Geoffroy) was able to parasitize both fresh and frozen eggs of *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), an invasive agricultural pest. Additionally, Zhao et al. (25) demonstrated that *Anastatus fulloi* Sheng and Wang was able to parasitize (>80%) *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae) eggs that were stored at -5°C and -18°C for 6 to 12 months, respectively. Similarly, results reported here indicate that *A. orientalis* can parasitize *L. delicatula* egg masses that have been exposed to -40°C. Collectively, these examples suggest that frozen host eggs (i.e., Pentatomidae, Saturniidae and Fulgoridae) may not affect the acceptance behavior of foraging *Anastatus* spp. females. If this is correct, deployment of frozen *L. delicatula* egg masses could be used as sentinels to determine if the resident natural enemy fauna (e.g., parasitoids) in non-invaded areas are capable of successfully locating, parasitizing, and developing within *L. delicatula* eggs. Field studies of this kind may provide useful information on the levels of naturally occurring biotic resistance incipient *L. delicatula* populations could experience when invading new areas. Frozen sentinel egg masses could be deployed monthly during spring, summer and fall, for example, at study sites of interest to document levels of egg parasitoid activity and identities of species attacking frozen *L. delicatula* eggs.

The proportion of male *A. orientalis* that emerged from *L. delicatula* egg masses stored for 8 months at 5°C was significantly greater than that observed for egg masses stored for < 1 month, 4, or 11 months at 5°C. This result may have occurred because either the quality of *L. delicatula* eggs were affected by location and time of field collection or the nutritional value of the *L. delicatula* eggs stored for 8 months at 5°C was not optimal for *A. orientalis*. However, it seems unlikely that location and time of collection was a factor because egg masses used in all experiments reported on here were randomly selected from the same field locations and stored in the laboratory (5°C: 60% R.H.) under similar conditions. Sex allocation theory predicts the preferential placement of female eggs into higher quality host eggs and males into lower quality host eggs as a strategy to enhance the fitness of female offspring (26, 27). Accordingly, Zhao et al. (18) found that the percentage of female *A. fulloi* progeny decreased when ovipositing females were provided with *A. pernyi* eggs stored at -5°C to 3°C for 12 months when compared to fresh laid eggs. However, results reported here indicate that the female sex ratio of *A. orientalis* emerging from egg masses stored at 5°C for eleven months was not significantly different to the sex ratio of the egg masses stored for <1 month. Consequently, at this time, there appears to be no reasonable biological explanation as to why *L. delicatula* egg masses stored for eight months at 5°C in this study produced significantly more males especially when compared to egg masses that were stored for longer periods (i.e., 11 months at 5°C). Consequently, the low proportion of females that emerged from eggs stored at 5°C for eight months could be an artifact caused by males with low mating performance. If this assumption is correct, this could have resulted in female *A. orientalis* ovipositing fewer fertilized eggs when compared to females used in other treatments that mated with males with higher mating performance. Additionally, male parasitoids with low mating performance might also explain the low parasitism rates observed for egg masses stored for eight months, especially in the 24 hours at -40°C treatment, where significant differences in parasitism rates on egg masses from different storage periods were observed.

Hind tibia length, as a measure of body size, is used to estimate the fitness of parasitoids and the assumption is that larger individuals have longer tibia and are therefore likely to exhibit greater levels of fitness (26). Female *A. orientalis* that emerged from egg masses (i.e., cold stored at 5°C for 4 months) frozen for 1 hour at -40°C had significantly longer hind tibia lengths when compared to females that emerged from *L. delicatula* egg masses stored for four months that were either not frozen or frozen at -40° C for 24 hours. This result suggests that *L. delicatula* egg masses stored for four months at 5°C and then frozen at -40°C for 1 hour maybe more suitable for *A. orientalis* larval development than similarly aged egg masses that are stored at 5°C and not frozen or frozen at -40°C for 24 hours and then provided to ovipositing females. Similar studies on hemipteran (i.e., Pentatomidae) egg parasitoids (i.e. *Trissolcus* spp. [Hymenoptera: Platygastridae]) found that offspring that emerged from frozen (i.e., host eggs were held at -20°C or -80°C up to 4 years) host eggs had shorter hind tibia lengths when compare to offspring that emerged from non-frozen eggs (27, 28). Importantly, the positive effect of freezing four-month-old *L. delicatula* egg masses for 1 hour at -40°C on offspring size maybe the first time this effect has been demonstrated. However, further studies will be necessary to confirm and explain this potentially novel finding.

In conclusion, results presented here indicate that freezing *L. delicatula* egg masses of varying ages (i.e., eggs stored at 5°C for < 1 month, 4, 8, and 11 months) at -40°C for 1 or 24 hours results in 100% egg mortality, *A. orientalis* females can successfully parasitize eggs frozen at -40°C for 1 or 24 hours, and the fitness of offspring maybe enhanced if larvae develop in four month old egg masses that are frozen at -40°C for 1 hour. These results have significant practical applications. First this finding suggests that *L. delicatula* eggs exposed to -40°C for 1 to 24 hours is an effective and fast way to kill eggs making them amenable for safe removal from quarantine facilities. It may be possible to store egg masses at -40°C for considerable time periods without a loss in quality. If *L. delicatula* egg masses are not carefully managed, current storage practices (i.e., long-term storage at 5°C) can result in egg deterioration and mortality due to moisture related problems, especially the growth of saprophytic fungi (only egg masses that had no fungal contamination were used in these studies). It is possible that the quality, durability, and suitability of *L. delicatula* egg masses stored at -40°C for varying time periods for parasitism by *A. orientalis* could enhance long term storage options. However, this possibility needs experimental verification as storage at -40°C may inadvertently result in eggs of poor quality (e.g., desiccation) which could make them unusable. Finally, sentinel *L. delicatula* egg masses frozen at -40°C are killed which potentially allows for field deployment in non-invaded areas to proactively assess levels of parasitism and predation and possible identification of resident natural enemy species capable of attacking eggs in advance of an anticipated incursion by this pest.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FG-M conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. MH obtained the funding, authored or reviewed drafts of the paper, and approved the final draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2022.937129/full#supplementary-material

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Oviposition selection in spotted lanternfly: impact of habitat and substrate on egg mass size and hatchability

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Oviposition strategies adopted by insects (e.g., habitat selection, substrate preference, egg size, clutch size, structure, arrangement, parental care) are critical to the survival and development of their eggs. The impact of habitat and oviposition substrate on spotted lanternfly egg mass size and hatchability was studied in Pennsylvania through laboratory observations and field monitoring in 2019 and 2021. Eggs were arranged in single layers of 1–13 columns (1–18 eggs/column) on surfaces of various types of oviposition substrates, with the longest column(s) in the middle of the egg mass. Egg mass size was positively correlated with column number, with a mean of 26.6–35.1 (0–105) eggs/egg mass for different samples. Significant differences in egg mass size were observed between study sites, with larger egg masses found at Wertz (44.8), Sam Lewis (40.6), Pinnacle (39.1), Marsh Creek (37.9), Susquehannock (34.5), and Memorial Lake (33.3) and smaller egg masses at Nolde Forest (25.0), Gordon (24.4), and Antietam (21.0). Significant differences were also detected between types of oviposition substrates with smaller egg masses found on American hornbeam (22.7). In general, more (31.6%–48.0%) eggs hatched in the field compared with the laboratory (10.0%). Egg hatch success was positively correlated with egg mass size, with the highest rates recorded on American beech, American hophornbeam, black birch, black cherry, black locust, hackberry, Norway maple, red maple, and sweet cherry at Wertz, Marsh Creek, Memorial Lake, and Pinnacle. Potential (positive or negative) impacts of tree-of-heaven density, initial infestation, treatment history, and incubation conditions are discussed.

KEYWORDS

***Lycorma delicatula*, egg mass structure, hatch success, Fulgoridae, invasive species**

Introduction

Insect eggs are vulnerable to mortality factors such as parasitoids, predators, pathogens, and unfavorable weather conditions in the field (1). Eggshells, oviposition sites, maternal secretions, and other built-in defense mechanisms provide protection to eggs in various environments (1–4). As a life stage which cannot actively defend itself, the escaping strategies for insect eggs include parental care, sociality, concealment, and egg mass formation (1, 5). For example, thick spumaline coating protects egg masses of some caddisfly species from predation by *Orthotrichia armata* Wells (Trichoptera: Hydroptilidae) larvae (6), whereas egg-stacking and scale-casing are used by *Lymantria dispar* (L.) (Lepidoptera: Erebidae) and *Ochrogaster lunifer* Herrich-Schäffer (Lepidoptera: Notodontidae) to prevent inner layer eggs from being parasitized by certain species (7–10). Oviposition strategies have profound impacts on egg survival.

The spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), a univoltine pest of tree-of-heaven (*Ailanthus altissima* (Mill.) Swingle [Sapindales: Simaroubaceae]) from China (11), was introduced to Berks County, Pennsylvania, in 2014 (12, 13). It is currently found in 11 states from Massachusetts to Indiana in eastern United States (14). Egg masses are laid on various types of substrates (e.g., surfaces of trees, shrubs, vines, stones, fence posts, and other non-living materials) from mid-September to early November in Pennsylvania, with American beech (*Fagus grandifolia* Ehrh. [Fagales: Fagaceae]), black birch (*Betula lenta* L. [Fagales: Betulaceae]), black cherry (*Prunus serotina* Ehrh. [Rosales: Rosaceae]), grapes (*Vitis* spp. [Vitales: Vitaceae]), Norway maple (*Acer platanoides* L. [Sapindales: Sapindaceae]), red maple (*Acer rubrum* L. [Sapindales: Sapindaceae]), sweet cherry (*Prunus avium* L. [Rosales: Rosaceae]), tree-of-heaven, and tuliptree (*Liriodendron tulipifera* L. [Magnoliales: Magnoliaceae]) as favorites (15–17).

The size and hatch success of *L. delicatula* egg masses have been reported in its native range of Asia (11, 18–20). Limited observations on egg mass characteristics and comparative hatching in the laboratory and the field were also carried out in North America as parts of related studies (16, 21). However, systematic studies in egg mass structure, size, and hatchability are still lacking for the better understanding of their impacts on *L. delicatula* population dynamics in the field. It is hypothesized that habitat and substrate play an important role in the oviposition selection of *L. delicatula*. The objectives of this study were therefore to 1) understand the basic structure of *L. delicatula* egg masses, 2) examine the impact of habitat and oviposition substrate on egg mass size, and 3) compare egg hatch success in the laboratory and the field.

Materials and methods

Study sites

This study was carried out at 11 mixed hardwood sites (~0.5 ha) in Pennsylvania between 16 and 80 km South and West of the initial introduction in Berks County. See Table 1 for location, type, structure, tree-of-heaven density, year of infestation, and treatment history for each study site. Study sites were at least 5 km apart from each other except Gibraltar North and Gibraltar South which were on the opposite sides of the same mountain ridge. Site Antietam was used in both 2019 and 2021. The number of tree-of-heaven trees with a diameter at breast height (DBH) >5 cm was recorded in each study site at the start of the field work. Northern spicebush (*Lindera benzoin* L. [Laurales: Lauraceae]) and summer grape (*Vitis aestivalis* Michx. [Vitales: Vitaceae]) were the most common understory species at all study sites.

Egg mass collection

Egg mass collection was carried out at six study sites (Antietam, Gibraltar North, Gibraltar South, Nolde Forest, Marsh Creek, and Wertz) in late April 2019. *Lycorma delicatula* egg masses are dark gray in color at the beginning (Figure 1A) and turn grayish white the next spring (Figure 1B). At each study site, the surfaces of live trees, shrubs, and vines were searched for *L. delicatula* egg masses. Egg masses found on the lower 2-m trunk of the tree (shrub/vine) were collected using a 1.27-cm bench chisel (Buck Brothers, Everett, WA). A rectangle was created first to surround the egg mass by cutting directly into the bark at 0.5 cm away from its outer margins. The egg mass on the surface was then dislodged by gently pushing the chisel under the bark rectangle upward from the lower end. Care was taken to ensure no eggs were accidentally missed, cut, squeezed, or otherwise damaged. Each dislodged egg mass was then held in a 50-ml centrifuge tube (VWR International, Radnor, PA) and labeled by collection date, study site, and type of oviposition substrate before being brought back to the laboratory for examination and incubation.

Egg mass marking in the field

Egg mass marking was performed at Antietam, Gibraltar North, Gibraltar South, Nolde Forest, Marsh Creek, and Wertz in 2019 and Antietam, Gordon, Memorial Lake, Pinnacle, Sam Lewis, and Susquehannock in 2021. Current generation egg masses found on the lower 2-m trunk of different types of oviposition substrates near the epicenter at each study site were circled with a yellow timber crayon (Dixon Ticonderoga, Heathrow, FL) in late April for monitoring.

Egg mass structure

Lycorma delicatula eggs are cylindrical with a diameter of 1.5 mm and height of 3.0 mm. They are usually laid in single

TABLE 1 Study site location, type, structure, tree-of-heaven density, year of infestation, and treatment history in 2019 and 2021.

Name	Latitude Longitude	Type	Structure	Tree-of-heaven density[a]	Year of infestation	Chemical treatment[b]	Herbicide treatment[c]
2019							
Antietam	40.35086 -75.87749	County park	South-facing upper slope dominated by mature black birch and black cherry	Low	2018	No	No
Gibraltar North	40.28670 -75.88703	State forest	North-facing middle slope dominated by mature black birch and tuliptree	Low	2018	Yes	Yes
Gibraltar South	40.28697 -75.89678	State forest	South-facing middle slope dominated by mature black birch and American beech	Low	2018	No	No
Marsh Creek	40.06542 -75.73059	State park	Level lakeside dominated by mature Norway maple and black cherry	High	2018	No	No
Nolde Forest	40.27140 -75.94782	State park	East-facing lower slope dominated by mature black locust and young black cherry	Low	2018	Yes	Yes
Wertz	40.31869 -76.11358	State forest	East-facing upper slope dominated by mature black birch and American beech	Medium	2018	No	No
2021							
Antietam	40.35086 -75.87749	County park	South-facing upper slope dominated by mature black birch and black cherry	Low	2018	No	No
Gordon	39.93546 -75.59956	Nature area	West-facing upper slope site dominated by mature red maple and white ash	Low	2018	No	No
Memorial Lake	40.41705 -76.59359	State park	Level lakeside dominated by young red maple and white ash	High	2020	No	No
Pinnacle	39.84534 -76.34342	State park	West-facing upper slope dominated by young black birch and red maple	Medium	2020	No	Yes
Sam Lewis	39.99316 -76.54415	State park	East-facing middle slope dominated by mature eastern white pine and red maple	Medium	2020	No	No
Susquehannock	39.80538 -76.28144	State park	East-facing upper slope dominated by mature hackberry and young pawpaw	Medium	2020	No	Yes

[a]By total number of tree-of-heaven trees (>5 cm in diameter) on site—low: <20, medium: 21~50, high: >50.
[b]Dinotefuran trunk spray on tree-of-heaven for L. delicatula control in adjacent areas in 2018.
[c]Triclopyr or glyphosate trunk hack-and-squirt for tree-of-heaven control in adjacent areas in 2018 (Gibraltar North and Nolde Forest) and 2020 (Pinnacle and Susquehannock).

layer masses with 5–10 columns (10–30 eggs/column) and covered by a layer of gray wax in Asia (11). To characterize the structure of the *L. delicatula* egg masses in North America, column number (Figure 1C) was recorded from left to right for egg masses collected in 2019 and those marked in the field in 2021, whereas egg number was counted for all egg masses (collected and marked) before hatch. All egg masses were counted again within 2 weeks after hatch completed to ensure accuracy. Direct count was possible for egg masses with little or no waxy cover on the top; however, for those with a thick waxy cover, wax removal with a #2 camel hairbrush (Grumbacher, Leeds, MA) was needed for exact enumeration. This procedure was only carried out after hatch when necessary to avoid potential influence on hatch success. The numbers of eggs and numbers of columns for each egg mass were categorized at intervals of 10 and 1, respectively. Size category, column category, and column size (no. eggs/column) were used in data analysis. Travel restrictions stemming from the COVID-19 pandemic forced cancellations of scheduled field works in 2020 and early 2021.

Egg hatch in the laboratory

Egg masses collected in 2019 were brought back to the laboratory for incubation inside a Percival incubator (model #DR-36VL, Percival Scientific, Perry, IA) at 22 ± 1°C, 40 ± 5% relative humidity (RH), and a 16:8-h photoperiod (light: dark) for 8 weeks (16). Egg mass and egg hatch success were monitored with the number of newly hatched nymphs recorded and removed weekly. Egg mass hatch success was calculated by dividing the number of egg masses with at least one hatched egg by the total number of egg masses at the study site, whereas egg hatch success was calculated by dividing the number of hatched eggs by the total number of eggs in the egg mass.

Egg hatch in the field

Egg hatch in the field was monitored weekly on the marked egg masses for 8 weeks from mid-May to early July in 2019 and 2021. Presence of white 1st-instar nymphs or open egg lids

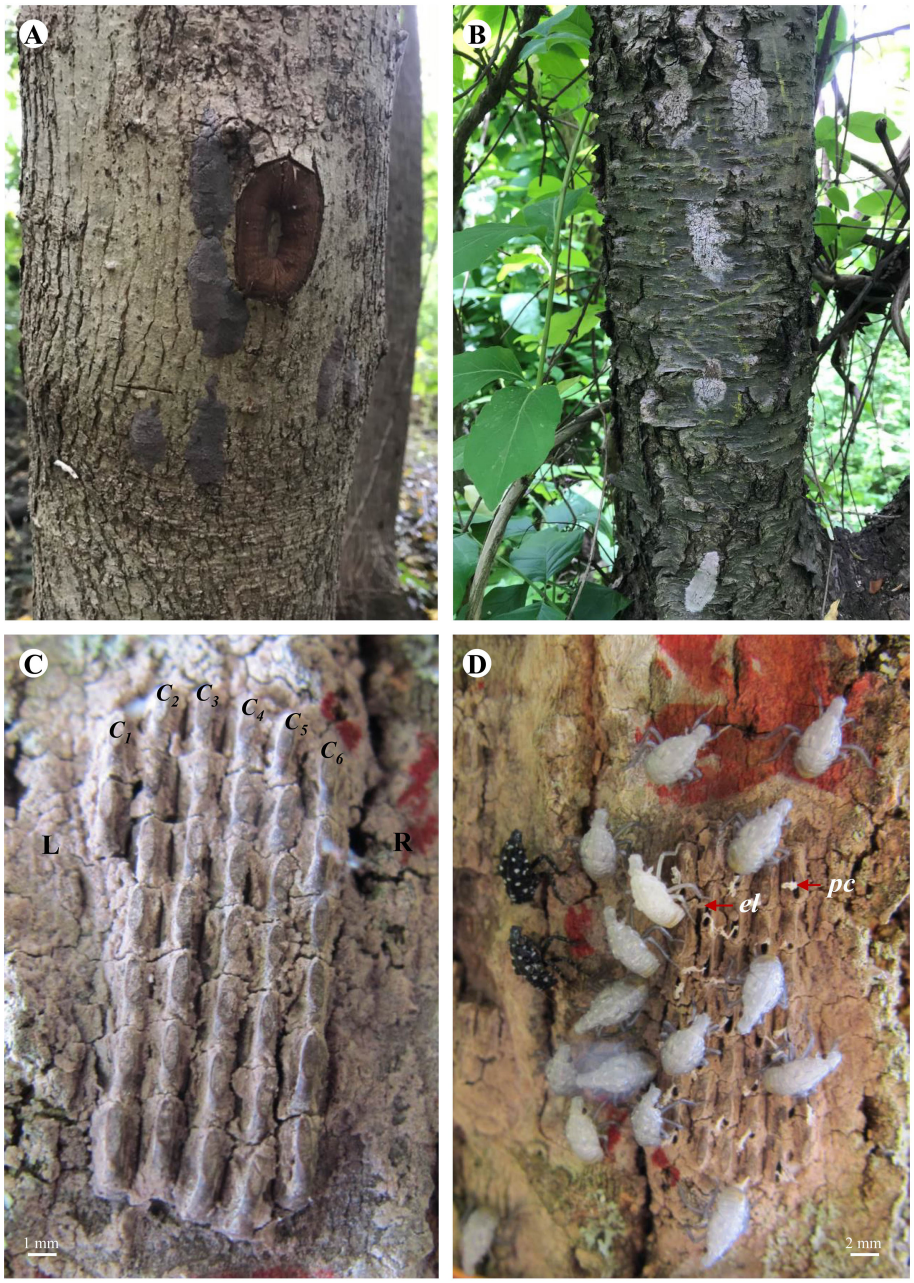

FIGURE 1
Lycorma delicatula egg masses in the field. **(A)** Newly laid on red maple. **(B)** Overwintered on black cherry. **(C)** Arrangement. **(D)** Hatching. L—left, R—right, C_1—column #1, C_2—column #2, C_3—column #3, C_4—column #4, C_5—column #5, C_6—column #6, *el*—egg lid, *pc*—pronymphal cuticle.

(opercula) and attached whitish/yellowish pronymphal cuticles (Figure 1D) indicates hatch success (20, 22). Hatch success was calculated as described before.

Data analysis

Data analysis was carried out in R (Version 3.3.3) (23). Egg counts per egg mass for field-collected and marked eggs in both years were subjected to Shapiro–Wilk normality test before analysis. If data were overdispersed, a negative binomial generalized linear (nbGLM in R) was used to detect the effect of study site or type of oviposition substrate. Kruskal–Wallis test was used to separate different columns based on eggs per column for egg masses collected or marked in the field. Significant effects were followed by pairwise Wilcoxon rank-sum test with a *P*-value adjusted by Benjamini–Hochberg method (24). A generalized linear model

(GLM) with binomial distribution was used to examine the frequency of size category, frequency of column category, correlation between egg hatch success and egg mass size, or effect of study site or type of oviposition substrate on egg hatch success in each year. A generalized linear model was used to fit egg number with column number in each year.

Results

In total, 300 egg masses were collected from 19 types of oviposition substrates at six study sites (50 egg masses/study site) in 2019, with most found on black birch, red maple, Norway maple, sweet cherry, tuliptree, black locust (*Robinia pseudoacacia* L. [Fables: Fabaceae]), tree-of-heaven, and black cherry (Table 2). Egg masses were also found on red oak (*Quercus rubra* L. [Fagales: Fagaceae]), black walnut (*Juglans nigra* L. [Fagales: Juglandaceae]), black willow (*Salix nigra* Marshall [Malpighiales: Salicaceae]), American hophornbeam (*Ostrya virginiana* (Mill.) K. Koch [Fagales: Betulaceae]), autumn olive (*Elaeagnus umbellata* Thunb. [Rosales: Elaeagnaceae]), northern spicebush, princess tree (*Paulownia tomentosa* (Thunb.) Steud [Lamiales: Paulowniaceae]), American beech, sassafras (*Sassafras albidum* (Nutt.) Nees [Laurales: Lauraceae]), shagbark hickory (*Carya ovata* (Mill.) K. Koch [Fagales: Juglandaceae]), and summer grape (Table 2).

In addition, 212 egg masses were marked in the field, including 120 egg masses on four types of oviposition substrates at six study sites (20 egg masses/study site, two types of oviposition substrates/study site, five plants/type of oviposition substrate, two egg masses/plant) in 2019, and 92 egg masses on 10 types of oviposition substrates at six study sites (4–20 egg masses/study site, 1–4 types of oviposition substrates/study site, 1–5 plants/type of oviposition substrate, 1–5 egg masses/plant) in 2021 (Table 3). The low population density at Antietam and Gordon in 2021 prevented more egg masses from being marked at those study sites. Tree-of-heaven was represented at all study sites in both years except Gordon in 2021, whereas black birch, black locust, and Norway maple were used in 2019, and American hornbeam (*Caprinus caroliniana* Walter [Fagales: Betulaceae]), black birch, black cherry, boxelder (*Acer negundo* L. [Sapindales: Sapindaceae]), hackberry (*Celtis occidentalis* L. [Rosales: Cannabaceae]), pawpaw (*Asimina triloba* (L.) Dunal [Magnoliales: Annonaceae]), red maple, shagbark hickory, and white ash (*Fraxinus americana* L. [Lamiales: Oleaceae]) were used in 2021 (Table 3).

Egg mass structure

Number of eggs in each *L. delicatula* egg mass ranged from 0 to 105 in Pennsylvania based on 300 egg masses collected from the field in 2019 (Figure 2A). Significant differences in frequency were observed among different size categories (Z-value = -11.820, $P < 0.001$). Three egg masses (one from Gibraltar North and two from Nolde Forest) contained no eggs. Most egg masses (91.3%) contained <50 eggs, 75.6% had 20–50 eggs, and 15.7% had <20 eggs/egg mass (Figure 2A). Only 1 egg mass had >100 eggs while 2 had >90, 8 with >70, 7 had >60, and 8 had >50 eggs (Figure 2A). Significant differences in frequency were also observed among different column categories (Z-value = -7.141, $P < 0.001$). Eggs were arranged in 1–13 columns within the egg masses. Most egg masses (88%) contained <7 columns while 77% had 3–7 columns and 11% had <3 columns (Figure 2B). One egg mass had 13 columns while 3 had 12, 2 had 11, 6 had 10, 10 had 9, and 14 had 8 columns (Figure 2B). In total, 10,115 eggs arranged in 1,644 columns were recorded from 297 egg masses with at least 1 egg, including 1,601 eggs (270 columns) at Antietam, 1,447 eggs (239 columns) at Gibraltar North, 1,683 eggs (288 columns) at Gibraltar South, 1,249 eggs (225 columns) at Nolde Forest, 1,893 eggs (286 columns) at Marsh Creek, and 2,242 eggs (336 columns) at Wertz. Column size ranged from 1 to 18 with a mean of 6.2 ± 2.6 eggs/column and differed significantly between columns ($\chi^2 = 179.610$, df = 12, $P < 0.001$). Significant differences were found between columns 2 and 1, 7; 3 and 1, 5, 6, 7, 8, 9; and 4 and 1, 6, 7, 8, 9 (Figure 2C). A significant positive correlation was found between column number and egg number for the egg masses (F = 676.700, df = 1, 298, $P < 0.001$) (Figure 2D), with total eggs in each egg mass increasing with the increase of columns in it.

Lycorma delicatula egg mass size ranged from 4 to 66 in Pennsylvania based on 92 egg masses marked in the field in 2021 (Figure 3A). No significant difference in frequency was observed among different size categories (Z-value = -0.448, $P = 0.654$). Most egg masses (93.5%) contained <50 eggs, 81.5% had 20–50 eggs, and 12.0% had <20 eggs/egg mass (Figure 3A). Only one egg mass contained >60 eggs while five had >50 eggs (Figure 3A). No significant difference in frequency was observed among different column categories either (Z-value = 0.932, $P = 0.351$). Eggs were arranged in 1–10 columns within the egg masses. Most egg masses (90.2%) contained <7 columns, while no egg masses contained <2 columns (Figure 3B). One egg mass had 10 columns while 3 had 9 and 5 had 8 columns (Figure 3B). In total, 3,288 eggs in 535 columns were counted from 92 egg masses, including 84 eggs (16 columns) at Antietam, 195 eggs (39 columns) at Gordon, 666 eggs (108 columns) at Memorial Lake, 781 eggs (132 columns) at Pinnacle, 690 eggs (118 columns) at Sam Lewis, and 812 eggs (122 columns) at Susquehannock. Column size ranged from 1 to 11 with a mean of 6.0 ± 2.4 eggs/column and differed significantly between columns ($\chi^2 = 111.380$, df = 9, $P < 0.001$). Significant differences were found between columns 2 and 1, 7, 8; 3 and 1, 6, 7, 8; and 4 and 1, 6, 7, 8 (Figure 3C). A significant positive correlation was found between column number and egg number for the egg masses (F = 84.820, df = 1, 90, $P < 0.001$) (Figure 3D), with total eggs in each egg mass increasing with the increase of columns in it.

Egg mass size

The mean (± SD) egg mass size was 33.7 ± 16.0 eggs/egg mass for the 300 egg masses collected in 2019. Significantly

TABLE 2 *Lycorma delicatula* egg mass collection by study site and oviposition substrate in 2019.

Substrate	Code	AT	GN	GS	MC	NF	WZ	Sub
Acer platanoides	Nm	1			32			33
Acer rubrum	Rm	4	2		18	6	25	55
Ailanthus altissima	Toh		4	4		4		12
Betula lenta	Bb	43	29	30				102
Carya ovata	Shag		1					1
Elaeagnus umbellata	Ao					4		4
Fagus grandifolia	Ab			1				1
Juglans nigra	Bw		3			4		7
Lindera benzoin	Spb			2				2
Liriodendron tulipifera	Tt		2	6		6		14
Ostrya virginiana	Hhb						4	4
Paulownia tomentosa	Pau		2					2
Prunus avium	Sc	1					21	22
Prunus serotina	Bc		3	1		6		10
Quercus rubra	Ro		3	6				9
Robinia pseudoacacia	Bl					13		13
Salix nigra	Wil					7		7
Sassafras albidum	Sas	1						1
Vitis aestivalis	Grp		1					1
Total		50	50	50	50	50	50	**300**

AT, Antietam; GN, Gibraltar North; GS, Gibraltar South; MC, Marsh Creek; NF, Nolde Forest; WZ, Wertz; Ab, American beech; Ao, autumn olive; Bb, black birch; Bc, black cherry; Bl, black locust; Bw, black walnut; Grp, summer grape; Hhb, American hophornbeam; Nm, Norway maple; Pau, princess tree; Rm, red maple; Ro, red oak; Sas, Sassafras; Sc, sweet cherry; Shag, Shagbark hickory; Spb, northern spicebush; Toh, tree-of-heaven; Tt, tuliptree; Wil, black willow.

larger egg masses were found at Wertz (Z-value = 3.624, $P < 0.001$) and smaller ones at Nolde Forest (Z-value = -2.619, $P = 0.009$) (Figure 4A). No significant difference in egg mass size was found between different types of oviposition substrates ($\alpha = 0.05$) (Figure 4A).

The mean (± SD) egg mass size was 26.6 ± 14.5 eggs/egg mass for the 120 egg masses marked in the field in 2019. Significantly larger egg masses were found at Marsh Creek (Z-value = 2.304, $P = 0.021$). No significant difference in egg mass

TABLE 3 *Lycorma delicatula* egg mass marking in the field by year, study site, and oviposition substrate.

Substrate	Code	2019							2021						
		AT	GN	GS	MC	NF	WZ	Sub	AT	GD	ML	PC	SL	SQ	Sub
Acer negundo	Box													5	5
Acer platanoides	Nm				10			10							
Acer rubrum	Rm										5	5	5		15
Ailanthus altissima	Toh	10	10	10	10	10	10	60	4		5	5	5	5	24
Asimina triloba	Paw												5	5	10
Betula lenta	Bb	10	10	10			10	40				5			5
Carpinus caroliniana	Ahb									3					3
Carya ovata	Shag									5					5
Celtis occidentalis	Hack													5	5
Fraxinus americana	Wash										5				5
Prunus serotina	Bc										5	5	5		15
Robinia pseudoacacia	Bl					10		10							
Total		20	20	20	20	20	20	**120**	4	8	20	20	20	20	**92**

AT, Antietam; GD, Gordon; GN, Gibraltar North; GS, Gibraltar South; MC, Marsh Creek; ML, Memorial Lake; NF, Nolde Forest; PC, Pinnacle; SL, Sam Lewis; SQ, Susquehannock; WZ, Wertz; Ahb, American hornbeam; Bb, black birch; Bc, black cherry; Bl, black locust; Box, boxelder; Hack, hackberry; Nm, Norway maple; Paw, pawpaw; Rm, red maple; Shag, shagbark hickory; Toh, tree-of-heaven; Wash, white ash.

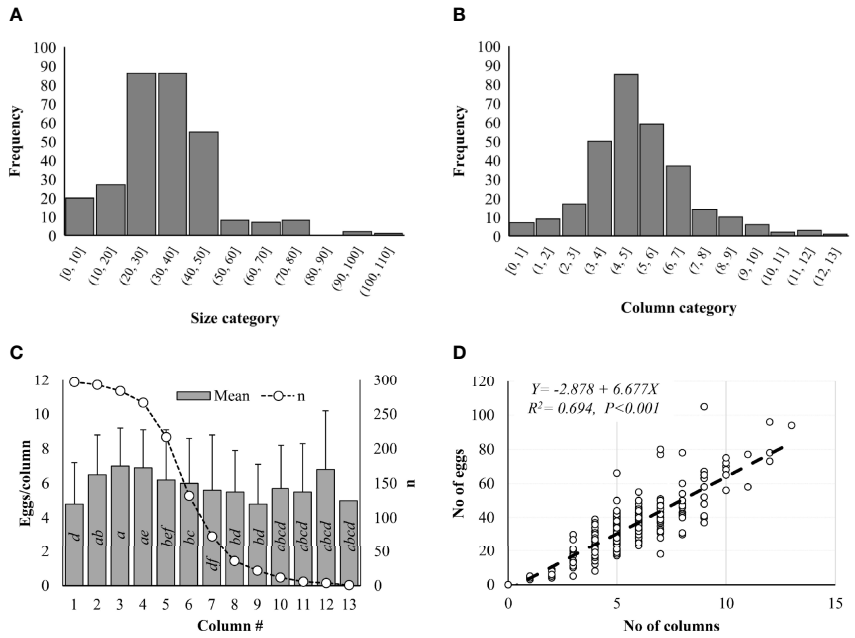

FIGURE 2
Lycorma delicatula egg mass structure in 2019. **(A)** Size category. **(B)** Column category. **(C)** Mean egg number per column. **(D)** Correlation between egg number and column number as indicated by the dash line. Means with the same lowercase letters are not significantly different (Wilcoxon rank-sum test, $\alpha = 0.05$).

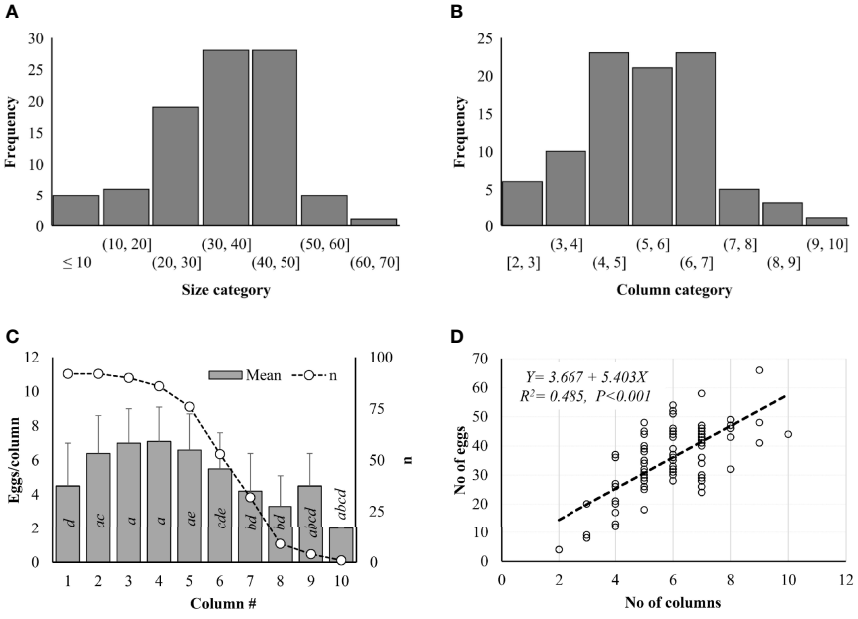

FIGURE 3
Lycorma delicatula egg mass structure in 2021. **(A)** Size category. **(B)** Column category. **(C)** Mean egg number per column. **D)** Correlation between egg number and column number as indicated by the dash line. Means with the same lowercase letters are not significantly different (Wilcoxon rank-sum test, $\alpha = 0.05$).

size was found between different types of oviposition substrates ($\alpha = 0.05$) (Figure 5A).

The mean (± SD) egg mass size was 35.1 ± 11.9 eggs/egg mass for the 92 egg masses marked in the field in 2021. Significantly larger egg masses were found at Sam Lewis (Z-value = 3.275, $P = 0.001$) and Pinnacle (Z-value = 3.080, $P = 0.002$). Larger egg masses were also found at Susquehannock (Z-value = 2.460, $P = 0.014$) and Memorial Lake (Z-value = 2.283, $P = 0.022$) (Figure 5B). Significantly smaller egg masses were found on American hornbeam (Z-value = -2.023, $P = 0.043$) compared with other types of oviposition substrates (Figure 5B).

Egg hatch in the laboratory

The three egg masses with no eggs were excluded from laboratory hatch study. Egg hatch started within 2 weeks after incubation and lasted for about 2 weeks for individual egg masses, with peak hatch occurring in the middle of the period. Only 39.7% of the egg masses contained at least one hatched egg in 2019, with the highest egg mass hatch success of 86% at Wertz, followed by Marsh Creek (64%), Gibraltar South (42%), Antietam (28%), Gibraltar North (14.3%), and Nolde Forest (2.1%). The mean (± SD) egg hatch success was 10.0 ± 18.5 (0–90)% for the egg masses, with a positive correlation between hatch success and egg mass size (Z-value = 15.880, $P < 0.001$).

Significantly higher egg hatch success was found at Wertz (Z-value = 14.967, $P < 0.001$) and Marsh Creek (Z-value = 10.856, $P < 0.001$) while significantly lower hatch success was found at Nolde Forest (Z-value = -5.801, $P < 0.001$) and Gibraltar North (Z-value = -2.063, $P = 0.039$) (Figure 4B).

Significant differences in egg hatch success were also observed between types of oviposition substrates, with lower rates on red oak (Z-value = -4.353, $P<0.001$), black cherry (Z-value = -4.262, $P < 0.001$), tuliptree (Z-value = -4.085, $P < 0.001$), black willow (Z-value = -3.852, $P < 0.001$), black birch (Z-value = -2.627, $P = 0.009$), tree-of-heaven (Z-value = -2.094, $P = 0.036$), and northern spicebush (Z-value = -1.993, $P = 0.046$) (Figure 4B). No eggs on autumn olive, black locust, black walnut, princess tree, sassafras, shagbark hickory, and summer grape hatched successfully (Figure 4B).

Egg hatch in the field 2019

In 2019, *L. delicatula* egg hatch was first observed at Marsh Creek on 21 May, followed by Gibraltar South, Nolde Forest, Wertz, Gibraltar North, and Antietam in the following days in the field. Hatch generally completed within 2–3 weeks for individual egg masses, with the last egg hatch observed on 1 July at Nolde Forest. Overall, 69.2% of the egg masses contained at least one hatched egg, with the highest egg mass hatch success of 90% at Marsh Creek, followed by Gibraltar South (75%), Gibraltar North (70%), Nolde

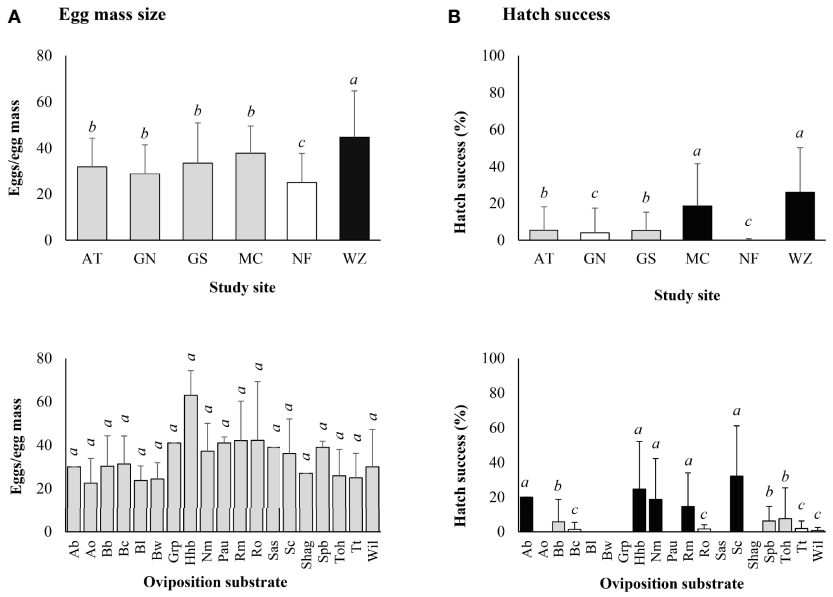

FIGURE 4
Lycorma delicatula. **(A)** Egg mass size and **(B)** egg hatch success by study site and type of oviposition substrate based on laboratory observations in 2019. AT—Antietam, GN—Gibraltar North, GS—Gibraltar South, MC—Marsh Creek, NF—Nolde Forest, WZ—Wertz. Ab—American beech, Ao—autumn olive, Bb—black birch, Bc—black cherry, Bl—black locust, Bw—black walnut, Grp—summer grape, Hhb—American hophornbeam, Nm—Norway maple, Pau—princess tree, Rm—red maple, Ro—red oak, Sas—Sassafras, Sc—sweet cherry, Shag—shagbark hickory, Spb—northern spicebush, Toh—tree-of-heaven, Tt—tuliptree, Wil—black willow. Means with the same lowercase letters are not significantly different (A-negative binomial generalized model, B-generalized linear model with binomial distribution, $\alpha = 0.05$).

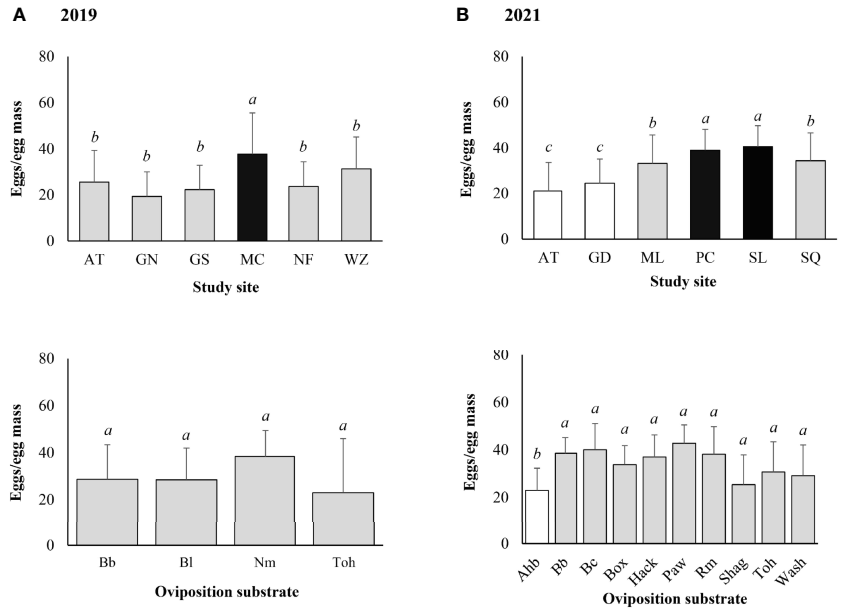

FIGURE 5
Lycorma delicatula egg mass size by study site and type of oviposition substrate based on field monitoring in **(A)** 2019 and **(B)** 2021. AT—Antietam, GD—Gordon, GN—Gibraltar North, GS—Gibraltar South, MC—Marsh Creek, ML—Memorial Lake, NF—Nolde Forest, PC—Pinnacle, SL—Sam Lewis, SQ—Susquehannock, WZ—Wertz. Ahb—American hornbeam, Bb—black birch, Bc—black cherry, Bl—black locust, Box—boxelder, Hack—hackberry, Nm—Norway maple, Paw—pawpaw, Rm—red maple, Shag—shagbark hickory, Toh—tree-of-heaven, Wash—white ash. Means with the same lowercase letters are not significantly different (negative binomial generalized model, $\alpha = 0.05$).

Forest (70%), Antietam (55%), and Wertz (55%). The mean (± SD) egg hatch success was 31.6 ± 30.9 (0–100)% for the egg masses, with a positive correlation between hatch success and egg mass size (Z-value = 4.478, $P < 0.001$).

Significantly higher egg hatch success was found at Wertz (Z-value = 8.447, $P < 0.001$), Nolde Forest (Z-value = 5.220, $P < 0.001$), Marsh Creek (Z-value = 3.784, $P < 0.001$), and Gibraltar South (Z-value = 2.374, $P = 0.018$). Significantly lower egg hatch success was found on tree-of-heaven (Z-value = - 8.187, $P < 0.001$) compared with other types of oviposition substrates (Figure 6A).

Egg hatch in the field 2021

Lycorma delicatula egg hatch in the field in 2021 was first observed at Pinnacle and Susquehannock on 24 May, followed by Memorial Lake, Sam Lewis, Antietam, and Gordon. Hatch generally completed within 2 weeks for individual egg masses, with the last egg hatch observed on 24 June at Gordon. Overall, 69.6% of the egg masses contained at least one hatched egg, with the highest egg mass hatch success of 80% at Pinnacle, followed by Antietam (75%), Memorial Lake (75%), Sam Lewis (75%), Gordon (62.5%), and Susquehannock (50%). The mean (± SD) egg hatch success was 48.0 ± 38.7 (0–100)% for the egg masses, with a positive correlation between hatch success and egg mass size (Z-value = 3.672, $P < 0.001$).

Significantly higher egg hatch success was found at Memorial Lake (Z-value = 4.277, $P < 0.001$) and Pinnacle (Z-value = 2.962, $P = 0.003$). Significantly lower hatch success was found on pawpaw (Z-value = -11.296, $P < 0.001$), white ash (Z-value = -7.417, $P < 0.001$), shagbark hickory (Z-value = -3.161, $P = 0.002$), boxelder (Z-value = -3.151, $P = 0.002$), and tree-of-heaven (Z-value = -2.425, $P = 0.015$) compared with other types of oviposition substrates (Figure 6B).

Discussion

While oviposition substrate played an important role in *L. delicatula* egg mass structure and hatchability (16), more focus should probably be on habitat structure as those with more tree-of-heaven trees generally supported larger egg masses with more successful egg hatch (Table 1, Figures 4–6). This kind of oviposition selection can be explained by the proximity to suitable habitat for offspring hypothesis (25). The ability to feed on a wild range of hosts and to disperse freely between different host species (16, 26) makes young *L. delicatula* nymphs nearly independent of the oviposition substrates, rendering the preference–performance hypothesis (27) unlikely as long as tree-of-heaven is available in the habitat for necessary nutrition acquisition and defense sequestration (28). On the other hand, optimal foraging theory (29) should also be explored to shed

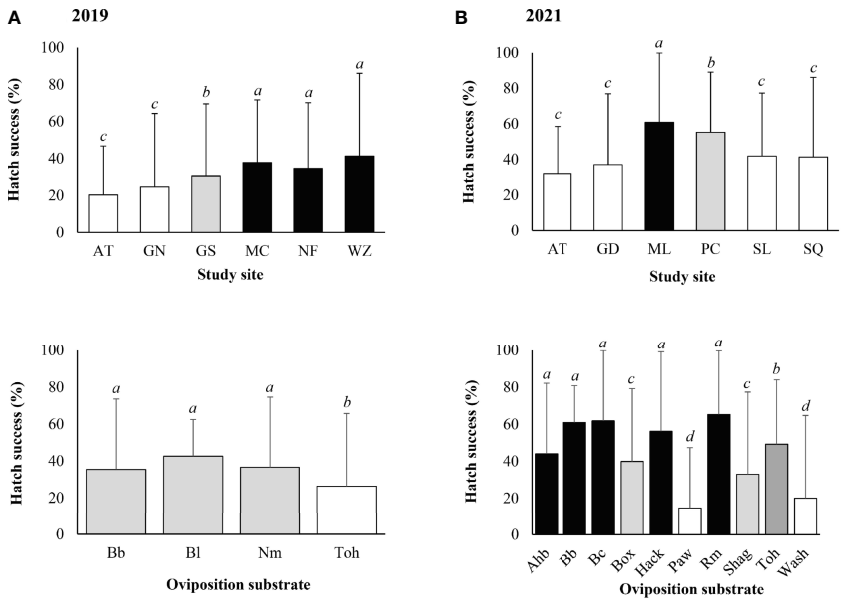

FIGURE 6
Lycorma delicatula egg hatch success by study site and type of oviposition substrate based on field monitoring in **(A)** 2019, **(B)** 2021. AT—Antietam, GD—Gordon, GN—Gibraltar North, GS—Gibraltar South, MC—Marsh Creek, ML—Memorial Lake, NF—Nolde Forest, PC—Pinnacle, SL—Sam Lewis, SQ—Susquehannock, WZ—Wertz. Ahb—American hornbeam, Bb—black birch, Bc—black cherry, Bl—black locust, Box—boxelder, Hack—hackberry, Nm—Norway maple, Paw—pawpaw, Rm—red maple, Shag—Shagbark hickory, Toh—tree-of-heaven, Wash—white ash. Means with the same lowercase letters are not significantly different (generalized linear model with binomial distribution, $\alpha = 0.05$).

light on the selective patterns on some tree species (e.g., maples) by late-stage adults as both feeding hosts and oviposition substrates (30, 31). Impacts of habitat and oviposition substrate on egg mass size have also been reported for parallel-banded leafroller moth (*Choristoneura parallela* (Robinson) [Lepidoptera: Tortricidae]) (32) and beet armyworm (*Spodoptera exigua* (Hübner) [Lepidoptera: Noctuidae]) (33).

Empty egg masses have been recorded for *L. delicatula* in a previous study (16). It is not yet clear how females decide to place a certain number of eggs in each column in the egg mass, and why no eggs are laid under the waxy cover in a few of them. In addition to low tree-of-heaven density in the habitats, chemical control of *L. delicatula* on tree-of-heaven in adjacent areas in the previous year might have a negative impact on the mean egg mass size at Nolde Forest in 2019, whereas a longer infestation history could have contributed to the smaller egg masses observed at Antietam and Gordon in 2021 (Table 1, Figures 4, 5). However, the potential impact of herbicide treatment of tree-of-heaven in adjacent areas at Pinnacle and Susquehannock in 2021 still needs to be examined (Table 1, Figure 5). The largest egg mass ever recorded contained 192 eggs (16). Comparable egg mass sizes (30–50 eggs/egg mass) were also reported before (11, 13, 16, 20, 34).

In general, egg hatch was less successful in the laboratory compared with that in the field (Figures 4, 6). However, this may change as laboratory rearing conditions improve in the near future. An egg hatch success of 20.5% was reported in the laboratory compared with 68.2% observed in the field in 2017 (16). In another study, 65.9% egg masses and 58.4% eggs hatched successfully at 15°C in the laboratory (21). On the other hand, egg hatch success dropped to 10.8% when held at 20°C constantly (21). A higher relative humidity and lower than 20°C incubation temperature in the laboratory may be needed to simulate field conditions in late May in southeastern Pennsylvania.

A difference in egg hatch success on different types of oviposition substrates has been reported before. About 80% of eggs on tree-of-heaven hatched whereas only 2%–3% of eggs on Japanese pagoda tree (*Styphnolobium japonicum* (L.) Schott [Fabales: Fabaceae]) and elms (*Ulmus* spp. [Rosales: Ulmaceae]) hatched successfully in the field in China (11). On the contrary, only 23% of eggs from tree-of-heaven hatched, whereas 79.6% of eggs from black locust hatched after 2 months of incubation in the laboratory in the United States (16). In Japan, egg hatch success was significantly reduced when wax cover was removed from the surface in the field (20). Oviposition substrates, waxy cover, collection disturbance, incubation conditions, and number of egg masses evaluated all contributed to the reported egg hatch success in the laboratory and the field (11, 16, 18–21).

Oviposition is a critical aspect of the reproductive biology for insects. The decision of when and where and the process of how

to lay the eggs have profound impact on the fitness of the species (35). Habitat structure, site conditions, host availability and quality, inter- and intraspecific competition, parasitoids, and predators all play a role in oviposition site selection (1, 4, 36–41). Reproductive success also depends on optimal allocation of available resources by females toward quantity (large clutch size) or quality (large eggs) (42). *Lycorma delicatula* eggs are relatively well protected from adverse abiotic conditions with thick eggshells and wax cover. No predators rely solely on them while only two species of parasitoids are recorded in the field (43–45). Habitat suitability, tree-of-heaven density, substrate conditions, and intraspecific competition should be the most important factors in oviposition selection for *L. delicatula*.

Information from this study is beneficial to the understanding of *L. delicatula* population dynamics and its management in the field in North America. Infestations usually start in suitable habitats with tree-of-heaven trees (11). Egg masses are mostly found on tree-of-heaven and a few neighboring species in the habitats at the beginning (16). Onsite chemical control of *L. delicatula*, herbicide treatment of tree-of-heaven, and fungal epizootics could interfere with egg mass size and hatchability. Management strategies should therefore focus on newly infested tree-of-heaven trees with egg mass survey extended to preferred substrates in the habitats. Hatch success in the field should be used to evaluate current-generation nymphal populations since those measured in the laboratory were generally lower and more variable depending on incubation conditions. The impact of other key factors (tree-of-heaven health, climatic conditions, natural enemies, and management activities) in *L. delicatula* population dynamics based on egg mass evaluation should be investigated.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

The author conceptualized the study; conducted the field work; collected and analyzed the data; wrote the draft; and reviewed and edited the final version of the manuscript.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Deciphering genome-wide transcriptomic changes in grapevines heavily infested by spotted lanternflies

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The spotted lanternfly, a newly invasive insect in the U.S. that is a great concern for the grapevine industry, produces damage on its host plants through aggressive feeding, using a piercing and sucking method to feed on the phloem of plants. In the eastern US, adult SLF can invade vineyards through fruit ripening until the end of the growing season; however, it is still unclear how prolonged late-season SLF feeding can affect the health of grapevines, as well as the host responses to this extensive damage. Thus, we have performed a comprehensive genome-wide transcriptome analysis in grapevines heavily infested by the spotted lanternfly, as it occurs in Pennsylvania vineyards, and compared it to other relevant transcriptomes in grapes with different degrees to susceptibility to similar pests. Among a variety of plant responses, we highlight here a subset of relevant biological pathways that distinguish or are common to the spotted lanternfly and other phloem feeders in grapevine. The molecular interaction between spotted lanternfly and the vine begins with activation of signal transduction cascades mediated mainly by protein kinase genes. It also induces the expression of transcription factors in the nucleus, of other signaling molecules like phytohormones and secondary metabolites, and their downstream target genes responsible for defense and physiological functions, such as detoxification and photosynthesis. Grapevine responses furthermore include the activation of genes for cell wall strengthening *via* biosynthesis of major structural components. With this study, we hope to provide the regulatory network to explain effects that the invasive spotted lanternfly has on grapevine health with the goal to improve its susceptibility.

KEYWORDS

transcriptome, plant-insect interactions, invasive species, spotted lanternfly, grapevine

1 Introduction

The spotted lanternfly (SLF), *Lycorma delicatula* (White), is a newly invasive insect of the U.S (1). Native to Asia, the first report of SLF being found in the United States was in 2014 where it was discovered in Berks County, Pennsylvania (2). The insect quickly dispersed to multiple counties across Pennsylvania, and has now invaded New Jersey, Maryland, Delaware, Virginia, and West Virginia, with individual sightings reported in further surrounding states (1). While in its native range the insect does not represent a pest species, in the U.S. SLF has the potential to become a greater threat, because it is a generalist, a robust phloem feeder, it lacks natural enemies, and thus can reach high populations, in the hundreds, on single plants, if not controlled by insecticides (1). Though *Ailanthus altissima* is a preferred host of SLF, the insect can feed on other trees such as black walnut, maple, fruit trees, and grapevines (1).

Damage caused by SLF on grapevine can be extensive, if SLF establishes in a vineyard in high numbers (3) and if the insects are not managed, or if the insects migrate from the surrounding areas in a vineyard multiple times per season. Economic losses are mainly related to increased use of insecticide, which is the only method currently available to control SLF population. Often SLF congregate on single vines (3) and their feeding, if unchecked, can reduce photosynthesis, sap flow, carbohydrates such as starch, micro and macronutrients and amount of nitrogen in storage tissues. Heavy infestations of SLF on grapevines have been noted to reduce vine health by reducing carbon assimilation and increasing competition for important resources involved in plant growth and production (unpublished data). Furthermore, high density of SLF on vines in the previous season can reduce the number of clusters per shoot the following spring and may reduce vine hardiness and increase winter injury susceptibility (https://extension.psu.edu/spotted-lanternfly-management-in-vineyards). At this point, nothing is known about the molecular mechanisms governing the impact of SLF on grapevines or other plants, or the molecular responses of plants to SLF.

Aside from damage caused by the abundant ingestion of plant sap, SLF can also cause wounding to stems and trunks *via* its piercing stylet (4) and this damage can be magnified when inflicted by high number of SLF. Interestingly, SLF feeding is characterized by dark feeding lesions that can be observed by necked eye when pealing the bark of infested plants. Other phloem-feeding pests such as aphids, mealybugs, and whiteflies are much smaller-bodied than SLF and SLF size is much more like the one of the destructive glassy winged sharpshooter (*Homalodisca vitripennis*, Germar), that can pierce directly woody tissues but that feeds on plant xylem instead of phloem. While most of the damage caused by smaller piercing sucking insects is attributed to the consumption of photoassimilates and sometimes to their ability to vector pathogens but not to wounding (5), not much is known about the direct impact of SLF on plants while breaching the plant cell wall and physical barriers. The presence of dark lesions at feeding sites suggests that plants react to SLF wounding by promoting oxidation and production of secondary metabolites, as in other plant:insect interactions, and this hypothesis would need to be verified (6).

The voracious feeding and gregarious nature of SLF also causes copious amounts of honeydew to be excreted, leading to excessive sooty mold growth, that can also reduce plant photosynthesis and, in the long-term, vigor (3). Since many microorganisms can grow in honeydew and since insects are often associated to a multitude of microbes in their secretions (gut and frass), it cannot be excluded that plant responses to SLF can be also mediated by plant:microbe interactions (7–10).

The interactions of insects and their host plants are known to be specific to the organisms involved (6), thus, it is difficult to predict what impact an invasive species, such as SLF, will have in a certain system. Our understanding of how SLF and grapevines interact is still limited, but advances in this area might help explain why grapevines responses to SLF are not efficacious at repelling the insect and could help identify what plant defenses are employed by grapevines against SLF. Generally, plant responses may include a variety of defenses against insect stress, often including both active and passive defenses (11). Active defenses such as alterations in plant structure, secondary metabolite formation, and plant hormone responses can be monitored by analyzing the transcriptome and associated gene regulation under insect attack (11, 12). These plant responses vary across types of herbivorous insect feeding, with significant differences seen between chewing insects versus piercing and sucking phloem-feeding insects (12).

Feeding by either chewing or piercing and sucking insects can induce regulation of genes involved in plant defense-related processes and repress the expression of genes responsible for photosynthesis and plant development (12). However, differences exist in plant hormonal response, specifically between the generally antagonistic jasmonic acid (JA)/salicylic acid (SA) pathways (11, 12). Attack by chewing insects has been shown to repress the SA pathway and upregulate JA production, while phloem-feeders elicit the opposite (5, 12). This difference may be attributed to the contrast in physical damage to the plants, with phloem-feeders causing less overall damage (5, 12). It is also worth noting that while in most reported cases of phloem-feeding insect attacks pathogenesis-response transcripts, proteins, and/or activities are elevated, this response is not associated with chewing insects (5).

In addition to hormone regulation, attack by phloem-feeding insects may lead to alterations in plant structures. These changes include cell wall thickening, lignification, stomatal closure, and formation of a waxy cuticle (11). Structural changes are induced through a variety of defense mechanisms interacting with each other in different ways. For example, lignin production is associated with the oxidation of

phenolic compounds by peroxidases, while peroxidases themselves are important enzymes involved in reactive oxygen species reduction (11). Plants have also been reported to respond to mealybugs and aphids with an increase in Ca^{2+} signaling and callose deposition to aid in repairing wounds and strengthening phloem cells by stomatal closure (5, 13).

While several studies have examined the effect of prominent phloem-feeding insects on a variety of plants at the transcriptomic level, there have been limited studies on the response of grapevines to phloem-feeding insects. In addition, in grapevines there has been reported a wide variation in plant responses dependent on the insect/host relationship, with specificity as narrow as plant variety (14). The present study aims to examine effect of prolonged SLF feeding on a *Vitis interspecific hybrid* 'Marquette', at a transcriptomic level, and to elucidate some of the mechanisms responsible for the detrimental effect reported in SLF infested vineyards.

2 Materials and methods

2.1 Plant material and experimental design

The study was conducted at the Penn State Berks Campus (Reading, Pennsylvania, USA; 40.364702° N, 75.976374° W) located in southeast Pennsylvania. The experimental material was twelve 6-year-old hybrid 'Marquette' vines grown on a custom-made substrate (field topsoil, perlite, and peatmoss mixed at a 1:1:1 proportion, and pH kept at 7.1) in 38L plastic pots. Pots were painted white to reduce radiative heating from growing under outdoor conditions. The pots were arranged in two parallel rows of six vines in each row. A completely randomized design was used to assign half of the vines (six) to a control treatment and the remaining six to an adult SLF treatment. All vines were covered with an insect barrier netting bag with zippers (1.3 m × 1.4 m, AgFabric, WellCo Industries, Inc., Corona, California, USA) to avoid SLF escape and entrance. Eighty adult SLF, collected from nearby woodlands were released inside each netting bag on vines assigned to SLF treatment. SLF were kept on the vines from August 19th through September 30th. Vines were monitored three times each week, and dead insects were counted and replaced with live ones. At the end of the experiment stem tissue which developed during the growing season (i.e., canes) was harvested from all vines and 10-15, 5 cm long cane pieces were randomly sampled from all areas of each vine to make a composite representative sample for each vine. These cane pieces were put in plastic Ziplock bags and transported to the laboratory (University Park, Pennsylvania, USA, 40.7982° N, 77.8599° W) inside coolers filled with dry ice. Upon arrival, the cane pieces were flash frozen in liquid nitrogen and immediately stored in a freezer at -80°C.

2.2 Sample processing and RNA extraction and quality

Cane pieces, stored in -80°C freezer, were used for extracting RNA. Sample bags containing cane pieces were taken out of the freezer, put on dry ice and peeled of their lignified outer bark to expose the green phloem tissue underneath. The phloem tissue was rapidly scraped off into a pre-chilled mortar and pestle. The scraped tissue was hand-ground into fine powder by pouring liquid nitrogen into the mortar and grinding using a pestle. About 50 mg of ground tissue was homogenized in a cetyltrimethylammonium bromide (CTAB) based buffer with a chloroform denaturation step and the RNA was selectively precipitated with LiCl following Blanco-Ulate et al. (15). RNA was cleaned up using a RNeasy Plant Mini Kit (Qiagen Sciences Inc, Germantown, Maryland, USA) including the DNase treatment on column. Purity of extracted RNA was measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Bioanalyzer (Agilent 2100 Bioanalyzer system, Agilent Technologies, Santa Clara, California, USA).

2.3 RNA sequencing, mapping and annotation

Extracted RNA was sent to the Genomics Core Facility of the Huck Institute of the Life Sciences at Penn State for sequencing where a unique dual indexed library was prepared from each sample using the TruSeq Stranded mRNA kit according to the manufacturer's instructions (Illumina, Inc., San Diego, California, USA). The concentration of each library was measured, and an equimolar pool of the libraries was made using the KAPA Library Quantification Kit Illumina Platforms (Kapa Biosystems, Inc., Wilmington, Massachusetts, USA). The library pool was sequenced using a NextSeq 550 High Output 75 nt single read sequencing run. Raw reads are deposited to NCBI under the BioProject accession no. PRJNA860209. This provided an average of ~58 million reads per sample. Sequences were then analyzed through a series of bioinformatics tools using Unix commands and R. In summary, the quality of the raw reads for all samples provided by the sequencing facility, were preprocessed and checked using Fastqc (16). Hisat2 (17) was used to align and assemble the sequences against the reference genome the *Vitis vinifera* (PN40024) genome assembly 12X.v2. Mapped sequences were then annotated using the *Vitis vinifera* VCost.v3 annotation version.

2.4 Differential gene expression (DGE) and gene enrichment analysis

Reads for the annotated genes per sample were counted by featureCounts (18). Finally, differential gene expression (DGE) patterns across treatments were analyzed by using the DESeq2 and edgeR package in the Bioconductor library (19).

Significant DEGs in the treatments were functionally characterized by using the annotation described in the Plant and Fungi data integration database (Grapevine reference genome assembly). However, due to the limited Gene Ontology (GO) information in the grapevine genome, we used grapevine gene IDs to find the best match ortholog genes (TAIR IDs) in *Arabidopsis thaliana* as described in the same database. To crosscheck and validate, reciprocal blast was also performed using orthology package in R that implements gene orthology inference using the reciprocal best hit (RBH) method as described by Drost et al. (20). These IDs were then used to conduct gene enrichment analysis using DAVID bioinformatics resources v6.8 (21).

3 Results

3.1 Sequence mapping and reads assembly

Sequence mapping and percent genomic alignment are summarized in Supplementary Table 1. For both treatments ('Marquette' grapevines infested with SLF (S) and uninfested controls (C)), the overall alignment percentage was 86-91%. We estimated the distribution of samples by sample distance matrix (SDM) and principal component analysis (Figures 1A, B). We found strong clustering of biological replicates for control and for SLF treatments, except for replicates S1 and S2. An analysis of variation in the dataset using principal component analysis showed similar grouping of replicates for both treatments. The low percent of variation with PC2 (6.2%) indicated that although S1 and S2 were placed lower than the other replicates in the same treatment group they were not highly different considering PC1, which explained 83% of the variation. To test if inclusion of S1 and S2 in the analysis might affect the differential expression, we plotted and compared each replicate among the treatments using scatter plots (Supplementary Figure 1). These plots didn't show any abnormal shape and distribution of gene expression for any pairwise comparison, so we considered all the replicates in the differential gene expression analysis.

3.2 Differential gene expression (DGE) analysis

We analyzed and assessed the variation of the read counts for each DEG between replicates by dispersion plot (Supplementary Figure 2A). Read counts for each gene were clustered around the ideal fitted line, with the dispersion decreasing as the means of the normalized reads count increases, indicating that the data was a good fit for the DGE analysis. Expression of the top 5000 genes based on their read counts (considering both treatments) was examined by hierarchical clustering heatmap (Supplementary Figure 2B). The majority of these top 5000 genes had higher signal ratios (Z-scores calculated from the read counts of each gene) in the SLF treatment, indicating that more upregulated genes were found in the SLF than control treatments. DESeq2 and edgeR were used to identify DGE filtering on Log2FoldChange ≥ 1.0

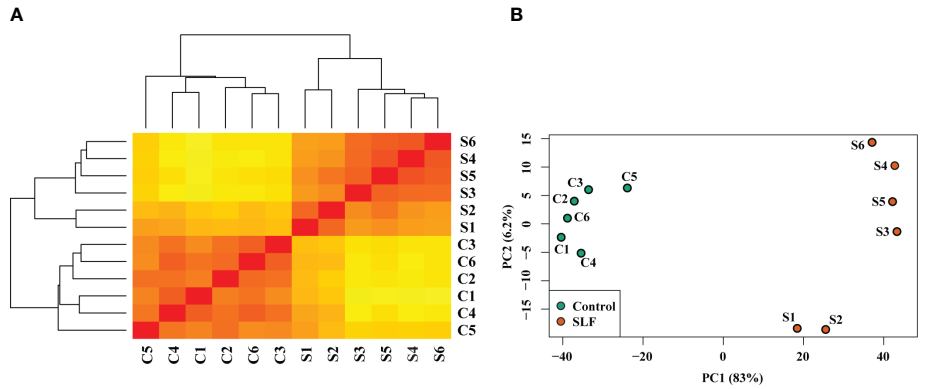

FIGURE 1
Sample distance matrix and principal component analysis of the treatments and replicates. **(A)** Dendrogram and sample distance matrix among the samples. Replicates for both C and S were clustered together and separated by treatment. Here red and yellow colors indicate, respectively, the closely and distantly related samples based on the read counts of DEGs. **(B)** Principal component analysis plot of relative distribution of the biological replicates and the treatments. PC1 (83%) and PC2 (6.2%) together explain approximately 90% variation of the samples.

and padj < 0.05, (indicated in violet and pink color, respectively), and are shown as a Volcano plot (Figure 2A). DESeq2 analysis yielded a total of 4,793 significantly DEGs, among which 3,497 genes were upregulated and 1,296 were downregulated (Supplementary Table 2). EdgeR returned 5,617 significantly DEGs, of which 3,929 and 1,688 genes, respectively were up and down regulated. Comparing the genes identified from both analyses revealed that 4,704 genes (82%) were common, while 89 (2%) and 913 (16%) genes were found respectively by only DESeq2 and by only edgeR, respectively (Figure 2B). All the genes that were found downregulated in DESeq2, were also captured by edgeR. Since almost all the genes captured by DESeq2 were also found by edgeR, we proceeded with the gene list identified with DESeq2 for functional analysis.

3.3 Gene enrichment analysis of the DEGs

We annotated the functions of the significant DEGs using the annotation described in the Plant and Fungi data integration database (Grapevine reference genome assembly). However, due to the limited Gene Ontology (GO) information in the grapevine genome, we used grapevine gene IDs to find the best match ortholog genes (TAIR IDs) in *Arabidopsis thaliana* as described in the same database. These IDs were then used to conduct gene enrichment analysis using DAVID bioinformatics resources v6.8., and the associated biological pathways (BPs), molecular functions (MFs), cellular components (CCs), and KEGG (KOs) pathways were retrieved. A total of 162 BPs, 91 MFs, 45 CCs, and 24 KEGG pathways were enriched with a False discovery rate (FDR) ranging from $3.0X10^{-9}$ to 0.9 (Supplementary Table 3). Among these, we found 33 BPs, 23 MFs, 31 CCs, and 15 KEGGs enriched with FDR < 0.05, which can be considered as the most probable pathways triggered by SLF infestation (Supplementary Table 3). Pathways were manually curated and sorted out the prospective biological pathways and KEGGs for a more comprehensive analysis (Figures 3A, B). BPs were grouped by their generic functions and assigned into major functional categories such as protein kinase, transcription factor, phytohormone signaling, photosynthesis and metabolic process, cell wall organization, and antioxidant (Figure 3A).

3.4 Analysis of DEGs elicited by SLF infestation

The aggressive group feeding nature of SLF can lead to wounding, which in turn may trigger plant defense responses and signaling involved in maintaining physiological homeostasis. However, effective host plant responses depend on the specific insect-plant interactions and how the plant perceives and orchestrates these signals. Therefore, in this study, we focused on the pathways related to insect-plant interactions, their signaling, host responses, and cellular homeostasis.

3.4.1 DEGs involved in insect-plant interactions and signal transduction

3.4.1.1 Signaling kinases

Plant responses to an insect begin with the recognition of plant-insect interplays occurring during the feeding time, such as the diverse mechanisms induced by oral secretions. For instance, herbivore-associated molecular patterns (HAMPs) could be recognized by plant cell wall receptors, resulting in

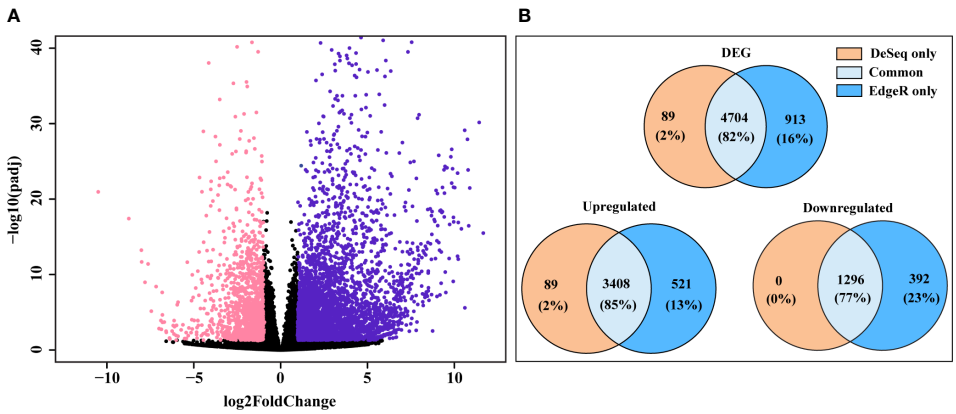

FIGURE 2
DEG found in DESeq2 and edgeR. **(A)** Volcano plot of significantly up and downregulated genes. X-axis and y-axis denote the Log2FoldChange and -log10 of padj values, respectively; where log2FoldChange ≥ 1.0 and padj < 0.05 were considered as significant and indicated in violet (upregulated) and pink color (downregulated). **(B)** Ven diagram of DEG, yielded from DESeq2 and edgeR, showing genes discovered by each analysis or genes found by both analyses.

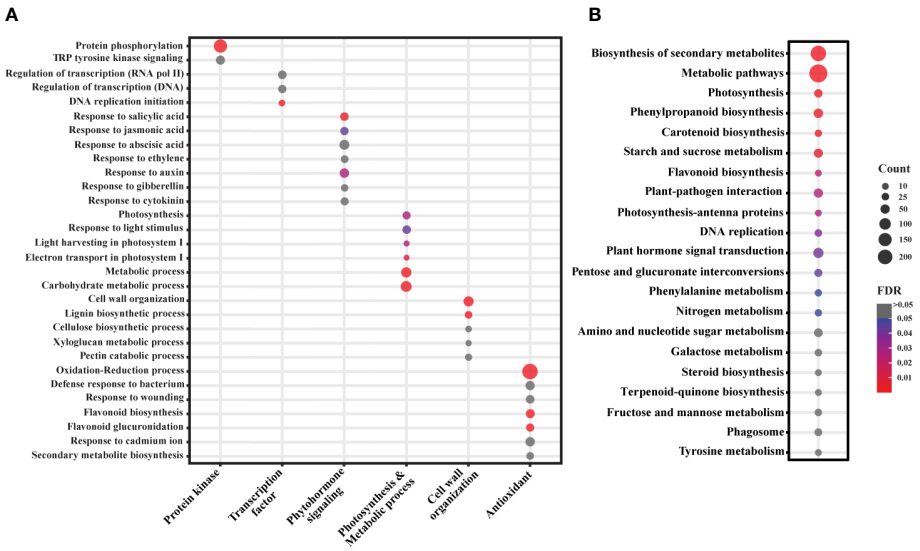

FIGURE 3
Gene enrichment analysis of the significant DEGs. **(A)** GO analysis of the significant DEGs. Selected BPs are categorized based on their functions in plants. **(B)** KEGG analysis of the DEGs. Color and bubble size indicate the false discovery rate (FDR) and the number of genes (count) belonging to each class, respectively.

the activation of signal transduction cascades carried by the secondary messenger molecules, such as cyclic AMP, cyclic GMP, inositol triphosphate, diacylglycerol, calcium, etc. In most cases, signal cascades start with the phosphorylation of related proteins mediated by protein kinases. 257 and 26 genes up and downregulated, respectively, related to protein phosphorylation (Supplementary Table 4). Among these, many signaling kinase genes, such as *LRR receptor kinase*, *LRR transmembrane protein kinase*, *NBS-LRR receptor kinase*, *S-locus protein kinase*, *Serine/Threonine receptor-like kinase*, *wall associated kinase*, and *FLG22-induced receptor-like kinase* showed enhanced expression under SLF infestation. Stimulation of protein kinase genes like *FLG22-induced receptor-like kinase* suggests presence of microbes, either deposited by SLF or exogenous microbes mobilized in the wounds. Our data also suggests that interchanges of signals triggered by protein kinases consequently induces the expression of transcription factors (TFs) in the nucleus, followed by activation of other signaling molecules like phytohormones and secondary metabolites, with their downstream target genes responsible for defense and physiological functions such as detoxification and photosynthesis.

3.4.1.2 Transcription factors regulation

TFs are the master regulators that control the expression of genes at transcriptional level under different physiological conditions. The past few decades have been productive in identifying the TFs that are involved in regulating diverse cellular functions. These TFs mostly belong to large gene families, and their regulatory networks often overlap and function together (22).

A total of 232 TFs, assigned to various functional categories/gene families, were differentially expressed in our data. Most (160) were upregulated under SLF infestation (Supplementary Table 5). TFs that are members of the *myb domain containing protein* family contained the highest number of DEGs (23) (Figure 4A). MYB proteins are one of the largest families of plant TFs that have been linked to many distinct functions, especially in regulating plant stress responses (22, 24). The other major TF families that have been associated with defense signaling are *basic helix-loop-helix* (bHLH), *ethylene-responsive-element-binding factors* (ERF), WRKY families, *NAC domain containing proteins* (NACs), *basic leucine-zipper (bZIP)*, and *zinc finger* (25). Each of these TFs were detected in our study, with most of them upregulated (Figure 4A). TFs involved in plant defense (17), phytohormones regulation (25), and both (24) were also differentially expressed (Figure 5 and Supplementary Table 5).

3.4.1.3 Phytohormone signaling

Phytohormones are small signaling molecules that are essential for the regulation of plant growth and development, and are deployed by plants as a universal strategy to defend against stresses (27, 28). It is well documented that SA and JAs, along with abscisic acid (ABA) and ethylene (ET), carry the

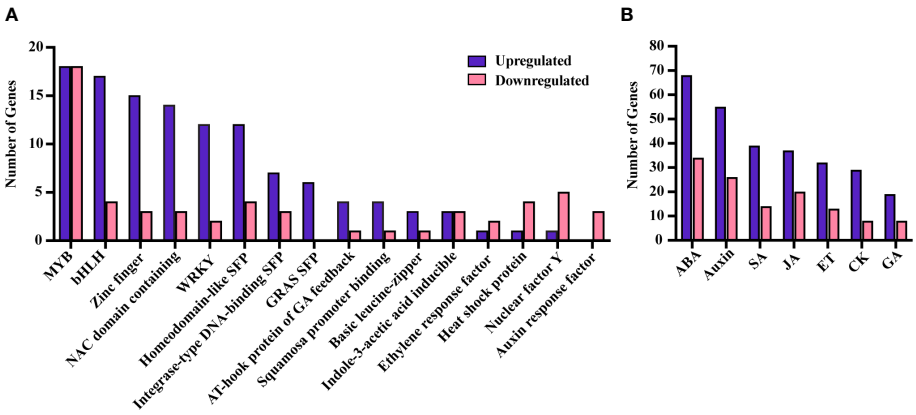

FIGURE 4
DEGs belonging to different TF families **(A)** and phytohormones **(B)**. SFP, super family protein.

major primary signals in modulating a wide range of adaptive immunity under stress conditions (27). However, more recently, the crucial roles of auxins and other phytohormones under stress conditions have also been reported (27). The direct involvement of plant growth regulators in plant defense suggests that the regulation of plant growth, development, and defense are intertwined and are part of a complex regulatory circuits of cross-communicating hormone signaling pathways.

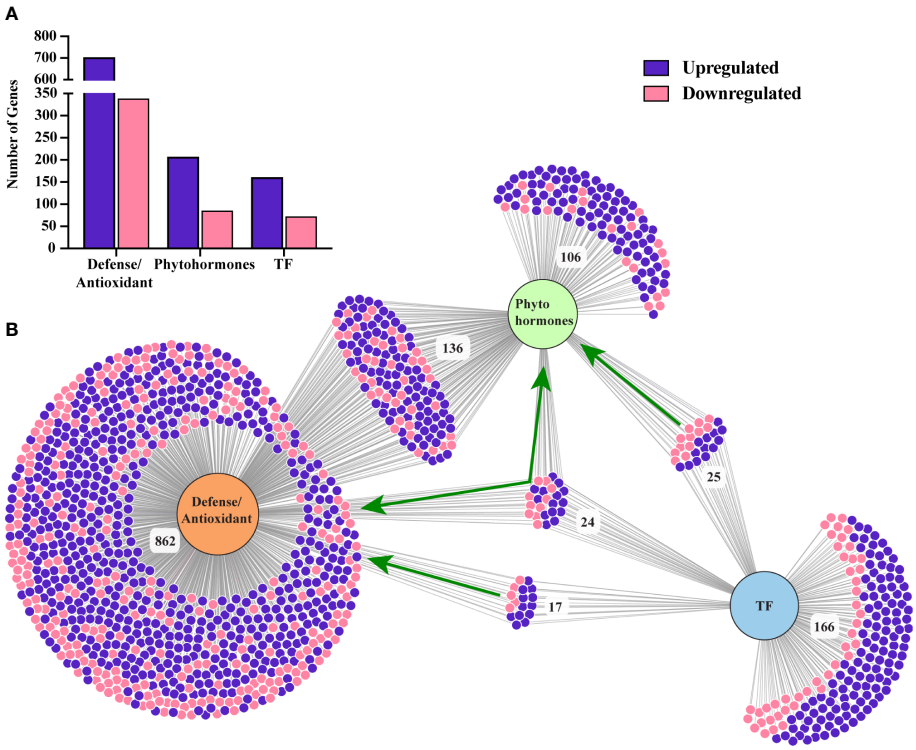

FIGURE 5
(A) Number of differentially expressed genes belong to plant defense, phytohormone signaling, and TFs. **(B)** A visualization of a number of common and distinct genes related to these three categories. Green arrows indicate the TFs related to plant defense, phytohormone and both. Figure was generated using a web-based visualization tool, DiVenn (26).

Genes related to all the major phytohormones were enriched in our study, with 206 and 85 unique genes up and downregulated, respectively, under SLF infestation (Supplementary Table 6). Among these, we found 136 genes were directly involved in plant defense (Figure 5; Supplementary Table 6).

Genes responsible for ABA signaling were highly enriched in the dataset, with 68 and 34 genes up and downregulated, respectively (Supplementary Table 6 and Figure 4B). ABA is commonly associated with plant growth and acts as a major regulator in abiotic stresses, however its involvement in biotic stresses is becoming more evident (29, 30). For instance, ABA both acts synergistically with JA under wounding or herbivorous insect attack, while also affecting resistance against necrotrophic pathogens (31, 32). Multiple copies of genes related to ABA, abiotic stress, and diverse cellular activities were upregulated in our study including BURP domain-containing protein (RD22), DREB2C, aquaporins, annexin 4, phospholipase D alpha and others (33-40). We also found differential regulation of several genes belonging to the ABC transporter G and B families which are necessary for wax transport to the cuticle and detoxification of xenobiotics (41, 42).

While SA, JAs and ET are naturally expected as these hormones are the primary regulators of inducible defenses, our data suggests inconclusive roles of these phytohormones under SLF infestation (Supplementary Table 6). However, we found that auxin biosynthesis and signaling related genes were the second highest enriched class of genes (Figure 4B and Supplementary Table 6). Auxin is associated primarily with plant growth and development, but also plays roles in plant defense *via* utilizing the secondary metabolite and TFs regulatory network. Thus, our data on phytohormones suggest that grapevines invest simultaneously on defense and in cellular homeostasis.

3.4.2 DEGs involved in cellular homeostasis and host responses or resource reallocation

3.4.2.1 Photosynthesis

Photosynthesis is part of the primary metabolic processes in plants and is a key indicator of their physiological condition. A total of 84 genes related to photosynthetic processes were differentially expressed under SLF feeding pressure (Supplementary Table 7). Among these, 77 genes were upregulated, with only 7 genes downregulated. We observed a strong upregulation of genes related to PSI reaction center subunits, PSII, phototropic-responsive NPH3, Rubisco, and light-harvesting chlorophyll binding (LHCB) proteins under SLF feeding pressure.

3.4.2.2 Cell wall reformation and stomatal closure

Our data suggest that cell wall reformation and stomatal closure are two other crucial events that may take place under SLF infestation. Genes that are reportedly involved in stem lignification, such as peroxidases and laccase (43) were enriched in our analysis (Supplementary Table 8). In addition, lignins, which are complex cell wall polymers, are produced by the oxidative polymerization of monolignols in assistance with plant oxidases, peroxidases, and/or laccases (44, 45). Out of 19 peroxidase and 19 laccase DEGs in grapevine, 13 and 12, respectively, were upregulated (Supplementary Table 8). Furthermore, we found enrichment of genes involved in the biosynthesis of major structural components of the cell-wall matrix and its organization. For instance, genes responsible for the formation of cellulose, xyloglucan, and pectin were significantly upregulated upon SLF feeding (Supplementary Table 9). This results a role for stimulation of cell wall reformation pathways under SLF infestation in grapevine.

Additionally, 76 genes that are categorized as 'response to cadmium ion' (Supplementary Table 10) were differentially expressed. Genes responsive to cadmium ion or any heavy metals induce callose deposition in the cell wall, which in turn may stimulate stomatal closure (46). Additionally, insect herbivores feeding on the vascular system can induce hormonal responses resulting in stomatal closure (47, 48). Differential expression of genes such as glutamate receptor (GLR) proteins and receptor kinases that are involved in stomatal regulation indicate the plants' promotion of stomatal closure as a response to SLF feeding (49-52).

3.4.2.3 Plant defense and detoxification

Our data showed that SLF infestation triggered defense responses in grapevine by inducing multiple defense pathways recognized for biotic and abiotic stresses. A total of 1039 unique DEGs responsible for abiotic and biotic stresses and parts of a plant's physiological immunity were assigned to defense/antioxidant category (Supplementary Table 8). The highest number of DEGs (363) belong to the oxidation-reduction process, where 263 and 100 genes were up and downregulated, respectively, under SLF infestation (Supplementary Table 8). Among them, the highest number of DEGs belong to the cytochrome P450 superfamily. These enzymes play a crucial role in detoxification of xenobiotics across animals, plants, insects, and microorganisms (53). Several *flavin-containing monooxygenase* and *glutathione S-transferase* DEGs that are involved in detoxification of toxic substances (54, 55) were also enriched in our data. Additionally, the upregulation (98 out of 120) of genes like *flavonoid 3'-monooxygenases, flavonone-3'-hydroxylase, flavonoid-3'-hydroxylase, flavonoid-3',5'-hydroxylase, UDP-glucose:flavonoid 7-O-glucosyltransferase, flavonol synthase,*

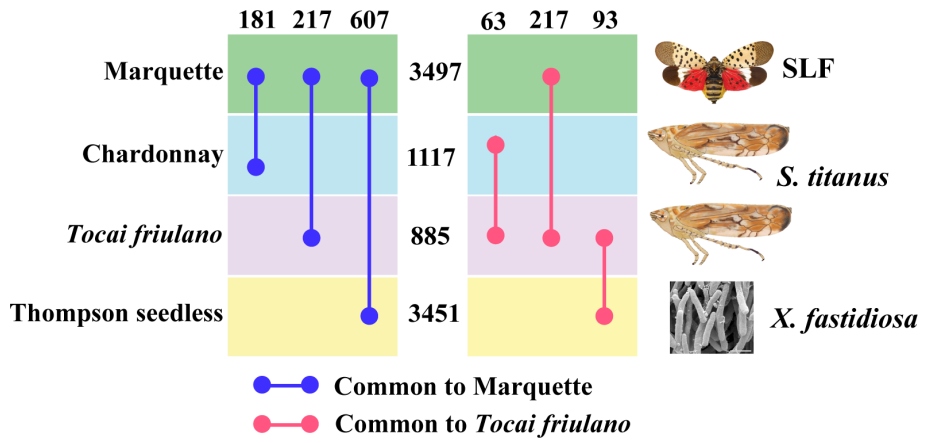

FIGURE 6
Comparative analysis of transcriptomes of different grapevine varieties under insect infestation and disease conditions. Numbers in-between horizontal boxes and on top indicate, respectively the total number of upregulated genes and the common upregulated genes between two varieties.

chalcone synthase, stilbene synthase, etc. support the idea of antioxidant pathway stimulation under SLF infestation (56).

3.5 Comparative transcriptomes analysis of grapevine varieties infested with similar pests

To put our results in the context of grapevine responses, we looked at other studies where grapevine was subjected to stress by insects similar to the SLF or by pathogens transmitted by similar insects. Surprisingly, not many transcriptomes that follow one of these two criteria have been published. We thus conducted a comparative analysis of transcriptomes using data from Bertazzon et al. (14) and Zaini et al. (57). Since there were not many common downregulated genes among the studies, we decided to conduct analysis only on the upregulated ones. Bertazzon et al. did a transcriptomic profiling on two grapevine varieties (Chardonnay and *Tocai friulano*) with different levels of susceptibility, former being the most susceptible to *Flavescence dorée*. This is one the most severe grapevine yellows diseases in Europe that is caused by phytoplasmas and transmitted by the leafhopper, *Scaphoideus titanus*. Authors carried out a comparative transcriptome analysis of both grapevine varieties in presence and absence of the vector and/or phytoplasmas. We used their data to sort out the genes that were significantly upregulated under insect infestation in both varieties (Figure 6). Our study on Marquette found a total of 3497 upregulated genes under SLF infestation, whereas Chardonnay and *Tocai friulano* had, respectively 1117 and 885 genes upregulated under leafhopper infestation (Figure 6). Among these, Marquette shared 181 and 217 common genes, respectively, with Chardonnay and *Tocai friulano*. On the other hand, Zaini et al. conducted a transcriptome analysis on grapevine var Thomson seedless, a susceptible variety to *Xylella fastidiosa*, the causal agent of Pierce's disease of grapevine under disease and control conditions. *X. fastidiosa* is a bacterium transmitted by leafhoppers and sharshooters, but the study did not involve insects. Authors found a total of 3451 upregulated genes under disease condition, among which, 607 genes were common to our study (Figure 6).

We then analyzed the biological pathways of the genes shared among these grapevine varieties, which showed that Marquette, Chardonnay, and Thomson seedless plants triggered more defense pathways related genes than *Tocai friulano* (Supplementary Table 11). Since *Tocai friulano* is a relatively less susceptible variety, we also looked for genes which are common to this variety and unique, to comprehend genes or pathways that could be related to tolerance. *Tocai friulano* shared 217, 63, and 93 genes, respectively with Marquette, Chardonnay, and Thomson seedless, whereas 510 unique genes that were upregulated under insect infestation and could constitute genes for tolerance (Figure 6). A more in depth and investigative study of these genes in the future will help unveiling the mechanism of tolerance in the grapevine against insect infestation. Our comparative analysis also suggests that susceptible varieties tend to allocate more resources than tolerant varieties, when challenged by insects feeding, suggesting that a reallocation of resources could be detrimental to grapevines, if it would divert resources from the regular metabolic pathways. Further studies would be needed to explore this possibility.

4 Discussion

Spotted lanternfly is a phloem feeding insect that uses piercing and sucking to feed on the stem and trunk of host plants (4). On infested grapevines, over 100 adult SLFs can be clustered on a single vine. The aggressive and group feeding nature of SLF can cause a depletion of plant resources and consequently may increase susceptibility to pathogen invasion (4). Given the circumstances, understanding how grapevines respond to 'heavy' attack by SLF at the transcriptional level will advance our knowledge on how SLF interacts and impacts the host plant. To do so, we compared comprehensive, genome-wide transcriptional changes in SLF-free and SLF-infested 'Marquette' grapevines. We decided to test the gene expression level after long term feeding since the SLF effect are noticeable only in the season following the prolonged feeding event. RNASeq data generated from phloem tissue after one and half months of SLF infestation suggests that grapevine simultaneously induces defense and maintains cellular homeostasis *via* signaling cascades initiated by protein kinases, TFs, and phytohormones.

Plant defenses consist of structural barriers such as wax, lignin, and cuticle, and immune responses that induce active or adaptive immunity under adverse conditions (11, 58). We found plant-pathogen interaction, protein phosphorylation, TFs, and plant hormone signal transduction were enriched according to GO categories and KEGG pathways analysis (22, 27). These pathways control the plant's physiological homeostasis and regulate the active defense response under stressors. The active defense response is a fine-tuned co-regulation of complex interchanges of signals triggered by plant-pathogen interactions orchestrated by series of signaling molecules like protein kinases, phytohormones, TFs, and activation of their downstream target genes. Many genes belonging to the categories of protein kinase, TF, and phytohormones were significantly expressed in our data. To categorize the differentially expressed TFs based on their functions we found that 66 out of 232 TFs were involved in plant defense and phytohormones regulation, whereas the rest may be involved in other physiological pathways.

Our results on phytohormone genes showed a rather noteworthy phenomenon. It has been reported that chewing herbivores are largely associated with the JA-mediated response, while phloem-feeding insects, such as SLF, are often associated with the SA-mediated response and a somewhat weaker JA response (59–61). However, we observed that similar number of genes from both pathways were induced by SLF feeding. Most of them were defense related TFs with a few downstream and signaling pathways related genes, such as PR-1 and LOX precursor 1. Therefore, SLF induced defense signaling connecting to SA or JA mediated pathways was inconclusive from our data.

Remodeling of the plant cell wall is a frequently reported phenomenon against pathogens or herbivores (62, 63) and is often associated with cell wall reinforcement (64) or the release of signaling molecules from the cell wall (65). KEGG pathways analysis of DEGs showed that biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, phenylalanine metabolism, and flavonoid biosynthesis were enriched by SLF infestation. The biosynthesis of phenylpropanoids begins with the conversion of phenylalanine to cinnamic acid by phenyl ammonia-lyase (PAL), leading to the formation of different forms of phenolics, including lignin (66). Enhanced expression of genes in the general phenylpropanoid pathway such as PAL, 4CL, C4H, peroxidase, and CCoAOMT strongly infer the stimulation of lignin biosynthesis under SLF feeding. We have also found peroxidases and laccase genes that reportedly function in lignification were enriched in our data (43–45) supporting the hypothesis of structural defense upregulation in response to SLF. Lignin plays a crucial role in plant defense against herbivores by physically restricting the entry of insects through increasing the robustness of cell wall. It also decreases the nutritional content in the area, thus reducing feeding by the herbivores (11). Additionally, we found upregulation of genes that are involved in the biosynthesis of the major structural components of cell-wall matrix and their organization, such as cellulose, xyloglucan, and pectin. We also observed DEGs responsible for callose deposition which may eventually stimulate stomatal closure. Plants regulate stomatal closure as a strategy for cell wall strengthening, as well as maintaining photosynthetic rate (46). This is one of the key adaptive response of plants against herbivores (67). Several insects use stomatal openings for feeding sites (68–70) and oviposition (71). Oral secretion from insects can induce herbivore-associated molecular patterns (HAMPs) that could result in stomatal closure (67). Moreover, insect herbivores feeding on the vascular system can induce hormonal response resulting in stomatal closure (47, 48). Differential expression of genes such as glutamate receptor (GLR) proteins and receptor kinases that are involved in stomatal regulation indicate the plants' promotion of stomatal immunity as a response to SLF feeding (49–52).

In this study, we found a significant upregulation of DEGs involved in photosystem I and II, such as phototropic-responsive NPH3, precursors for chlorophyll pigment synthesis, ferredoxin, and enzymes involved in photosynthesis such as RuBisco and LHCB. An increase in photosynthesis related genes could be the result of the plant's strategy to maintain physiological homeostasis, a result of SLF sequestering large amounts of photosynthates, or it could be related to the increased demand for components of the cell wall.

Furthermore, all the major classes of DEGs in oxidoreductase families were enriched in our data, with the highest number of genes belonging to cytochrome P450 superfamily. These enzymes play a crucial role in detoxification and also protect plants by enhancing antioxidant activity (53, 72, 73). Enrichment of flavin-

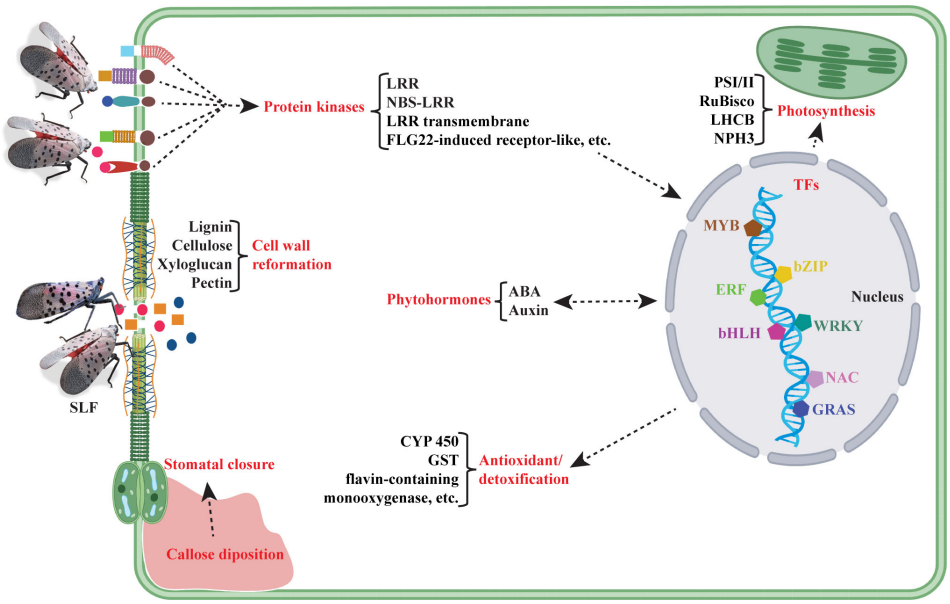

FIGURE 7
Molecular model of events occurring in hybrid *Vitis vinifera* 'Marquette' under SLF infestation, based on transcriptomic data. Here red color text indicates the categories of molecules or crucial events that are possibly happening upon SLF feeding, whereas black color text denotes the essential genes or pathways.

containing monooxygenase and glutathione S-transferase genes also suggests these activities under SLF feeding.

To summarize the complex and intertwining patterns of gene expression, we constructed a molecular model of events that may happen under SLF infestation (Figure 7). This study suggests that interactions between SLF and grapevines activate signaling molecules like protein kinases, TFs, and phytohormones. These in turn activate the downstream target genes responsible for various metabolic functions and defense, such as photosynthesis, cell wall reformation, stomata closure, and antioxidation/detoxification.

In conclusion, we conducted an experiment to evaluate the transcriptional response of heavy infestation of SLF on grapevine. Extensive changes in gene expression, particularly in pathways associated with biosynthesis of lignin and other structural components of cell-wall matrix, and antioxidant/detoxification indicate that grapevine likely responds to SLF feeding through remodeling of cell-wall and detoxification. Patterns of SA and JA response indicate that SLF attack elicits novel pathway interactions and suggests that future studies should explore more regarding the phytohormone signaling. We also carried out comparative transcriptomes analysis of grapevine varieties infested with similar pests. Our analysis suggests that under insect infestation, susceptible varieties tend to allocate more resources than tolerant varieties. Reallocation of resources, especially channeling off resources from the regular metabolic pathways, consequently, might be detrimental to grapevines.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/ PRJNA860209.

Author contributions

MC and CR designed experiment. SK and CK-W performed RNA extraction. MI conceptualized data curation and analysis pipelines. MI performed data analysis and generated figures. MI, CK-W, and SK prepared original draft. MC, CR, JL, and MI wrote, reviewed, and edited manuscript. CR and MC supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2022.971221/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Scatter plots for each replicate of the treatments **(A)**: Control, **(B)**: SLF. No replicate showed any abnormal distribution of gene expression for the pairwise comparison.

SUPPLEMENTARY FIGURE 2
Dispersion and hierarchical clustering heat map of each gene among the replicates. **(A)** Black dot and blue circle designate, respectively, the mean of normalized read counts and variation of a gene. Strongly clustered data points around the red line suggested that data were well distributed and fit for differential gene expression (DGE) analysis. **(B)** Hierarchical clustering heat map of differentially expressed genes among SLF (S) and control **(C)** treatments. Z-scores calculated from the read counts of each gene are shown in a blue-yellow color scale, where blue and yellow represent higher and lower read counts, respectively. Each column and row, respectively represents the replicates and a differentially expressed gene.

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Responses of adult spotted lanternflies to artificial aggregations composed of all males or females

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Spotted lanternflies (SLF) *Lycorma delicatula* are economically important invasive planthoppers discovered in North America in 2014. SLF are gregarious, but how they locate each other, or who finds whom and when, is poorly understood. Here we describe adult SLF behavior and phenology on their preferred host, *Ailanthus altissima*, under field conditions, in the context of both aggregation and mate-location, since SLF demonstrated aggregation prior to mating. We documented aggregation behavior of adults and found we could manipulate free-living SLF populations in both number and sex ratio by the placement of confined populations of SLF males or females on trees. Trap capture of arriving SLF was significantly higher on trees with confined SLF aggregations than on control trees, and was corroborated with photographic data, demonstrating the manipulation of attraction and aggregation behavior. Sex ratios of trapped SLF arrivals were significantly more male-biased on trees with confined males and more female-biased on trees with confined females, evidence that the male- and female-biased sex ratios observed on trees naturally can be explained by sex-specific conspecific signals. SLF sex ratios shifted over time in the same pattern over two consecutive years. A mark-release-recapture study over time found that 1) SLF behavior is density dependent and strongly influenced by natural populations, 2) released females were captured significantly more on trees with caged females, particularly prior to mating, and 3) released males were captured significantly more on trees with caged females starting at mating time. Photographic data revealed that most clustering behavior (a measure of courtship) of free-living SLF began on trees with caged females during mating time, but not on trees with caged males or controls. We describe adult male and female SLF phenology whereby 1) aggregation behavior occurs, 2) males and females arrive at different times, 3) females began to aggregate several weeks prior to mating, 4) males subsequently joined aggregations at the time of mating, and 5) aggregation continued into oviposition. Population density and aggregation behavior were found to be key factors in their natural history which can be

manipulated, providing a foothold for future research. Possible mechanisms for future exploration are discussed.

KEYWORDS

aggregation, sex ratio, attraction, trapping, pheromones, phenology, reproductive biology

Introduction

Spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae) (hereafter, SLF), is a phloem-feeding invasive pest, with a broad host range, that has spread to numerous U.S. states since its first detection in eastern Pennsylvania in 2014 (1–3). With populations expanding relatively unchecked, they occur in large numbers, and their intensive feeding causes direct damage to and even death of host plants, particularly grapevines, posing a significant threat to the grape industry (4, 5). Indirect damage occurs when heavy SLF feeding in trees causes honeydew to rain down from the canopy, coating the understory, and promoting the growth of sooty mold which blocks photosynthesis, killing understory plants. Large SLF populations in urban and suburban areas, and the accumulation of their honeydew on patios, cars, and other outdoor items, in turn attracting stinging insects, impact outdoor activities and create a nuisance to humans. Around the time of mating, swarms of adult SLF take flight and have entered aircraft, manufacturing and packing factories, and food-processing facilities, and in some cases have rendered products unusable, causing problems for businesses (KM pers. obs., G. Parra, pers. comm.). Furthermore, cryptic SLF egg masses are deposited on outdoor objects, including timber, plant nursery stock, toys, furniture, tiles, rocks, vehicle wheel wells, shipping containers, and train cars, making them excellent hitchhikers and facilitating their spread to new areas (5). Thus, SLF threatens numerous industries, worth billions of dollars, through direct and indirect feeding damage, disruption of commercial activities due to their presence in large numbers, and quarantines restricting movement of infested goods. Until its invasion in the U.S., little information was available on SLF biology, and even less on its reproductive biology. In the last 8 years, researchers have begun to fill the knowledge gaps and develop tools to control this pest outbreak.

Although SLF are polyphagous, they have a strong association with tree-of-heaven *Ailanthus altissima* Swingle (Mill.) Swingle (Sapindales: Simaroubaceae) (3, 4, 6). In Pennsylvania, adults oviposit between the end of September and early November when they die, eggs overwinter, and nymphs start to emerge in the end of May or early June (3, 7). Each of the four nymphal stages lasts approximately two weeks, and the first adults emerge in the end of July. Nymphs are highly active, mobile, gregarious, and polyphagous, but as they develop, their diet becomes more specialized on their preferred host *A. altissima* (3, 6, 8). Adults are long-lived, and in the first six weeks prior to the observation of mating, described as "Early", they predominantly can be found feeding (7, 9). About halfway into Early, near the end of August, large aggregations start to appear on *A. altissima* with honeydew accumulating and, at the bases of the most heavily-infested trees, becoming white and frothy, and emitting a strong smell of fermentation (2, 10). It is at this time when large numbers have also been observed to take flight (9, 11) and sex ratios have been observed to become strongly skewed, with mostly males on some trees and mostly females on other trees (12, 13). Mating is first observed in mid-September, marking the beginning of a stage called "Mid", and a week or two later the first egg masses start to appear, marking the beginning of a stage called "Late" (7).

Tools for early detection typically combine powerful attractants, such as pheromones or kairomones, with effective traps (14). Numerous kairomones were recently identified for SLF (15), but no pheromones have been identified for SLF or any planthopper (5), although this may be due to lack of investigation. Bioassay studies produced evidence of possible pheromone use in SLF (MFC, unpublished) (16). Evidence to suggest that SLF may actively aggregate has also been found recently (17, 18). If aggregation or mating behavior in SLF is mediated by a pheromone, it could lead to the discovery of powerful attractants. Thus, our research efforts aim to determine: 1) where, when, and how adult SLF find each other, 2) if adult aggregation is actively taking place, 3) which sex releases signals and which sex responds to them, and 4) the timing and physiological state required by SLF to release these signals so that we can collect, study, and exploit them.

We sought to answer the question "Who finds whom and when?" under field conditions. Thus, in 2020, we conducted an experiment in the field using artificial aggregations of either male

or female SLF adults confined in sleeve cages on trees, with circle trunk traps placed above them to capture the naturally occurring SLF responding to the confined populations. This experiment was designed to measure the number of naturally occurring adult SLF males and females arriving in response to aggregations of each sex, as well as marked-released-recaptured SLF with an equal opportunity to reach a tree with an artificially confined male or female aggregation. Based on resulting observations in which the trees with the artificial aggregations on them appeared to have triggered aggregation behavior of free-living SLF, the experiment was repeated in 2021 with the addition of control trees that had empty sleeves, and the collection of photographic data.

Materials and methods

Sleeves and traps

Experiments, detailed in sections below, were performed in the field in 2020 and 2021 with blocks of either two or three trees, respectively. Sleeves containing either males or females were placed around tree trunks, and in 2021 there were also control trees with no SLF inside the sleeves. A circle trunk trap was placed above each sleeve (Great Lakes IPM, Vestaburg, MI) (19) with the bottom edge placed at breast height. Traps collected arriving SLF into a bag rather than a jar, which was found to be significantly more efficient at capturing SLF (20). A pesticide strip was placed in each trap bag and refreshed every six weeks to prevent escapes and predation (Vapona II 2,2-dichlorovinyl dimethyl phosphate (10%), Hercon Environmental, Emingsville, PA) (19). Two field experiments in consecutive years (2020 and 2021) tested the cumulative effects over time of placing artificial aggregations of males or females on paired trees in low density field sites. In both experiments, the artificial aggregations were confined within custom sleeves (76 cm tall) enclosed around trunks of *A. altissima* trees. The top of each sleeve started 2-3 cm below the bottom of the circle trap which captured free-living SLF that arrived on the tree trunk. Sleeves were constructed by first placing three layers of foam batting (BugBarrier; Environmetrics Systems USA, Inc., Victor, NY) around the trunk at the top and bottom margins of the sleeve to provide space between the sleeve and the trunk for the SLF inside to move around. Chicken wire was placed over the batting, followed by tulle mesh over the chicken wire. These were all secured to the tree at the top and bottom using zip ties, and the vertical seam in the tulle was closed using Velcro in 2020 (Velcro Companies, Inc., Manchester, NH), and yellow lab tape in 2021 (Research Products International, Mt. Prospect, IL) (Figure 1). At the beginning of the first week, sleeves were stocked with groups of live field-collected males or females (numbers and details described for each year below). At the beginning of each subsequent week, sleeve contents were checked, and if some died or escaped, they were replaced with newly captured SLF of the designated sex. Sleeves on the control trees in the 2021 blocks

FIGURE 1
A photograph from 2020 showing two *Ailanthus altissima* trees with sleeves containing adult spotted lanternflies, *Lycorma delicatula* (SLF). One sleeve contained males and the other contained females. Natural aggregations of free-living adult SLF accumulated beneath both sleeves (arrows). The nearby *A. altissima* trees of similar size (circled) had no SLF aggregations.

contained no SLF. If a sleeve or trap was found damaged, the whole block was excluded from analysis for that week. Weekly trapping was conducted from August 10 to October 26 in 2020, and from August 17 to November 3 in 2021, for a total of 11 weeks of trapping each year with the start date staggered by one week (Table 1).

Capture of naturally occurring SLF on trees with artificial aggregations - experimental design in 2020

Rural field sites were located on private properties with forest edges in Warren County, NJ, selected for their pairs of similarly-sized and spaced *A. altissima* trees, as well as the presence of low density populations of SLF. This was determined in the early spring by visual inspection of each site by two experienced scouts, and finding only 1 egg mass or 1-10 nymphs in 15 min of searching. Seven sites in Warren County, NJ were selected to establish 10 blocks, each containing a pair of *A. altissima* trees spaced 2 to 3 m apart. In 2020, the average difference in diameter at breast height (DBH) between male- and female-sleeved trees in each pair was 4.2 cm, with the male-sleeved tree being the larger tree in 5 blocks, and the smaller tree in the other 5 blocks. The average tree DBH (± SE) was 18.6 (± 1.6) cm. Data from one block was discarded in week 3 due to weather knocking down a trap (Table 1). In 2020, each block consisted of two sleeved trees, one containing 40 adult male SLF and one containing 40 adult female SLF to answer the question "who finds whom and when?" based on the number of naturally occurring adult male and female SLF captured each week on male- or female-sleeved trees.

Capture of naturally occurring SLF on trees with artificial aggregations - experimental design in 2021

A second experiment, conducted in 2021, attempted to duplicate the first experiment, but with the addition of a third *A. altissima* tree to each block, outfitted with a trap and an empty sleeve which served as a control to demonstrate what a normal wild, or naturally occurring, population would look like. The purpose of adding the control trees was to assess whether the presence of artificial aggregations resulted in wild aggregations. Field sites in 2021 consisted of a mix of private properties and state wildlife management areas, with forest edges. In 2021, 11 blocks were initially established on six rural properties; nine were in Sussex County, NJ and two were in Warren County, NJ. During the study, two blocks in Warren County, were abandoned due to bear activity. However, two additional blocks were established mid-study in Sussex County. Since other blocks already had established sleeves, the sleeves in the newly added blocks were allowed to establish for one week prior to data collection, resulting in a total of 9 or 10 blocks each week (Table 1). As in 2020, paired male and female trees in 2021 were 2 to 3 m apart except for one pair that was 3.5 m apart. The control tree represented either the third point on a triangle with the other two trees, or the third in a line if a suitable tree in the triangle position could not be found. Sites in 2021 were selected not only for their presence of triplets of similarly-sized and spaced *A. altissima*, but also the presence of low density populations of SLF. Prior to the experiment in 2021, populations were sampled with circle traps set on June 30, 2021, and captures of 30-40 SLF per site over a 5-week period indicated a low initial population density. In 2021, the average

TABLE 1 A description of the timing of spotted lanternfly, *Lycorma delicatula*, activities in the field, and numbers of trapping block replicates per week per year.

			Trapping blocks (N)		
Stage	**Week**	**Date Range**	**2020**	**2021**	**Primary activities observed on *A. altissima***
Early-1	1	8/10 – 8/17	10		Adults recently emerged, feeding
	2	8/17 – 8/24	10	10	Feeding continues
Early-2	3	8/24 – 8/31	9	10	Feeding continues, aggregations form, sex ratio sharply changes to female-biased
	4	8/31 – 9/7	10	9	Flight behavior increases
Mid	5	9/7 – 9/14	10	9	Sex ratio shifts back again, first observation of courtship and mating in the field
	6	9/14 – 9/21	10	10	Courtship and mating increases, first observation of oviposition in the field
Late-1	7	9/21 – 9/28	10	9	Oviposition increases, courtship and mating continue
	8	9/28 – 10/5	10	9	Oviposition increases and courtship and mating decreases
Late-2	9	10/5 – 10/12	10	10	Oviposition continues and courtship and mating decreases
	10	10/12 – 10/19	10	10	Oviposition continues and courtship and mating taper off
Late-3	11	10/19 – 10/26	10	10	Oviposition becomes most observed behavior
	12	10/26 – 11/2		10	Oviposition continues, death with freezing temperatures

Events denoting key physiological shifts, such as the first observations of mating and freshly oviposited egg masses, occurred approximately 5 calendar days later in 2021 than in 2020. Consequently, stage designations are slightly offset in the two years, but for purposes of labeling we use the stage designations from 2020.

difference in DBH between male- and female-sleeved trees within each block was 1.6 cm, with the male sleeve being on the larger tree in half of the blocks and on the smaller tree in the other half of the blocks. Control trees were on average 4.1 cm DBH larger than the other trees in their blocks. The average DBH (± SE) of all trees used in 2021 was 19.7 (± 0.8) cm. The numbers of egg masses deposited inside the sleeves was noted weekly.

Photographic data on SLF clusters

To record SLF that may have landed on trees without entering traps (see Figure 1), in 2021 a photograph of each tree was taken weekly from August 10 when sleeves and traps were first set up until October 27. Each photograph encompassed the tree trunk from the ground to just above the trap on any side where any SLF were seen. For each tree photograph, the total numbers of free-living SLF, and the numbers of clusters of free-living SLF, defined by two or more SLF physically touching each other, were quantified.

Marked-released-recaptured SLF adults

In addition to investigating movements of naturally occurring SLF with respect to the artificial aggregations at low density sites, in both years a second study was superimposed at the same time and place, in which a known number of marked male and female SLF were released on the ground, halfway between the male-sleeved and female-sleeved trees, and their responses were recorded given their known starting point and an equal probability of arriving at either tree. Equal numbers of males and females were released each week, but in 2020, weekly releases varied between 10-25 of each sex (average of 16.4) released per block. In 2021, 15 SLF of each sex were released weekly between each male- and female-sleeved tree pair. Since the density of SLF naturally occurring on trees was an uncontrollable factor with the potential to influence where marked SLF arrived, and SLF density was found to contribute to SLF orientation in the field (MFC, unpublished) (21), the relative SLF density between the trees in each pair was taken into consideration in the final analysis. Each week, the number of naturally occurring SLF per cm circumference caught on each tree was counted and categorized into one of eight categories (<0.1, 0.1-0.5, 0.5-1, 1-2, 2-3, 3-6, 6-9, and 9-12 SLF per cm circumference of the tree at breast height). For each week, if one tree fell into a different density category than the other tree in its pair, they were considered to have different densities: higher and lower. If they were in the same density category, the trees in the pair were considered to have the same density. For each release, the combination of these density categories with the male sleeve vs. the female sleeve choice, were considered in the analysis of which tree in each pair the released SLF chose. Therefore, the following density-sleeve treatments were compared: higher-female vs lower-male, lower-female vs higher-male, or same-female vs same-male.

Insects

At the beginning of each week, adult SLF were collected and sexed, sleeves were restocked, trap bags were changed, and SLF were marked and released. SLF were collected from *A. altissima* growing nearby (<30 km) private properties that were heavily infested with SLF and were free from pesticides. This ensured sleeves had the correct number of live SLF in them at the beginning of each trapping period. For the mark-release-recapture experiment, equal numbers of male and female SLF were dusted with fluorescent powder dye (DayGlo Color Corp., Cleveland, OH) and released on the ground halfway between the trees with male and female sleeves. A different color dye was used each week to determine how long ago the recaptured SLF had been released.

Data analysis

The total naturally occurring SLF captured and their sex ratio (percent male), for the entire season on the paired male- and female-sleeved trees in 2020 were examined using a matched paired T-test ($\alpha = 0.05$). Sex ratio data in 2020 were normally distributed, but season totals of males, females, and total SLF were not. Therefore, log transformation was used to normalize the data for the analysis of season totals. In 2021, with the addition of a third treatment to each block, totals for the entire season were log transformed, and sex ratios were arcsin-square-root-transformed, which normalized the data, which was then analyzed using ANOVA and Tukey means separation ($\alpha = 0.05$). Back-transformed data are reported.

Weekly catch of males, females, and sex ratio was examined to expose patterns or changes over time. For this, a Wilcoxon test was used because data were not normally distributed due to many zeroes ($\alpha = 0.05$). In 2021, for weeks showing significance, a Wilcoxon test was conducted on each pair with Bonferonni correction ($\alpha = 0.025$).

Photographic data in 2021 were also not normally distributed. Data were consolidated into three periods based on the dominant behavioral activity, feeding (weeks 1-5), mating (weeks 6-9), or oviposition (weeks 10-12), and the number of clusters were compared by these time intervals, and by sleeve treatments, using Wilcoxon test and Bonferroni corrections (α=0.025). If

found to be significant, Wilcoxon pairwise comparisons were performed, also with Bonferroni corrections (α=0.0125). The same analysis was conducted for total number of SLF per tree. All above analyses were conducted using JMP (v. 10.0.0).

For the mark-release-recapture study, due to low numbers of recaptured SLF, data for 2020 and 2021 were combined and grouped into three 4-week time periods as follows. Early-1 and Early-2 corresponded to the first four weeks of data collection from August 10 to September 7 when feeding was the primary activity and mating had not yet been observed in the field. Mid and Late-1 corresponded to the second four weeks of data collection from September 7 to October 5 when mating was observed in the field and was the primary activity, but it included the beginning of oviposition. Finally, Late-2 and Late-3 corresponded to the final four weeks of data collection from October 5 to November 2, when oviposition was the primary activity in the field, courtship and mating activity tapered off, and adults began to die (Table 1). The *post hoc* analysis categorized the treatments into groupings based on whether one tree in a pair had higher, lower, or the same naturally occurring SLF background density relative to the other tree in the pair that week, as described above. Because each insect released equidistant between two trees had an equal chance of arriving at either tree, a chi-square test was used to test the null hypothesis that released male and female SLF would arrive at the male-sleeved and female-sleeved trees with equal frequency (α = 0.05 with G $\geq$ 3.84) (22).

Results

Phenology

A general phenology of observed activities is described in Table 1 with definitions of the adult phases, names given to each two-week period, and the number of replicates acquired in each week and year. Developmental stages in 2021 lagged behind those in 2020 by approximately 5 calendar days.

Mating in the field was first observed on September 8 and 13, in 2020 and 2021, respectively, marking the onset of the "Mid" stage. Approximately one week later, on September 16 and 20, in 2020 and 2021, respectively, the first freshly oviposited egg masses were observed in the field, and mating activities began to overlap with oviposition activities.

Capture of naturally occurring SLF on trees with artificial aggregations

In 2020, 13,567 free-living SLF were captured. Over the course of 2020, there were no significant differences in total SLF, males, or females captured on trees with sleeved males as with sleeved females, but total sex ratios differed significantly, as detailed below (Figure 2). Seasonal changes in trap capture of free-living total, male, and female SLF in 2020 can be seen in Figure 3 (A, B, and C, respectively), for each treatment. At the beginning of the season, numbers of naturally occurring SLF captured per trap per week started out lower than the numbers within the sleeves, but increases of males (starting week 6) (Figure 3B) and females (starting week 3, and again in week 9) (Figure 3C) caused a surge in total SLF captured per week, exceeding the numbers in the sleeves in 2020 (Figure 3A). Over time, although males were captured significantly more on trees with male sleeves during weeks 1-8 (Figure 3B), there was no clear indication of which sex found the other sex for mating in 2020.

In 2021, which included control trees and sites with lower background densities than the prior year, 4,519 free-living SLF were captured. In 2021, significantly more males were captured on trees with male sleeves (86.6 ± 16.4) than on control trees (33.3 ± 14.2), and the number of males caught on trees with female sleeves (95.8 ± 47.2) did not differ from the other two treatments (P = 0.016, 0.128; F-ratio = 5.10, 1.79; df = 2, 10). Significantly more females were captured on trees with female sleeves (90.5 ± 29.1) than control trees (46.5 ± 22.9), and the number of females caught on trees with male sleeves (58.6 ± 23.9) did not differ from the other two treatments (P = 0.012, 0.012; F-ratio = 5.56, 3.25; df = 2, 10). In total, significantly more SLF were captured on trees with male or female sleeves than on control sleeves (P = 0.010, 0.083; F-ratio = 5.80, 2.05; df = 2, 10) (Figure 2). Thus, the artificial aggregations drew significantly more SLF to those trees than controls, and a pattern of males locating male sleeves, and females locating female sleeves, was seen.

Seasonal changes in trap capture of free-living total, male, and female SLF in 2021 can be seen in Figure 3 (D, E, and F, respectively), for each treatment. As seen in 2020 (Figures 3B, C), in 2021 there was a sharp influx of males (starting week 6) (Figure 3E) and females (starting in week 4) (Figure 3F). The influx of females diminished in week 7 on male and control sleeves, but was sustained on female sleeves thereafter (Figure 3F). The influx of males occurred on both male and female sleeves, but not on control sleeves, and was sustained until week 12 (Figure 3E). In both years, the influx of females occurred in Early-2, followed by the influx of males during Mid, when mating started.

By looking at the numbers captured over time in 2021, it was again not obvious which sex attracted the other for mating, because the significant values indicated that males were more attracted to male sleeves, and females were more attracted to female sleeves. However, a difference in the behavior between males (Figure 3E) and females (Figure 3F) appears as a trend

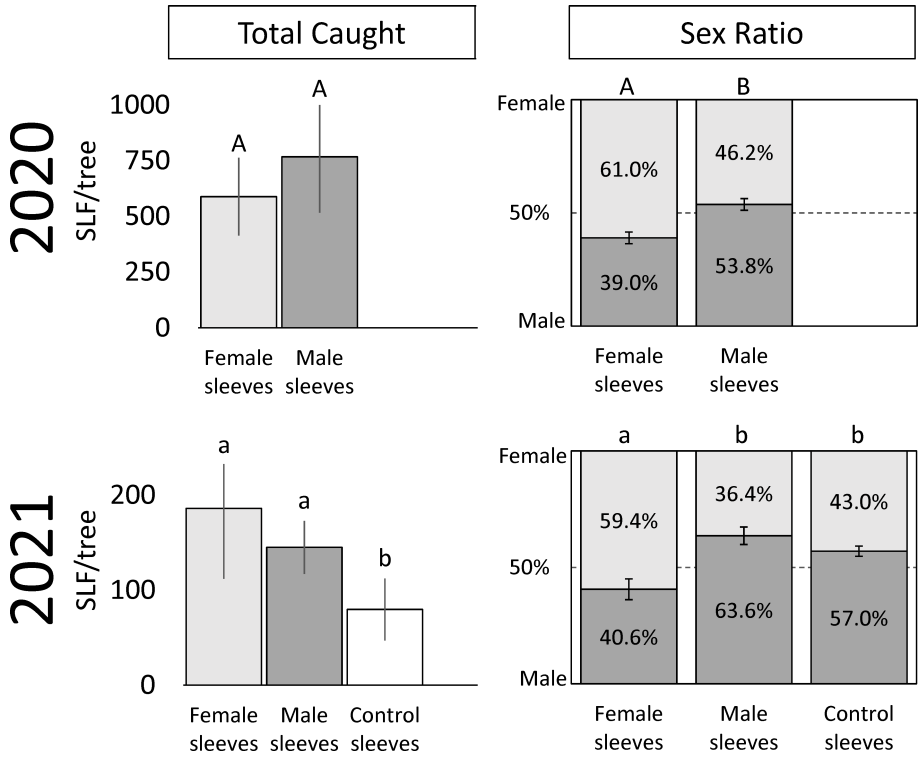

FIGURE 2
The average numbers (± SE) of total free-living adult spotted lanternflies, *Lycorma delicatula* (SLF) captured, and their overall sex ratios, on trees outfitted with sleeves containing artificial aggregations of either SLF males or females, or containing no SLF (control sleeves) over the entire trapping period in 2020 and 2021. Within each measured variable (total SLF caught and sex ratio) and year, bars with the same letters do not differ significantly.

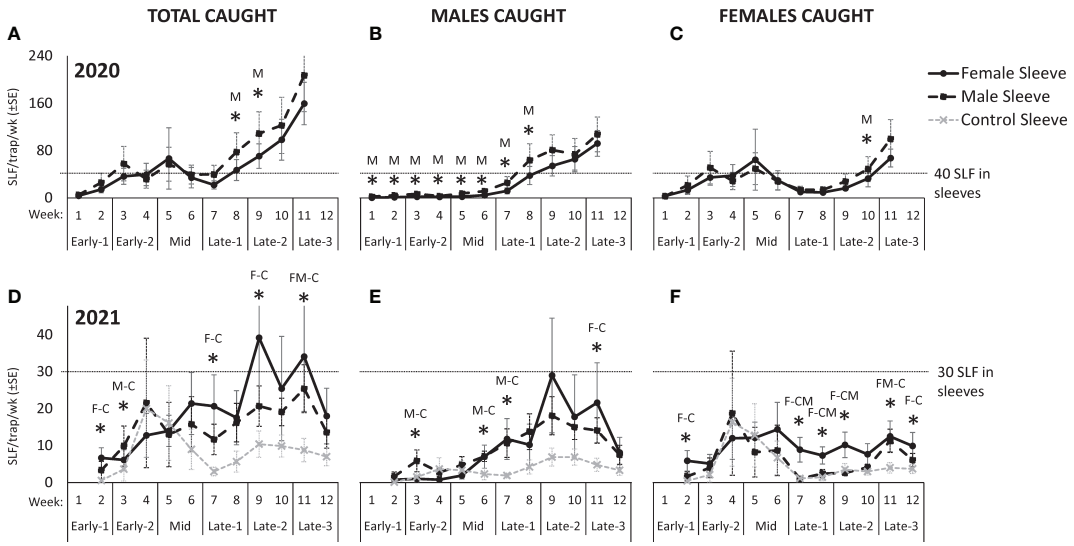

FIGURE 3
The weekly average numbers (± SE) of total, male, and female, respectively in 2020 **(A–C)** and 2021 **(D–F)**, of free-living adult spotted lanternflies, *Lycorma delicatula* (SLF) captured on trees outfitted with sleeves containing artificial aggregations of SLF males (M) (black dashed squares) or females (F) (black solid circles) or control sleeves containing no SLF (C) (gray dashed x). Asterisks indicate significant differences between treatments. In 2020, the letter represents which sleeves caught more. In 2021, letters of sleeves that were significantly different are separated by a dash.

over time starting in week 6 in 2021 (Mid), in that both male and female sleeves attracted more males than control sleeves (Figure 3E), but only female sleeves attracted females, not male sleeves or control sleeves (Figure 3F). This trend suggests that females aggregated on all three treatments prior to mating and with females after mating, but males started locating aggregations (not control trees) around the time that mating started (week 6).

Sex ratios of naturally occurring SLF

In 2020, trees with sleeved males had significantly higher sex ratios of captured SLF (53.8% male) than trees with sleeved females (39.0% male) (Paired t-test; $P = 0.0187$, t-ratio = 2.86, df = 9) (Figure 2). Similarly, the time sequence and total season sex ratio data suggested that each sex was most attracted to its own sex in 2020 (Figures 2, 4). A similar pattern was found in 2021, where trees with sleeved males had significantly higher sex ratios (63.6% male) than trees with sleeved females (40.6% male), and control sleeves (57.0% male) which differed from female-, but not male-, sleeved trees ($P = 0.004$, 0.001; F-ratio = 7.44, 4.88; df = 2, 10) (Figure 2). In both years during Early-2, the sex ratio on female sleeves was less than 10% male. Even though female-sleeved trees were more female biased than male-sleeved trees, in both years the sex ratios of each treatment shifted over time in a similar pattern, from more female- to more male-biased, then converging to approximately 50% at the end of the season (Figure 4).

Photographic data on SLF clusters

The numbers of free-living SLF and clusters of SLF on each tree photograph were compared for differences between sleeve treatments in each time period, and between time periods in each sleeve treatment ($\alpha = 0.025$). The number of clusters changed over time for all treatments (Figure 5A): control sleeves ($P < 0.001$, chi-square = 18.441, df = 2), female sleeves ($P < 0.001$, chi-square = 15.69, df = 2), and male sleeves ($P < 0.001$, chi-square = 31.29, df = 2). Pairwise comparisons for each significant factor showed that for control sleeves, there were significantly more clusters during oviposition time than feeding time ($P < 0.001$, Z = -3.86, df = 2) and mating time ($P = 0.012$, Z = -2.49, df = 2); for female sleeves, there were significantly fewer clusters during feeding time than mating time ($P = 0.001$, Z = 3.24, df = 2) or oviposition time ($P < 0.001$, Z = -3.96, df = 2); and for male sleeves there were significantly more clusters during oviposition time than either feeding time ($P < 0.001$, Z = -4.68, df = 2) or mating time ($P = 0.001$, Z = -3.13, df = 2) (Figure 5A). During feeding and oviposition time, there were no differences between sleeve treatments, but during mating time there were significantly more clusters on female sleeves than on male sleeves ($P = 0.010$, Z = -2.59, df = 2).

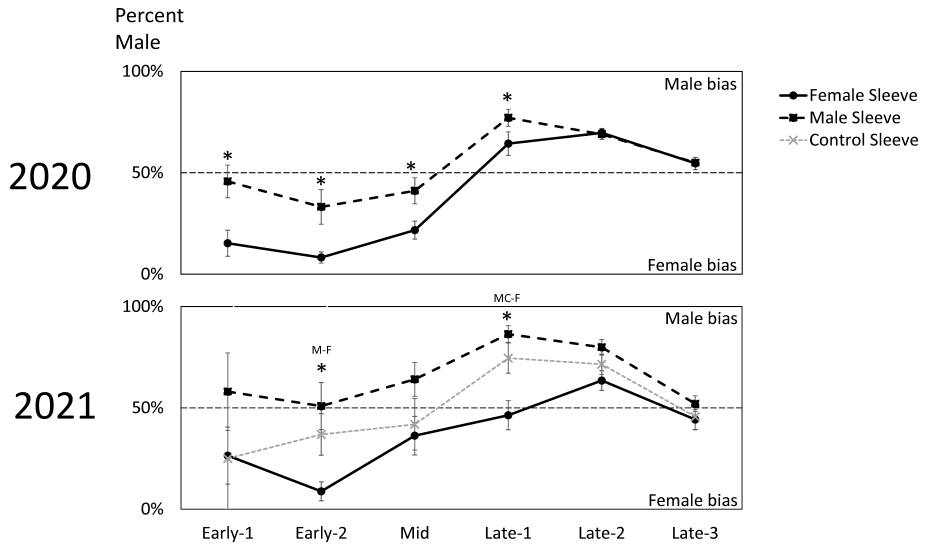

FIGURE 4
The biweekly average sex ratios (± SE) of naturally occurring adult spotted lanternflies, *Lycorma delicatula* (SLF) captured on trees outfitted with sleeves containing artificial aggregations of SLF males (M) (black dashed squares) or females (F) (black solid circles) or control sleeves containing no SLF (C) (gray dashed x) in 2020 and 2021. Asterisks indicate significant differences between treatments. In 2021, letters of sleeves that were significantly different are separated by a dash.

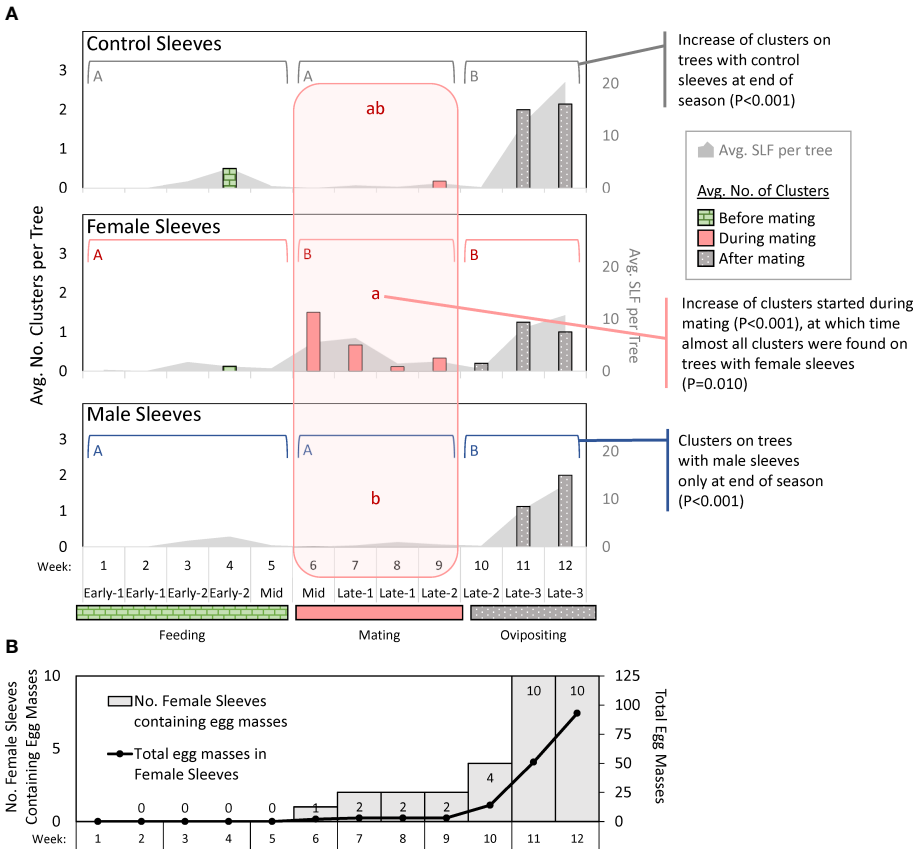

FIGURE 5
Graphs in **(A)** show weekly average numbers of clusters (columns) of adult spotted lanternflies, *Lycorma delicatula* (SLF) photographed on trees outfitted with control sleeves containing no SLF or sleeves containing artificial aggregations of 30 SLF females or males in 2021. Brackets with different uppercase letters signify differences between time intervals during which feeding, mating, or oviposition was the primary activity. The lowercase letters in the shaded vertical area signify differences between sleeve treatments during the mating period. No other period had significant differences between sleeves. The gray shaded areas represent the average numbers of free-living SLF per tree in photographs (secondary y-axis). The bottom graph **(B)** shows the number of female sleeves with egg masses (shaded), and the total number of egg masses in those sleeves (line) over time in 2021.

The same analysis was conducted on the total number of SLF per tree in photographs, and there were no differences between sleeve treatments for any time period (α = 0.025). It was, therefore, removed from the model. Time period differed significantly in the overall model ($P < 0.001$, chi-square = 62.44, df = 2), and the number of SLF was different between feeding (score mean = 98.6) and mating (score mean = 126.4) ($P < 0.001$, Z = 3.69, df = 2), feeding and oviposition (score mean = 174.7) ($P < 0.001$, Z = -7.77, df = 2), and mating and oviposition ($P < 0.001$, Z = -4.02, df = 2).

Egg masses inside sleeves

In 2021, no egg masses were deposited inside any control sleeves, and a free-living female SLF entered through a hole in one male sleeve and deposited a single egg mass in that sleeve in week 11, which was still present in week 12. Inside female sleeves, there were no egg masses during weeks 1 through 5. The total (and average) number of egg masses accumulating inside all female sleeves from week 6 to 12, respectively, were 2 (0.2), 3 (0.3), 3 (0.3), 3 (0.3), 14 (1.4), 51 (5.1), and 93 (9.3). In weeks 6-9, only two female sleeves contained egg masses. In week 10, only four sleeves contained egg masses. In weeks 11 and 12, all female sleeves contained egg masses (Figure 5B).

Mark-release-recapture of SLF adults

In the two years combined, a total of 6,630 SLF were captured, marked, and released, and 1,514 of those were recaptured (22.8% total recapture rate). In 2020 and 2021, 24.8% and 20.6% of marked-released SLF were recaptured, respectively (Table 2). The vast majority of SLF were

recaptured in the first week of their release, but 14% and 4.2% of marked males and females, respectively, were recaptured in the weeks that followed (Table 2). The proportion of trees that caught lower numbers of unmarked SLF per week than the number of SLF inside the sleeves for Early, Mid/Late1, and Late2/Late3, were 87%, 68%, and 27%, respectively for 2020, and 92%, 86%, and 85% in 2021, indicating that most of the time the naturally occurring SLF population density was low compared to the aggregations within sleeves. Timing, relative background density, and the sex of the artificial aggregation contained within the sleeve all played a role in what choices were made by marked-released-recaptured SLF (Figure 6). Figure 6 compares marked SLF responses, given a choice between two trees, taking into consideration differences in the natural SLF density occurring on the two trees within each pair, and which direction the difference was with respect to the contents of the sleeves. Relative background density interacted with sleeve choice, in that the trees with the higher relative background densities were chosen significantly more. A given tree did not have the same relative background density designation each week, thus background populations of SLF and their tree preferences fluctuated, but they did have the same sleeve designation (male or female) each week. By comparing the significant choices of marked-released SLF on the higher density trees (controlling for weekly relative background density changes), significant preferences were revealed. During Early, Mid, and Late, marked females significantly preferred trees with the higher background density when associated with sleeves containing females (Figures 6A–C), but not sleeves containing males (Figures 6D–F). Early males showed no preference for either sleeve coupled with the higher density tree (Figures 6J, M). During Mid, marked-released males significantly preferred the higher density tree when coupled with sleeves containing females (Figure 6K), but not when coupled with sleeves containing males (Figure 6N). During Late, marked males preferred the higher density trees regardless of the sex within the sleeve (Figures 6L, O). All other combinations resulted in no preference. Neither males nor females at any time demonstrated a sleeve preference when their background densities were equal. The naturally occurring average weekly SLF background density (wild SLF per cm circumference of the tree) for the nine comparisons is displayed in Figure 6.

TABLE 2 The total number of marked adult spotted lanternflies, *Lycorma delicatula* (SLF) in 2020 and 2021 combined that were recaptured, the overall recapture rates of females and males during 4-week time periods, and the number of weeks after which different proportions of recaptures occurred.

	Females	Males
Total SLF recaptured	789	695
Overall recapture rates (%)		
Early1-Early2	27.3	20.4
Mid-Late1	23.7	20.2
Late2-Late3	21.2	22.5
Weeks after release (%)		
1	95.7	86.1
2	2.0	7.4
3	1.1	3.5
4+	1.1	3.1

Discussion

Our main objectives were to determine who finds whom and when among SLF, and if we can use artificial aggregations to manipulate natural aggregations. The experiments demonstrated that, overall, adult SLF oriented significantly to confined artificial aggregations of other adult SLF. Specifically, caged aggregations of males drew significantly more free-living male SLF than controls, and caged aggregations of females drew significantly more free-living female SLF than controls. As such, in both 2020 and 2021, sex ratios (percent male) of free-living SLF were significantly more male-biased on male sleeves and female-biased on female sleeves (Figure 2). This evidence suggests that the natural male- and female-biased sex ratios that have been previously reported on different trees (12), likely form at least partly in response to sex-specific conspecific signals. The fact that the female sleeve treatment had significantly more female-biased sex ratios than male or control treatments, which were similar to each other, suggests that signals produced from females aggregating on *A. altissima* attracted more females. Such signals could be derived from the insects themselves, or from an interaction between the insects and their host plant. Logistical considerations precluded adding an additional treatment to test mixed-sex artificial aggregations, which is also of interest. However, due to the abrupt shift we have repeatedly observed in naturally occurring sex ratios from relatively unbiased during Early-1, to extremely male- or female-biased on different trees in the same vicinity which we use to characterize the Early-2 phase, it was decided that measuring SLF responses to same-sex aggregations was the primary question for this study.

Looking at the capture data over time, some interesting trends and differences are revealed (Figure 3). In both years, free-living females started arriving and becoming captured in large numbers on all treatments during Early, approximately two weeks prior to mating (Figures 3C, F). Captures of arriving males started to surge two weeks later during Mid, when mating began, and only on trees with artificial aggregations (Figures 3B, E). This difference in arrival time between naturally occurring females and males is reflected in the sex ratio shifts over time seen in both years (Figure 4), where sex ratios were more female-biased during Early. Around mating time (Mid), sex ratios approached 50% (Figure 4), and arriving females started showing a significant preference for confined

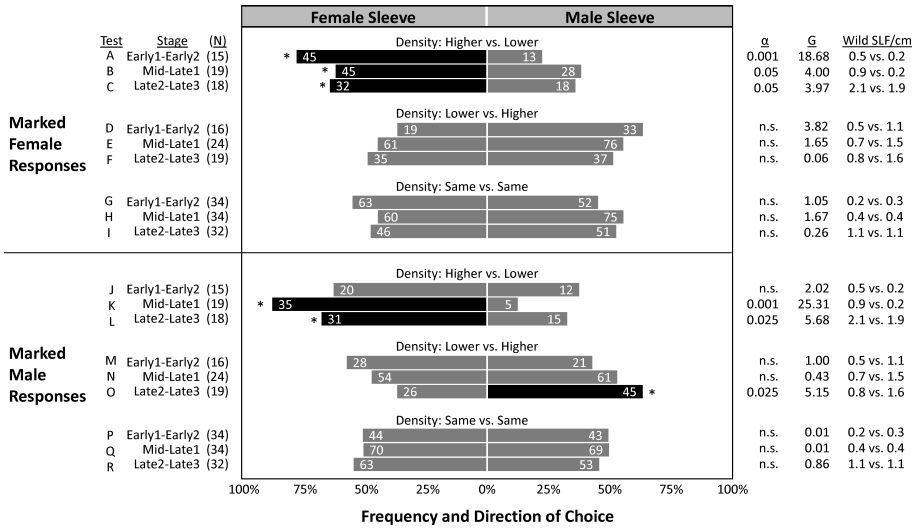

FIGURE 6
The frequency of choices made by marked-released-recaptured adult spotted lanternflies, *Lycorma delicatula* (SLF) (x-axis) of different ages in the field. In 2020 and 2021, groups of marked female and male adult SLF were released weekly halfway between pairs of trees outfitted with sleeves containing artificial aggregations of either SLF females (left) or males (right). Pairs of trees were categorized *post hoc* based on their relative naturally occurring unmarked SLF population density (wild SLF/cm circumference of the tree caught per week) relative to that on the other tree in each pair. The numbers in each bar indicate the total number of marked-released SLF that were recaptured over each 4-week trapping period. Asterisks and black shading indicate the choices that deviated significantly from predicted (Chi square test). The numbers of releases in each category are shown as (N). Critical alpha levels of significance, test statistic G, and average naturally occurring densities (number of wild SLF per cm circumference) on the female-sleeved vs. male-sleeved trees being tested are shown in columns on the right. Alpha greater than 0.05 indicates no significant difference (n.s.).

females over confined males or controls (Figure 3F). After oviposition was observed (Late), female arrivals somewhat diminished (Figures 3C, F) and male arrivals continued to increase (Figures 3B, E), resulting in a male bias during Late (Figure 4).

In the mark-release-recapture experiment, we cannot remove the influence that the naturally occurring population may have had on marked SLF, nor can we separate the effects of the sleeves on that naturally occurring population. However, we can analyze tree pairs based on the combination of those factors and look at their combined effects on the choices of marked SLF. In doing so, attraction to the tree in the pair with the higher density natural SLF population was observed as interacting with the sleeve treatments, in which marked-released adult SLF distinguished between sleeves containing either males or females only when that sleeve coincided with the tree with the higher background density. Corresponding with the timing of the natural surge in female arrivals, marked-released Early females significantly and most strongly preferred higher density trees only when combined with female aggregations (Figure 6A) but not with male aggregations (Figure 6D). This significant attraction of marked females to higher density trees with confined females, but not confined males, continued into Mid and Late, but was most pronounced during Early. No significant preferences were found in marked Early males. Corresponding with the timing of the later surge in males during mating time, marked-released Mid males significantly and most strongly preferred higher density trees when combined with confined females (Figure 6K) but not with confined males (Figure 6N), showing a strong attraction of marked Mid males to Mid females. Males during the Late stages, when natural populations were higher and sleeved females were unlikely to have been sexually receptive (as indicated by oviposition inside sleeves), significantly chose the tree with the higher background population density regardless of the sleeve contents (Figures 6L, O). When the higher background population density was on the male trees, there was little effect of the sleeves on choices of marked SLF. Curiously, in the absence of background population density differences between trees (Figures 6, G–I, P–R), sleeve contents had no effect on choices of marked SLF, leaving some unanswered questions as to why sleeves containing males or females were able to influence the naturally occurring population, but not marked individuals released midway between paired trees. Thus, the results do not explain all of the observed behaviors and additional work is still needed to fully decipher how SLF make decisions when locating each other for mating or aggregation. From the significant trapping results of naturally occurring SLF and marked-

released SLF captured over time, it appears that females locate females for aggregation and feeding, and males locate female aggregations for mating. Aggregation in insects is not defined by a single set of behaviors or mechanisms, and although attraction can play a role in aggregation, at the other end of the spectrum aggregation can result from random movements combined with arrestment (23, 24). Thus, a variety of different behavioral mechanisms may result in aggregation. Although these field experiments describing SLF aggregations over time in response to artificial same-sex aggregations provide key information about who finds whom and when, and demonstrate that aggregations can be manipulated, more work is needed to determine how aggregations are initiated or the mechanisms used to aggregate.

In 2020, the free-living SLF population density became much higher than the numbers of SLF in the sleeves, likely influencing the results that year (Figure 3A). However, the lower population densities in 2021 allow a look at SLF responses with less influence from naturally occurring populations (Figure 3D). In 2021 over time, especially after week 5 (Mid), more males were caught on male sleeves than controls (Figure 3E), and more females were caught on female sleeves than control or male sleeves (Figure 3F). The presence of the control trees in 2021 revealed a trend that, once mating had begun, males consistently oriented to both male and female sleeves more than controls (Figure 3E), but females oriented to female sleeves, not male sleeves or controls (Figure 3F). Thus, attraction was not symmetrical between sexes in that males were attracted to both males and females but females were attracted to only females. This likely resulted in the observed male- and female-biased populations of SLF on different trees. Such SLF sex ratio biases in the weeks leading up to mating have previously been described in natural populations (12, 13). The asymmetry in attraction speaks to the complexity of this system, suggesting multiple signalers and receivers, with potentially multiple sensory modalities involved, and illustrates how SLF attraction and aggregation behavior will not be fully conveyed by simple explanations.

Our field data on long range attraction corroborates results from laboratory walking olfactometer bioassays testing attraction to SLF-derived volatiles, giving evidence to suggest these behaviors may be mediated by pheromones to some degree (10, 16). Olfactometer studies found that male SLF were attracted to volatiles only from male-produced honeydew, and although not significant, females trended towards attraction to honeydew from females, but not males (10). In olfactometer studies on SLF body volatile extracts, we found that Early males were attracted to body volatiles from both sexes, but females were not (16). In that study, Mid males were able to distinguish between the body volatiles of Mid males and females and were attracted only to the volatile extracts from females. Therefore, a proposed set of mechanisms for the observed field attraction of males and females at different times is starting to materialize in which both body volatiles and honeydew volatiles from male and female SLF may play sex-specific roles in attraction for the purposes of aggregation and mating. This does not exclude the possible use by SLF of other conspecific communication mechanisms or signals, such as the release of plant damage volatiles from feeding activity, or substrate vibrations, which are commonly used by other members of Hemiptera to form aggregations or locate mates (25). However, substrate vibrations are limited spatially in that the signaler and receiver typically must already be on the same substrate, and signals attenuate beyond a few meters (26–28).

The trapping studies did not evaluate arrestment or aggregation behavior because they measured differences in the numbers of SLF that arrived on tree trunks, which is a measure of attraction. What happened after SLF arrived, such as arrestment or courtship, could be captured by the photographic data, which provided snapshots of their positions and behavior over time. Photographs informed us of where and when clustering, our measure of courtship, took place. This was defined as groups of two or more SLF that were physically in contact, often positioned in parallel or in groups, with bodies touching. Clustering during mating time was almost exclusively on trees with female sleeves (Figure 5A). Superficially, this side-by-side pairing of male and female SLF during mating time (see 9, 12) appears similar to whitefly courtship behavior in which a combination of a short range sex pheromone and substrate vibrations are employed (29–31). In the final two weeks of the study, when egg masses had been deposited in all female sleeves (Figure 5B), the naturally occurring population of SLF increased on all sleeve treatments, as did clustering (Figure 5A). During this time, the increased numbers of free-living SLF on trees may have exceeded any effects of the sleeved SLF. It is unclear what drove this increase in SLF and clustering when oviposition was well underway. It is possible that females, having fed and mated, left depleted trees seeking oviposition sites, and that aggregation continues to occur throughout this process. If so, it could explain why egg masses can also be observed in clusters (KM, pers. obs.). Although snapshots of clustering behavior and SLF on trees showed an increase in all sleeve treatments by week 11, this was not reflected in weekly trapping data which indicated that female sleeves still captured the most SLF, followed by male sleeves, and then controls at that time (Figures 3D–F). What guides SLF behaviors during their oviposition period should be investigated further, but it was not the focus of this study.

The scarcity of data currently available on fulgorid chemical ecology can be attributed to a lack of exploration. Until the recent invasions of SLF in Korea (2004), Japan (2008), and the United States (2014) (32), fulgorid chemical ecology had been neglected in the literature. There are numerous examples in the literature of pheromone use within the three major suborders of Hemiptera. Most examples are in Heteroptera (true bugs) (see

reviews by 33, 34), and some are known from Sternorrhynca which includes aphids (35), whiteflies (29), scales (36), mealybugs (37), and psyllids (38). Pheromone use has even been documented in the suborder to which SLF belongs, Auchenorrhyncha, which contains cicadas, treehoppers, leafhoppers, planthoppers, and spittle bugs (39), although it is widely understood that this suborder relies heavily on sound or substrate vibrations to locate mates (25). More research describing the sensory ecology of SLF is critical to the success of any control program.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MC secured funding, conceived and designed the experiments, oversaw the experiments, analyzed the data, and wrote the manuscript. KM conducted the field experiments, oversaw and coordinated the field work, handled field logistics, and collected the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Development of rearing methodology for the invasive Spotted Lanternfly, *Lycorma delicatula* (Hemiptera: Fulgoridae)

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Lycorma delicatula, White (Hemiptera: Fulgoridae), spotted lanternfly, is a univoltine, phloem-feeding, polyphagous and invasive insect in the USA. Although a primary host for this species is *Ailanthus altissima*, tree of heaven, *L. delicatula* also feeds on a wide range of hosts important to the USA including cultivated grapevines. Due to the need for classical or augmentative biological control programs to reduce impacts of *L. delicatula* across invaded areas, we developed a laboratory-based rearing protocol for this invasive species. Here, we evaluated the use of *A. altissima* apical meristems, epicormic shoots, and fresh foliage cut from *A. altissima* as a food source for rearing newly hatched *L. delicatula*. On these sources of plant material <20% of *L. delicatula* developed into adults and no oviposition occurred. However, when young, potted *A. altissima* trees were used as a food source, >50% of *L. delicatula* nymphs developed to the adult stage under natural daylengths and temperatures ranging from 20–25°C. The addition of wild grapevine, *Vitis riparia*, did not increase survivorship or reduce development time. To elicit mating and oviposition, adults were provided with *A. altissima* logs as an oviposition substrate and maintained under shortened daylengths and reduced nighttime temperatures (12L:12D and 24°C:13°C). This resulted in 2.12 egg masses deposited per female, which was 4× more than when adults were maintained in standard rearing conditions (16L:8D and 25°C). Based on these experiments, we present a protocol for reliably rearing *L. delicatula* under laboratory and/or greenhouse conditions.

KEYWORDS

Lycorma delicatula, colony, Ailanthus altissima, rearing, phloem-feeding

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Introduction

Lycorma delicatula White (Hemiptera: Fulgoridae), spotted lanternfly, is an invasive planthopper first detected in the USA in Berks County, PA in 2014 (1, 2). *Lycorma delicatula* has continued to spread and establish populations across Eastern states (3). *Lycorma delicatula* is univoltine with four nymphal instars; first instar nymphs emerge from overwintered egg masses in the spring. Nymphs develop throughout the late spring and summer and begin to emerge as adults in July (4, 5). Adult populations feed heavily in the late summer and reproduce throughout fall, generally dying off during hard frosts (4, 5).

Lycorma delicatula is a polyphagous phloem feeder with over 100 host plants reported globally (6). Tree of heaven, *Ailanthus altissima* (Mill.) Swingle (Sapindales: Simaroubaceae), is often referred to as the primary or preferred host of *L. delicatula*, although it is not obligatory for completion of their development (7–9). Damage caused by *L. delicatula* phloem feeding has been reported on grapevine and peach trees in its invaded range in South Korea (4). Commercial vineyards in PA, USA have suffered losses despite rigorous insecticide regimes for this pest (4, 10). Recent studies on *L. delicatula* feeding effects on young peach trees, point to an increase in frost damage susceptibility after *L. delicatula* infestation (11). *Lycorma delicatula* also causes nuisance problems and indirect plant damage as they produce large amounts of honeydew as they feed, which coats vegetation, enabling growth of black sooty mold (4).

Eradication efforts have focused on *A. altissima* management and removal (12), with longer term solutions targeting biological control agents (13–16). Indeed, an egg parasitoid *Dryinus sinicus* Olmi (Hymenoptera: Dryinidae), and a nymphal parasitoid *Anastatus orientalis* Yang & Choi (Hymenoptera: Eupelmidae), both from *L. delicatula*'s native range, are being evaluated for suitability within the USA as part of a classical biological control program. Rearing parasitoids, however, requires a continuous supply of the appropriate lifestage of the target host.

As *L. delicatula* is univoltine, establishing and maintaining a productive colony can be challenging. Information on such variables as diapause during the egg stage and nutritional needs for L. delicatula is still emerging (7–9, 17). Here, we evaluated several sources of *A. altissima* plant material and abiotic conditions promoting *L. delicatula* development and survivorship and different substrates for the promotion of mating and oviposition under laboratory conditions to generate standardized methods for maintaining a colony of *L. delicatula*. Our goal was to develop rearing methodology that is feasible and flexible for a range of research and biological control programs.

Materials and methods

Field collection of *Lycorma delicatula*

Lycorma delicatula egg masses for rearing studies conducted in quarantine facilities in Fort Detrick, MD and Blacksburg, VA were collected from host trees in a quarantine zone in Winchester, VA (within a 1-mile radius of 39°12'40.5"N 78°09'18.3"W). Sections of tree bark or branches harboring egg masses detached from trees were carefully handled and sized to fit into sealed Ziploc bags which were subsequently placed in sealed coolers and transported to quarantine greenhouses in accordance with APHIS permits P526P-18-03369 and P526P-18-02138, respectively. Additionally, nymphs and adult *L. delicatula* were collected from *A. altissima* in Winchester, VA, placed in mesh cages which were sealed in coolers, and transported to Fort Detrick, MD, where a quarantine greenhouse was used for additional rearing-related studies in accordance with APHIS permit P526P-18-03369. Insects for used for rearing studies from 2016-2018 at the Forest Pest Methods Laboratory, were collected from infested sites in Berks County, PA. Egg masses and the bark containing the mass were carefully chipped from host trees, placed in plastic boxes with mesh for ventilation. Boxes were double contained in a sealed 50 gallon barrel or sealed cooler, for transport to Buzzards Bay, MA. The egg masses were taken into quarantine, separated, dried in a laminar flow hood and stored in an environmental chamber (5° 0:0 (L:D) 65% RH) until they were removed and used for rearing studies. All collections were in accordance with APHIS permits P526P-17-04376.

Lycorma delicatula development and survivorship on *Ailanthus altissima* diet preparations

Three *A. altissima* plant diet materials were evaluated: 1) epicormic shoots generated on bolts of *A. altissima* >5 *cm* diam and placed in water and Maxi-Gro (General Hydroponics, Santa Rosa, CA) until shoots emerged ~ 4 weeks later; 2) apical meristems generated on bolts <5 cm diam and placed in water and Maxi-Gro to promote shoot and foliage propagation ~4 weeks later and 3) freshly cut branches from *A. altissima* in the field that included full leaves and woody stems (<5 cm diam) placed in water and Maxi-Gro. For rearing trials, plant material was cut to 50 cm in length, immediately placed in a container of water and Maxi-Gro and sealed in place using Parafilm (Bemis Company Inc., Neenah, WI) to prevent insects drowning in the water source (see Figures 1A, B). Two containers of each type of plant material (epicormic shoots, apical meristems or freshly cut branches) were placed in separate cages (W32.5 × D32.5 × H77.0 cm, 680 μm aperture mesh,

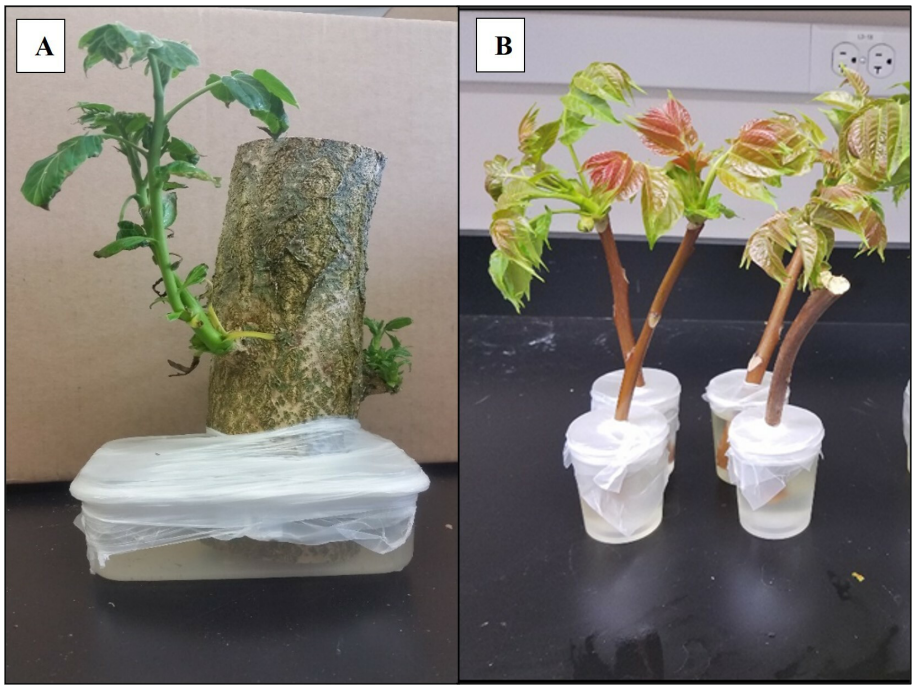

FIGURE 1
(A) Bolt of A altissima producing epicormic shoots in a container of fertilizer. **(B)** Apical meristem bolts of A altissima with foliage in a container of fertilizer. Photo credit: Mauri Hickin.

BugDorm-4S3074 Insect Rearing Cage, MegaView Science Co., Taiwan) and newly hatched (<72 h) *L. delicatula* nymphs were introduced. Survivorship and development were recorded every 2-3 d until adulthood, and plant material was changed as needed, generally once per week. All trials were conducted in quarantine facilities at USDA-APHIS, Buzzards Bay, MA (2016-2018), USDA-ARS, Fort Detrick, MD (2019), and Virginia Tech., Blacksburg, VA (2019). Across all locations five cages of containing epicormic shoots, seven cages containing apical meristems, and ten cages containing freshly cut branches were evaluated; all cages had starting numbers of 20 – 50 nymphs per cage.

Additionally, a preliminary trial evaluating the possibility of rearing *L. delicatula* nymphs on young, potted *A. altissima* was also conducted at Fort Detrick, MD in 2019. Newly hatched nymphs were placed in cages containing *A. altissima* (<1 year old, ~30 cm tall) trees. While nymphal starting numbers, survivorship, and developmental stage were not recorded during this pre-trial, the number of adults produced was documented.

Lycorma delicatula development and survivorship on potted *A. altissima* and *V. riparia* plant diets

In 2020, a study using potted plants based on 2019 preliminary trial was conducted. *Ailanthus altissima* trees were grown from seeds extracted from samaras collected in the field in October 2019. Samaras were stratified in a refrigerator at 5 – 7°C for 60 – 90 d, after which the wings were removed from seeds, and seeds then allowed to soak in water at room temperature (~22°C) for 18 h. Seeds were then planted into flat trays containing 5 cm deep potting mix (Pro-mix Premier BK25 Mycorrhizae, Premier Horticulture Inc., Quakertown, PA), and placed in a growth chamber (25°C, 16:8) to germinate. After 4 weeks, seedlings were transplanted into 6.5 L pots and moved to a greenhouse for maintenance with optimal conditions being 22 - 25°C with 16:8 L:D. Additionally, native grapevine, *Vitis riparia* Michx. (Vitales: Vitaceae), was purchased (Cold Stream Farm, Free Soil, MI) and planted into 0.6 L pot with potting soil. *V. riparia* vines were maintained at a length <50 cm under greenhouse conditions described above until use in rearing studies.

Two cohorts of nymphs were used. The first cohort included nymphs that emerged from eggs collected in Northampton and Lehigh counties, PA in October 2019, held at a constant 15°C until hatch in January 2020. The second cohort was comprised of eggs collected in Winchester, VA in February 2020, held at a constant 10°C for 2 months, and then at 25°C for 2-3 additional weeks until hatch in May 2020. For the first cohort, 43 1st instar nymphs and for the second cohort, 27 mixed 1st and 2nd instar nymphs were introduced per cage. Cages (W32.5 × D32.5 × H77.0 cm, 680 μm aperture mesh, BugDorm) contained either a

single *A. altissima* in a 6.5 L pot or an *A. altissima* in a 6.5 L pot and a *V. riparia* plant in a 0.6 L pot. Tracking development of the first and second cohorts began 24 January and 18 May 2020, respectively. All trials were conducted in a quarantine greenhouse at Fort Detrick, Frederick, MD (see Figures 2A, B for environmental conditions) under natural daylengths supplemented with strip lighting (T-5 High-Output Fixture - 54 W 2-Lamp, FarmTek, Dyersville, IA) set to 16L:8D. For each cohort, four cages containing *A. altissima* alone and four cages containing *A. altissima* plus *V. riparia* were assessed for survivorship and development of *L. delicatula* every 3 – 4 d. Plants were changed as needed, approximately every three weeks during 1st – 3rd nymphal instars and every two weeks thereafter. Once 4th instars molted to adults, they were transferred to corresponding cages held in an environmental chamber to monitor mating and oviposition.

Data from the first and second cohorts were analyzed separately, and all statistical analyses were performed using JMP software v.16.0 (SAS Institute, Cary, NC, USA). For each cohort, the effect of diet on the amount of time spent in each lifestage and total development period for *L. delicatula* were analyzed using an independent two-sample t-test.

Conditions Necessary to Elicit Oviposition

In September 2019, assessment of abiotic conditions necessary to elicit successful mating and oviposition in the quarantine greenhouse and growth chambers was conducted. Sources of adults used in these trials included: 1) adults reared from field-collected eggs on *A. altissima* trees (1 cage, 17M: 18F), 2) adults reared from late instar (3rd and 4th) nymphs collected from the field in Winchester, VA (1 cage, 19M: 20F), and 3) adults collected from the field in Winchester, VA (5 cages, 20-25 insects per cage, 1M: 1F). Cages containing adults from field-collected eggs and from late-instar nymphs were held in an environmental chamber at 12L:12D and 24°C:13°C to simulate

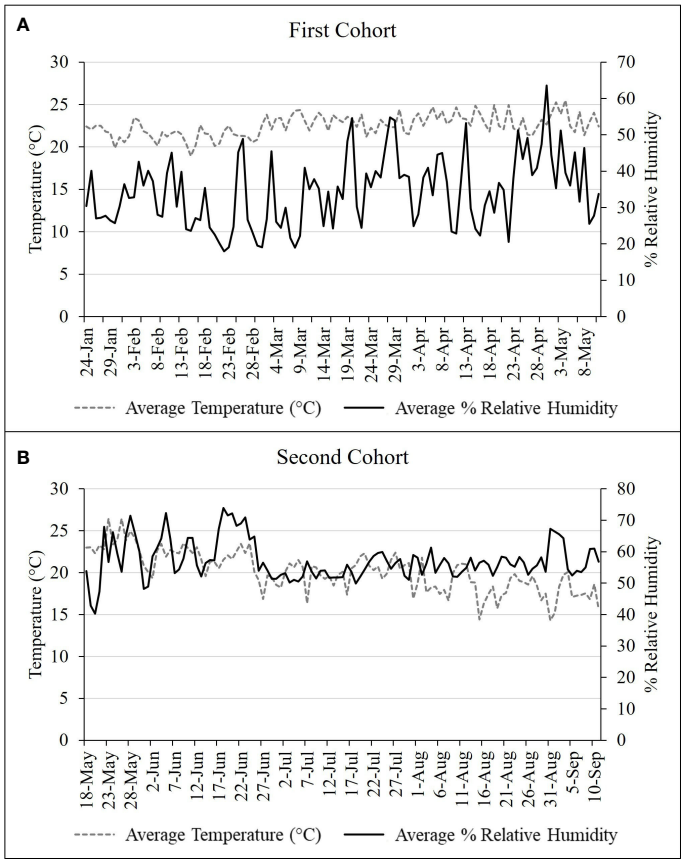

FIGURE 2
Daily temperature and humidity readings for **(A)** first and **(B)** second nymphal cohort study conducted in 2020 from January – May and May – September, respectively.

natural conditions in late summer and fall. Cages containing field-collected adults were held within the greenhouse space underwent temperature and humidity as shown in Figure 3 and natural light conditions, which ranged from 12L:12D in mid-September to 9.75L:13.25D at the end of November. Each cage (W47.5 x D47.5 x H93.0 cm, 680 µm aperture mesh, BugDorm-4S4590 Insect Rearing Cage, MegaView Science Co., Taiwan) was provided with the following substrates to promote egg laying: one potted *A. altissima* and one potted *V. riparia* (also serving as food sources), one *A. altissima* log (approx. 30 cm length, 8 cm diameter), one red maple, *Acer rubrum* L. (Sapindaceae: Sapindales) log (approx. 30 cm length, 8 cm diameter), and an open sided box constructed from two sheets of balsa wood (15 x 15 cm) separated by 2 cm spacers. Every 2-3 d, cages were inspected for fresh egg masses and dead adults were recorded and removed. Oviposition data were recorded from 26 September – 12 December 2019, concluding when the final female died. Data collected included oviposition date, substrate used, and number of egg masses per live female.

In 2020, adults produced from the two cohorts of nymphs were moved to cages containing their respective plant diets within an environmental growth chamber. This chamber was maintained at 65 ± 5% RH, 16:8 L:D, and 24 ± 3°C from July – September. From 1 October – November, the temperature was reduced to 18 ± 3°C but all other parameters remained the same. Each cage contained ~ 20 adults (~1M: 1F ratio) and was provided with one *A. altissima* log (approx. 30 cm length, 8 cm diameter); the first cohort of nymphs resulted in one cage of adults from each diet combination and the second cohort of nymphs resulted in three cages of adults from each diet combination. Cages were inspected for new egg masses every 2 – 3 d and dead adults were recorded and removed.

Lycorma delicatula survivorship and development from no-chill egg masses

A subset of the egg masses deposited on *A. altissima* logs in Fall 2019 were held in an environmental chamber at 12L:12D and 24°C:13°C with logs propped up diagonally within a cage (W32.5 x D32.5 x H77.0 cm, 680 µm aperture mesh, BugDorm) that contained a single potted *A. altissima* plant. After nymphs hatched on 2 January 2020, logs were removed, and chamber conditions were changed to 65% RH, 16:8 L:D, and 25°C, and a *V. riparia* plant was added. Survivorship and development of the hatched *L. delicatula* were monitored in this cage every 3 – 4 d until all insects died; plants were replaced when wilting occurred. As this was a preliminary trial, we did not record the total number of egg masses on logs or eggs per egg mass, therefore we provide descriptive results of documented egg hatch under no-chill conditions and nymphal development from hatched eggs.

Results

Lycorma delicatula development and survivorship on *Ailanthus altissima* diet preparations

Across all cut *A. altissima* plant material diets, a higher percentage of *L. delicatula* survived and developed on apical meristem compared with epicormic shoots or freshly cut branches (Figure 4). For trials conducted in VA quarantine facilities, 20% developed to the adult stage on both apical meristem and freshly cut branches, with 9.5% reaching adulthood on epicormic shoots (Figure 4A), which resulted in

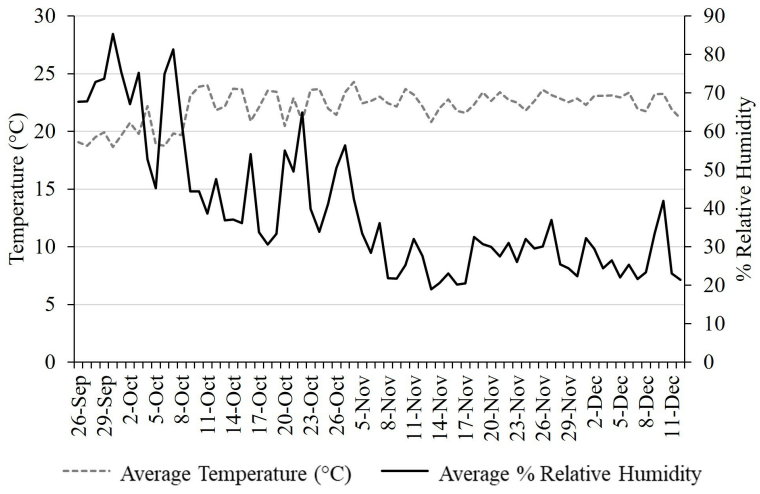

FIGURE 3
Daily temperature and humidity readings for L. delicatula oviposition study conducted 26 September – 11 December 2019.

a total of 21 *L. delicatula* adults from 126 hatched nymphs across all treatments. Trials conducted in MD and MA quarantine laboratories yielded no adults from the epicormic shoot diet, and those fed either apical meristem or freshly cut branches yielded fewer than 10% adults from the nymphal cohort (Figures 4B, C); these trials yielded 4 and 10 adults in the MD and MA facilities, respectively. In a pre-trial using potted *A. altissima* as a food source in MD quarantine facilities, 33 adults were produced.

Lycorma delicatula development and survivorship on potted *A. altissima* and *V. riparia* plant diets

The first nymphal cohort developed to the adult stage in a significantly shorter period of time on *A. altissima* plants alone compared with those reared on mixed diets of *A. altissima* and *V. riparia* plants (t = -6.96; df = 69.9; $P < 0.001$) (Figure 5A). The single host diet also had a significant effect on developmental time. Nymphs reared on a single host diet spent more days in the 2nd (t = 7.78; df = 285.7; $P < 0.001$), and 3rd (t = 2.15; df = 184.8; $P = 0.017$), instar lifestages and fewer days in the 4th (t = -5.28; df = 83.3; $P < 0.0001$) instar lifetage compared to mixed diet reared nymphs (Table 1). The percentage of hatched nymphs that developed to the adult stage was similar for both diets with 26.6% on *A. altissima* with *V. riparia* (46 adults total) and 29.8% on *A. altissima* alone (50 adults total).

For the second cohort, there was no significant difference in development time from hatch to adult between those reared on diets of *A. altissima* alone and those on *A. altissima* with *V. riparia* (t = 0.76; df = 107.6; $P = 0.77$) (Figure 5B). Diet had a significant effect on developmental time of nymphal instars. Nymphs spent fewer days in the 3rd (t = 4.20; df = 149.7; $P < 0.0001$) instar lifestage and more time in the 4th (t = 3.51; df = 111.7; $P < 0.001$) instar lifestage (Table 1). The percentage of hatched nymphs that developed to adults was comparable on both diets and greater than the first cohort: 59.8% on *A. altissima* with *V. riparia* (64 adults total) and 52.8% on *A. altissima* alone (57 adults total).

Conditions necessary to elicit oviposition

In 2019, a total of 133 egg masses were deposited by females, with each female depositing between 1-3 egg masses, for an average of 2.12 egg masses per female. Among substrates, 51% of all egg masses laid were deposited on *A. altissima* logs, 18% on *A. rubrum* logs, 19% on potted grape plants (principally on vines), and 12% on either the balsa wood structure or the cage structure itself. No egg masses were deposited on the live *A. altissima*. Oviposition began on 10 September and continued until 29 November with peak oviposition occurring in mid-October with 34 egg masses laid over a 4-day period (Figure 6).

In 2020, two egg masses were deposited in a cage containing four females with *A. altissima* and *V. riparia* (both on 19 October) and 10 egg masses were laid over two cages containing a total of 20 females with *A. altissima* alone from 4 November – 1 December, resulting in 0.5 egg masses per female for both diet treatments.

Lycorma delicatula survivorship and development from no-chill egg masses

A total of 13 nymphs emerged from a single egg mass under no chill conditions. Of these, five *L. delicatula* developed to the adult stage: four males and one female. The first male emerged 84 d after hatch and the female 102 d after hatch. The female adult survived for 88 d and did not oviposit. The male adults survived 40 ± 14 d.

Discussion

Here we demonstrated that *L. delicatula* can be reared from newly hatched first instar nymphs through to the adult stage under laboratory conditions using potted *A. altissima* trees while other *A. altissima* diet preparations did not result in reliable development to the adult stage. This approach is similar to rearing techniques for other insect species that require active vascular tissue for feeding, e.g., glassy-winged sharpshooter, *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae), with colonies provided host plants that generally need weekly replacement (18).

We also found that by reducing daylength from 16L:8D to 12L:12:D and providing *A. altissima* logs as a substrate, adult females would reliably oviposit under laboratory conditions. Indeed, females provided with these shorter daylength conditions to mimic those found in nature from mid-September onward deposited 4× as many eggs as those held at typical 16L:8D long-day conditions often used for standard insect colony maintenance. As *L. delicatula* is univoltine, and eggs are the overwintering lifestage, daylength appears to be an important cue for eliciting oviposition. Eggs are coated with a waxy material that provides an apparent barrier for protection (19), and females may be unwilling to oviposit too early to ensure eggs remain intact and well-protected. Moreover, as eggs are typically deposited on natural substrates, with *A. altissima* being the most common natural substrate (5), providing natural substrates in our rearing system appeared to also be critical to eliciting oviposition. Most eggs were deposited on *A. altissima* logs, though eggs were also deposited on *V. riparia* grapevines and *A. rubrum* logs.

Conversely, nymphal cohorts likely require longer daylengths to complete development. In the 2020 rearing experiments, the second cohort of nymphs had twice as many

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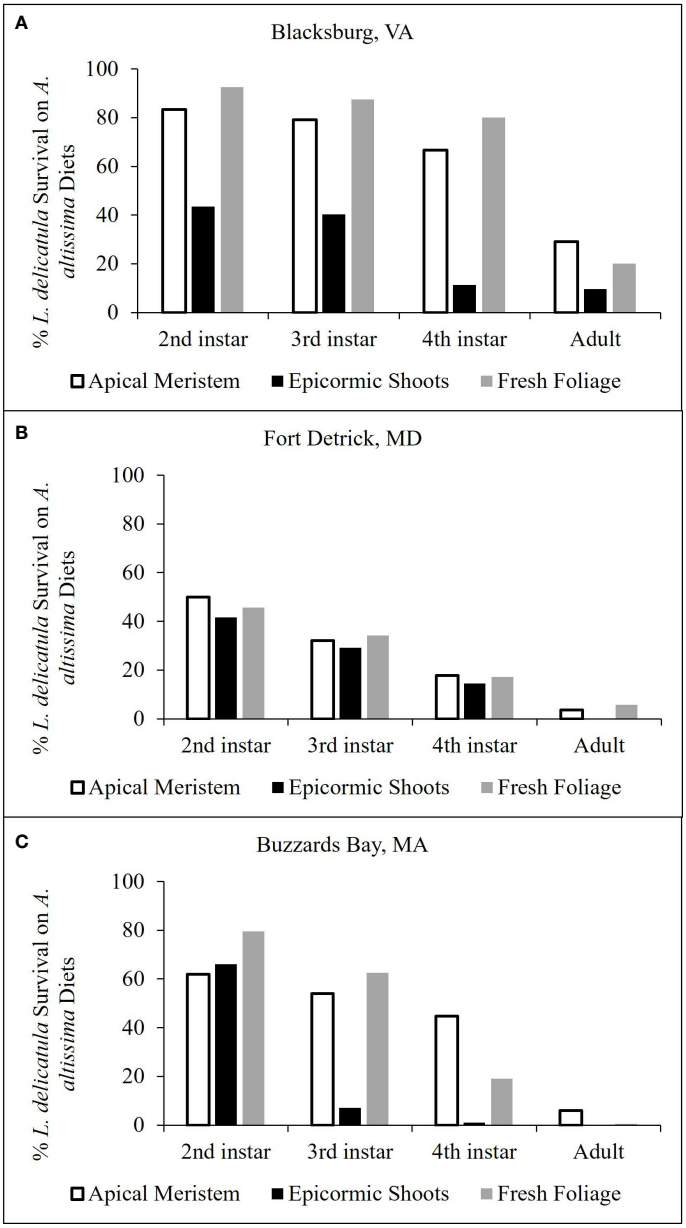

FIGURE 4
Percentage of surviving L. delicatula for each lifestage when 1st instars were reared on **(A)** altissima apical meristem, epicormic shoots, and fresh foliage. Results presented from experimental trials conducted in Blacksburg, VA **(A)**, Fort Detrick, MD **(B)**, and Buzzards Bay, MA **(C)**.

L. delicatula develop into adults than the first cohort; both cohorts were held at comparable temperatures, 20 – 25°C, but the first cohort was hatched and reared January – May (9.5L:14.5D – 14.5L:9.5D) and the second cohort during the time of year when wild *L. delicatula* in the region develop, May – September (14.5L:9.5D – 12L:12D). This would suggest that the natural daylengths of late spring and summer during this period is beneficial to nymphal development, particularly when compared with winter and early spring conditions. Degree day studies show that all mobile lifestages of *L. delicatula* survive and develop with temperatures between 15 and 30°C, and developmental rates increase with temperature within that range (20). Our rearing studies also show that longer daylengths are needed to support nymphal development, and variable humidity within that range did not seem to have a negative impact.

Lycorma delicatula egg masses undergo a prolonged period of chilling in nature throughout the winter months,

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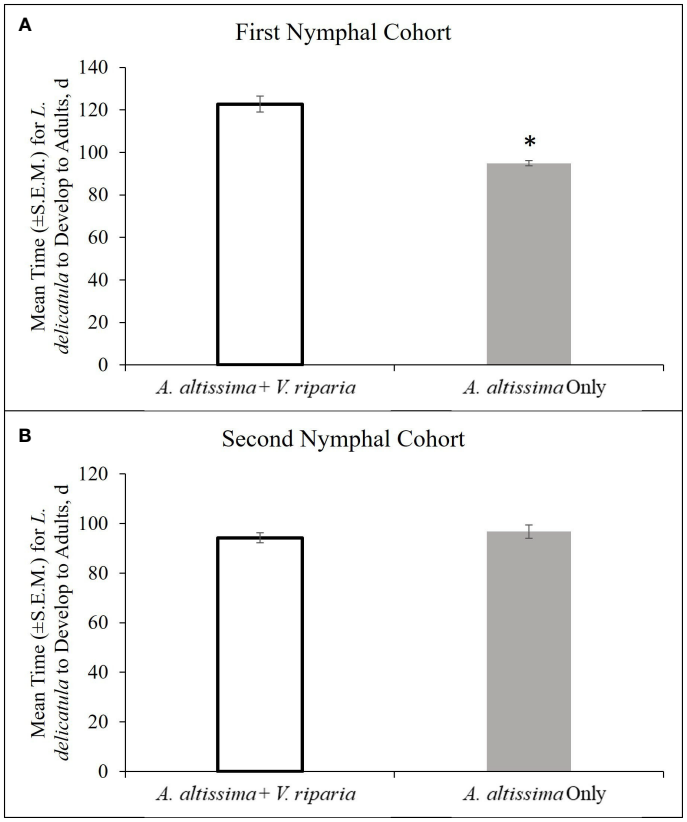

FIGURE 5
Developmental time (d ± S.E.M.) for L. delicatula from the first nymphal cohort (24 January – 8 May 2020) **(A)**, and second nymphal cohort (18 May – 10 September 2020) **(B)**.

although diapause requirements for *L. delicatula* are understudied and likely include daylength as a vital cue. However, egg masses can be held at a constant 15°C with no chill period resulting in >50% hatch with comparable results to when egg masses were held at 10°C for an 84 d chill period and moved to 25°C for hatching (17). Thus, it does not appear that a chilling period (≤10°C) is required for *L. delicatula* embryo development. A study has shown that a 7 d chill period (5 or 10°C) is not sufficient for most *L. delicatula* eggs to fulfil diapause requirements, although a small number were able to develop (17). Recent unpublished data (MK) has shown that holding eggs at an alternating temperature regime mimicking a colder climate can delay hatch of fall collected eggs until June, resulting in ~70% egg hatch and more rapid nymphal development at lower temperatures (when compared to nymphs hatched at 15°C). Use of the alternating regime can

TABLE 1 Mean number d (+S.E.M.) spent by Lycorma delicatula in each nymphal lifestage when reared on A. altissima with Vitis riparia and A. altissima alone. * signifies significant difference in development time between diets.

Treatment	First Instar First Cohort	Second Instar	Third Instar	Fourth Instar
Ailanthus altissima + Vitis riparia	20.4 ± 0.4	16.3 ± 0.3	20.7 ± 0.9	33.1 ± 1.9
Ailanthus altissima Alone	21.2 ± 0.4	19.7 ± 0.3*	22.8 ± 0.5*	22.1 ± 0.9*
	Second Cohort			
Ailanthus altissima + Vitis riparia	–	–	27.5 ± 1.1	38.1 ± 1.4
Ailanthus altissima Alone	–	–	22.2 ± 0.7*	45.8 ± 1.7*

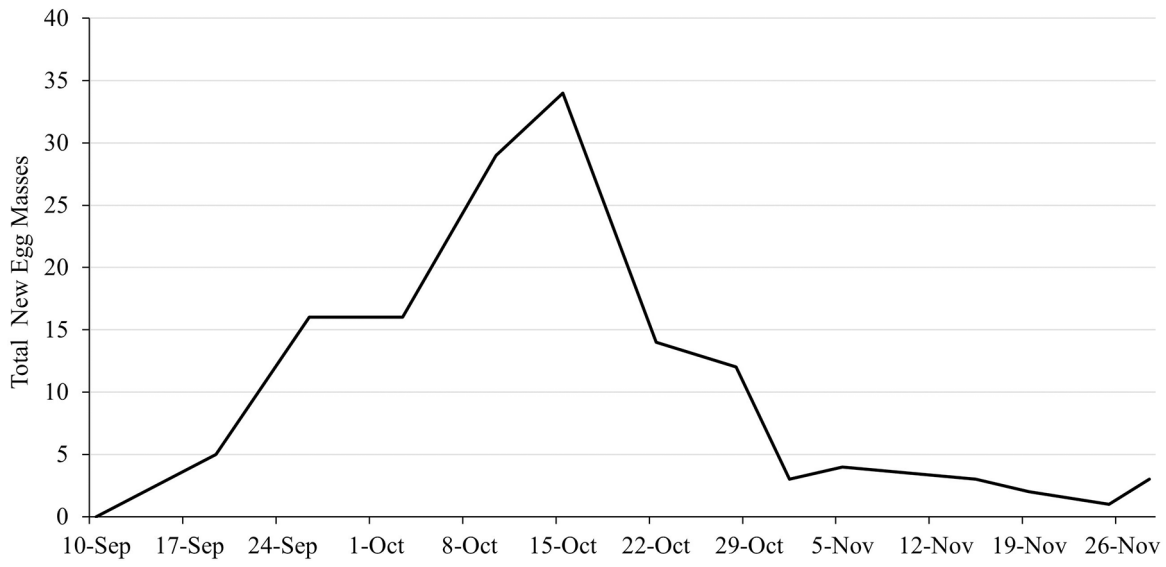

FIGURE 6
Non-cumulative phenology of egg mass deposition by female L. delicatula 10 September – 26 November 2019.

prolong the period egg masses remain viable and provide hatch to work with later in the year. In our studies, egg masses held at 12L:12D and 24C:13°C yielded limited *L. delicatula* hatch. While this was relatively rare, it does raise the possibility that *L. delicatula* could establish in regions that were previously deemed unsuitable due to a lack of environmental chilling (21).

Although it is well established that *L. delicatula* exhibit a broad host range during earlier lifestages and a narrower range during late instar and adult stages (2, 22), *A. altissima* appears to be a preferred host throughout their development (8, 22). However, both greenhouse and field cage experiments have demonstrated that *L. delicatula* can develop on other hosts without the presence of *A. altissima*. In large field cages, *L. delicatula* have successfully developed to adulthood and reproduced when provided with weeping willow *Salix babylonica* L. (Malpighiales: Salicaceae), silver maple *Acer saccharinum* L. (Sapindales: Sapindaceae), and river birch *Betula nigra* L. (Fagales: Betulaceae) (7, 9). *Lycorma delicatula* have successfully completed development to the adult stage on single host diets of *J. nigra*, black walnut, and *Vitis vinifera* L. (Vitales: Vitacae) (Elsensohn et al. in prep., 11) in greenhouse trials. Interestingly, survivorship and development varies among *Vitis* spp. Here, we included *V. riparia* which had no real impact on survivorship and development of *L. delicatula* when combined with *A. altissima* compared with a diet of *A. altissima* alone. In other studies, *V. rotundifolia* could not support development or survivorship of *L. delicatula* as a single host diet, and its inclusion with *A. altissima* seemed to have no impact (11). However, when *V. vinifera* is used as a single host, development and survivorship to the adult stage occurred, and survivorship increased when combined with *A. altissima* (Elsensohn et al. in prep). Thus, host diet selection is critical to any rearing system for *L. delicatula*, and in this case, *V. vinifera* is the only *Vitis* species that supports strong development and survivorship.

Summary

To successfully rear *L. delicatula* in the laboratory or greenhouse (Figure 7), eggs collected in the fall prior should be held at a constant 15°C for 90 – 100 d and eggs collected following significant chilling should be held at 10°C for 60 – 80 d. (17). To promote hatch, up to 15 egg masses should be placed in a typical insect rearing cage and provisioned with at least one 30 cm height potted *A. altissima* at 25°C and 16L:8D. Egg hatch should begin in ~7 – 14 d. *Ailanthus altissima* can be grown from stratified seeds germinating in ~4 weeks at 25°C, with subsequent potted plants reaching the appropriate 30 cm height in 8 – 10 weeks at ambient greenhouse conditions. Once hatch has occurred, 30 nymphs should be transferred to new cages containing a single *A. altissima*. Plants should be replaced at approximately 1–3-week intervals based on lifestage with 4th

FIGURE 7
Graphic displaying the optimized method for rearing L. delicatula including **(A)** growing A altissima from seed, **(B)** rearing SLF from egg to adult, and **(C)** eliciting SLF oviposition. .

instar nymphs and adults requiring every one to two weeks. Temperatures should be maintained at ~20–25°C at daylengths natural to late spring and summer months. To elicit mating and oviposition, adults should be maintained in cages with no more than 20 individuals and provisioned with an *A. altissima* plant as a food source and bolt as an oviposition substrate at 12L:12D and 24°C:13°C.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/projects/Development_of_rearing_techniques_for_spotted_lanternfly/146295

Author contributions

All authors conceived, facilitated, and designed the research. SJ, AD, DL, MH, LS, and LN conducted the experiments. LN analyzed the data and conducted statistical analyses. LN and TL wrote the manuscript. TL, TK, JG, MK, and DP secured funding. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Volatiles from male honeydew excretions attract conspecific male spotted lanternflies, *Lycorma delicatula* (Hemiptera: Fulgoridae)

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The spotted lanternfly (SLF), *Lycorma delicatula* (Hemiptera: Fulgoridae), is a generalist phloem feeder that produces copious amounts of honeydew, which in turn coats the understory. These insects form large aggregations covering the trunks of some trees, while similar trees nearby mysteriously seem unattractive. We investigated whether volatiles from SLF honeydew are attractive to conspecifics by collecting honeydew from the field and testing it for SLF attraction in a two-choice olfactometer. We found that honeydew excreted by adult male SLF was significantly attractive to male SLF, but not female SLF. Although the honeydew excreted by adult female SLF did not significantly attract male or female SLF, both sexes showed a positive trend towards attraction in response to female honeydew in the olfactometer. Analysis of the headspace volatiles of honeydew was conducted, and numerous semiochemicals were identified. Five of which, 2-heptanone, 2-octanone, 2-nonanone, benzyl acetate, and 1-nonanol, were tested in two-choice behavioral assays against a blank control. Benzyl acetate and 2-octanone were attractive to both sexes, whereas 2-heptanone was only attractive to males, and 2-nonanone only to females. The remaining compound, 1-nonanol, repelled females, but not males. Although honeydew has been reported as a source of kairomones for some natural enemies, this may be the first report of sex-specific attractants for conspecific insects found in the honeydew volatiles of a planthopper.

KEYWORDS

semiochemicals, pheromones, kairomones, honeydew, aggregation

Introduction

The spotted lanternfly, *Lycorma delicatula*, (Hemiptera: Fulgoridae) (SLF) is an invasive species in Northeastern United States. Although their preferred host plant is *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae) (1), they have a broad host range, including economically important species such as grapes, fruit trees and hardwood species (2). SLF causes damage by extensive phloem feeding and a large volume of honeydew secretion. This heavy feeding behavior, particularly during the adult stage, has devastated some vineyards in Pennsylvania (3). SLF has spread to numerous states and threatens agricultural, residential, and industrial areas despite the establishment of a restrictive quarantine zone in Pennsylvania and tripling applications of insecticides (4). Tools for non-insecticide control of this pest are in the early stages of development, such as the potential use of biological control agents like fungal pathogens (5), or parasitoid wasps (6). To implement any broad-scale control program, the distribution of the pest must first be determined. Therefore, our efforts have been aimed at the development of traps and semiochemical lures in order to develop survey, detection, and mass trapping tools (7–9).

Planthoppers perceive and respond to host plant volatiles (7, 8, 10), but little is known about the role of insect-produced volatiles such as pheromones in fulgorids. Recently, however, we documented evidence suggesting pheromone use may occur in SLF. Mid (during mating time) male SLF were attracted to extracts of Mid female SLF in laboratory behavioral bioassays (11). In field studies, aggregation behavior was generated in wild populations by placing groups of male or female SLF on trees in sleeves, and the sex ratio of the arriving SLF was biased toward the sex of SLF within each sleeve. Females, particularly before mating, were strongly attracted to sleeves containing female SLF, and Mid males were strongly attracted to sleeves containing Mid females. Courtship behavior was mainly observed during Mid on trees with confined females (12). Honeydew is produced by all phloem-feeding hemipteran insects, such as aphids (13), whiteflies (14), mealybugs (15), and planthoppers (16). For predators and parasitoids that attack hemipterans, volatile chemicals from honeydew are perceived as a kairomones, facilitating host habitat discovery by parasitoid wasps (17), coccinellids (18), chrysopids (19), mirid bugs (20), and flies (21, 22). Chemicals associated with honeydew may also serve as an oviposition stimulus for natural enemies (18, 23). The prospect of SLF honeydew emitting semiochemicals is clear from our observations of a variety of visiting hymenopterans that use it as a food source (24). Placing confined groups of SLF on trees generated aggregations of wild SLF in the field (12). Since their honeydew is produced in copious amounts (24), it was logical to investigate the potential role honeydew volatiles may play in the process of conspecific SLF attraction and aggregation. We hypothesized that SLF honeydew releases semiochemicals that inform other SLF about host resources, aggregations, or mates. Therefore, we sought to test whether volatiles from SLF honeydew were somehow involved in SLF attraction. Thus, this study aimed to 1) evaluate how volatiles from SLF honeydew influence SLF behavior, 2) identify any behaviorally active components and 3) define their behavioral function.

Methods

Timing

Developmental rates vary between year, location, and microclimate, and depend on local meteorological conditions such as degree days (25, 26). The adult stage of SLF is relatively long-lived, with eastern Pennsylvania typically seeing adult emergence in the end of July, mating in September, followed by oviposition, and finally death in late October or early November, a period spanning approximately 15-16 weeks. It is, therefore, necessary to break down the lengthy adult stage into shorter periods defined by their physiological state as it pertains to their behavior. Thus, the three time periods previously described in (7), "Early", "Mid", and "Late" were used. The onset of each adult phase was defined by the first field observation of its corresponding physiological state: adult emergence (Early), mating (Mid), and oviposition (Late). The calendar dates of these phases vary slightly depending on differences in latitude and climatic conditions at different field sites, and were based on the contemporary field observations at the collection sites. In 2019, start dates for adult phases were 22 July for "Early", 8 September for "Mid", and 22 September for "Late".

Field collection of honeydew and insects

On a weekly basis, honeydew samples were collected from SLF feeding on *A. altissima* in the field in Lehigh County, PA. Woody, sun-exposed branches were carefully selected away from overhanging branches to reduce honeydew falling from above. Custom mesh sleeves (tulle, 30 cm L x 60 cm circ) were wrapped around branches (5-7 cm diam), with three layers (7 cm thick) of foam batting (Bug Barrier, Envirometrics Systems Inc., Victor, NY) at the ends to space the tulle from the branch, secured by zip ties, and closed lengthwise with lab tape (Research Products International, Mount Prospect, IL). Wearing gloves, aluminum foil (20 by 40 cm, Reynolds Consumer Products, Lake Forest, IL) was suspended like a hammock below the branch inside of each sleeve for honeydew collection (Figure 1). A group of either 20 male or 20 female adult SLF were placed inside each sleeve and allowed to feed and produce honeydew for 48 h. In this way, honeydew of known age, from a known number and sex of SLF,

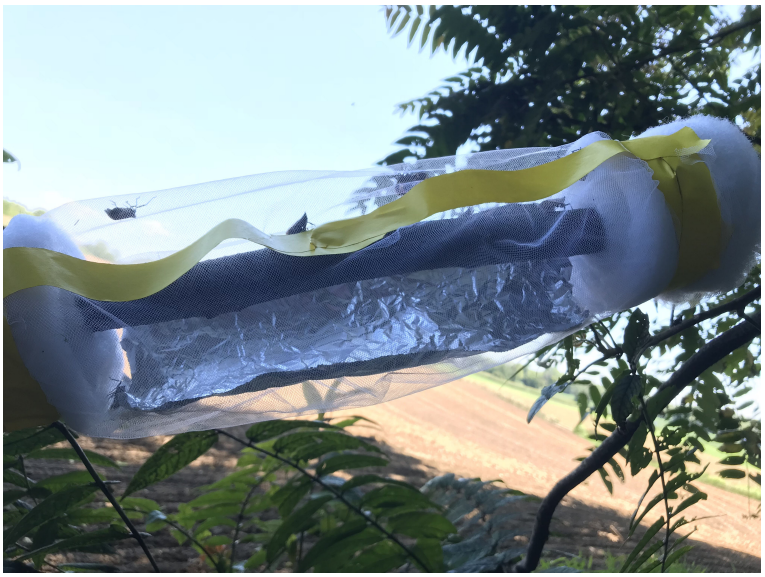

FIGURE 1
A custom mesh sleeve containing a foil "honeydew hammock" for SLF honeydew collection.

was collected on the foil. Four foil "honeydew hammocks" in sleeves were installed per week: two with males and two with females. After 48 hours, foil hammocks were removed, folded with the honeydew inside, and individually placed into single-use pre-baked oven bags which were tied tightly closed (Turkey size, Reynolds Consumer Products, Lake Forest, IL). Oven bags had been pre-baked at 150°C for 4 hours to remove volatile contaminants such as caprolactam (27). These bags containing the honeydew-laden foil were immediately placed in a cooler with dry ice. In addition, a control piece of foil which was not exposed to honeydew or SLF was handled and packaged in the same way and placed into the cooler, in case volatile compounds were inadvertently transferred to the foil during the handling and shipping process. The cooler was shipped overnight to the USDA Forest Pest Methods Laboratory (FPML) (formerly Otis Laboratory) in Buzzards Bay, Massachusetts. Each week, honeydew hammocks were set up on Monday, retrieved and shipped overnight on Wednesday, received by the FPML on Thursday morning, and used immediately upon arrival for volatile collections and behavioral bioassays (see below). This occurred weekly between 19 August and 27 September, 2019, which spanned Early, Mid, and Late phases.

Every Monday, live SLF were captured from the field and shipped overnight (as per conditions set by permits USDA P526P-15-00152 and PA PP3-0123-2015). The live insects were received on Tuesday at the FPML insect containment facility for use through Friday of the same week in behavioral bioassays and electrophysiology. There they were housed in cages (24.5 × 24.5 × 63 cm, Bugdorm, Megaview Science Co., Ltd., Taichung City, Taiwan) in an environmental chamber at 25 C with 16:8 L:D, and fed freshly cut *A. altissima* branches maintained in hydroponic solution (Maxigrow, GenyHydro Inc., Sebastopol, CA, prepared according to label).

Honeydew standardizing and headspace volatile collections

It was necessary to standardize the amount of honeydew used in behavioral bioassays using filter papers. Thus, the amount of honeydew that could saturate a 5 mm x 10 mm piece of filter paper was used in bioassays. All handling was done wearing gloves. Prior to use, a filter paper (Whatman, grade 1, 12.5 cm circles, China) was cut into 5 mm x 10 mm rectangles and washed by soaking them in a beaker containing 100 ml of hexane for 5 min and allowed to air dry on clean foil. Upon arrival of honeydew samples in the laboratory, one at a time, each frozen foil honeydew hammock was removed from dry ice and its oven bag, unfolded, and the foil was wiped with a pre-washed 5 mm x 10 mm piece of filter paper held by a clean pair of forceps until it became saturated. This filter paper was immediately tested for attraction in the y-plate olfactometer in a different room (described below). Additional filter papers were used to collect as much of the remaining honeydew as possible from the foil using the same technique until there was none left. These remaining filter papers, laden with crude honeydew, were used to collect and analyze the volatile headspace components of the honeydew. They were placed inside a clean glass Pasteur

pipette, and the wide end of the pipette was covered with aluminum foil. An absorbent solid phase microextraction fiber (SPME, 23 ga 100 µm polydimethylsiloxane, Supelco Inc., Bellfonte, PA) was selected because of its sensitivity in detecting minute amounts of volatile molecules such as insect semiochemicals, whereas preliminary attempts using other volatile collection techniques lacked such sensitivity. The SPME fiber was inserted through the narrow end of the pipette and was exposed to the headspace of the honeydew-laden filter papers for 2 h at 22°C. This process was repeated for each foil hammock and the control.

Analysis of honeydew volatiles

Each SPME fiber was desorbed in the injection port of an Agilent 7890B gas chromatograph (GC) coupled with an Agilent 5977A mass spectrometer (MS) (EI mode, 70 eV with a scanning range of 40.0–450.0 m/z), using a DB-5MS capillary column (Agilent, 30 m×0.25 mm i.d., 0.25 µm film thickness) in splitless mode, with helium carrier gas at constant flow rate of 1 ml/min. The injection port temperature was 280°C, and the oven temperature was held at 40°C for 1 min, ramped at 10°C/min to 300°C, then held for 25 min. Tentative identifications of the honeydew volatile components were made by comparing mass spectra with those in the mass spectral library database (Enhanced ChemStation, MSD Chemstation, Data Analysis software vF.01.00.1903, and NIST, v11, Agilent Technologies, Santa Clara, CA). Close matches were confirmed by obtaining and injecting authentic standards and comparing their Kovat's indexes (KI), retention times, and mass spectra to ensure they matched. Compounds that were also present in controls are not reported. Peak areas representing the total ion abundance for each peak were used to calculate the percent (ratio) of each identified compound over all SPME volatile collections combined for each sex (4 Early, 2 Mid, and 1 Late). The sum of peak areas for each compound was divided by the total sum of all 13 compounds for males and for females to calculate ratios.

Antennal responses to volatiles

Gas chromatography coupled with electroantennographic detection (GC-EAD) is a common electrophysiological technique used to determine which compounds in a natural volatile collection can be detected by an insect antenna (28). However, the quantity of volatile material collected from honeydew headspace was not enough for use in GC-EAD, since, compared to known amounts of injected standards, we estimate that the average peak size of honeydew headspace volatile compounds collected by SPME fibers was approximately 8 ng. Instead, antennal responses to synthetic standards of identified components were recorded using an Agilent 6890 GC, fitted with an HP-5MS column (30 m × 0.320 mm I.D. × 0.25 µm film, Agilent Technologies, Inc., Santa Clara, CA, USA) in splitless mode. The injector and programmed temperatures were the same as those described for the GC-MS. At the end of the GC column, effluent was split 1:1 (glass Y-connector, Restek, Corp., Bellefonte, PA), with half carried to a flame ionization detector (FID) at 250° C, and the other half carried out of the GC *via* a temperature-controlled arm (Syntech Temperature Controller, Kirchzarten, Germany) at 150°C, and delivered into an L-shaped glass odor delivery tube (11 mm diam.), which delivered the effluent over the antenna. Charcoal-filtered, humidified air passed through the odor delivery tube at 0.3 L/min.

An SLF head was mounted onto a ground electrode in the form of a custom-pulled glass capillary filled with Ringer's solution (8). Adult SLF have soft and fleshy antennae (29) which collapse when the integument is penetrated, hindering early attempts at GC-EAD. Therefore, the apical tip of the arista was removed with a razor blade, and brought into contact with the glass capillary recording electrode, such that the remaining portion of the arista was enveloped in the electrode. Electrodes were positioned using custom micromanipulators (Signatone Corp., Gilroy CA, USA) secured magnetically to a steel platform (Syntech, Kirchzarten, Germany). Antennal signals were amplified using a Dam 50 differential amplifier (World Precision Instruments, Sarasota, FL, USA), passed through Hum Bug 50/60 Hz noise elimination (Quest Scientific, North Vancouver, BC, Canada), and integrated with a two-channel signal acquisition interface (IDAC-2, Syntech, Hilversum, The Netherlands). Data were collected and analyzed using GCEAD/2014 software (Syntech, Version 1.2.5, Kirchzarten, Germany). The electrophysiological activity of both male and female antennae to synthetic compounds was determined by injecting 100 ng/ul of each compound, delivering 50 ng to the antenna and 50 ng to the FID. All synthetic compounds were manufactured by Sigma-Aldrich, Inc. (St. Louis, MO), except (*Z*)-3-nonenyl acetate which was manufactured by Bedoukian Research, Inc. (Danbury, CT).

Behavioral bioassays

The responses of male and female SLF when presented with a choice between a volatile stimulus and no stimulus (control arm) was evaluated using custom Teflon® Y-plate dual-choice olfactometers [Supplementary File; for descriptions, see (7, 8, 30)]. Stimuli being evaluated were either (1) a honeydew-laden filter paper, or (2) 1 mg of synthetic compound. Each Y-plate was 28.6 cm long x 21.6 cm wide and 3.8 cm tall, with a channel 5.1 cm wide cut in the shape of a Y, with the choices at a 90° angle from each other. A disposable sheet of clear acetate (Apollo, Lincolnshire, IL) was affixed to the top and bottom of the plate using electrode gel (Spectra 360, Fairfield, NJ) and served as the ceiling and floor of the bioassay, and were

discarded at the end of each session. Filtered, humidified air flowed through the olfactometer at 24 cm/s. Prior to their use in the olfactometer, SLF were allowed to acclimate individually inside release cages at 25°C for 30-60 min in the walk-in environmental chamber where bioassays were to be conducted. Each session of bioassays started with a newly cleaned Y-plate bioassay apparatus with all new disposable parts. At the beginning of each session, five SLF were tested individually without volatile stimuli to ensure there was no contamination or other bias in the olfactometer. In addition, dedicated control sessions were conducted using the identical protocols used for semiochemical testing, but without chemical stimuli, in order to document the baseline activity for SLF males and females under these conditions. Each insect was individually released and allowed three min to make a choice, which occurred when the insect walked halfway up one of the two arms of the olfactometer. Insects that did not make a choice in three min were counted as non-responders. Each bioassay session, evaluating a particular choice of treatment and control, tested up to 20 individual SLF composed equally of males and females in alternating order, ensuring that both sexes were offered exactly the same stimuli. The next session used a clean Y-plate, tested five more individual SLF without stimuli, then tested the stimulus and control with directions reversed. After each session, Y-plates and parts were washed with Alconox and ethanol 95%, dried overnight, and disposable parts were discarded and replaced. If the five control insects were found to have a bias (more than 1 response in either direction), that Y-plate was immediately replaced with a clean Y-plate, and the biased Y-plate was cleaned again before use.

In bioassays testing standardized honeydew-laden filter paper (described above) for attraction, a single piece of hexane-cleaned filter paper was placed in the upwind section of one arm of the olfactometer as a control, and the single (5 mm x 10 mm) piece of filter paper laden with honeydew was placed in same position of the other arm. In this experiment, each week consisted of two sessions: one testing 10 males and 10 females, alternating, to honeydew produced by males, and the other testing 10 males and 10 females, alternating, to honeydew produced by females. For each of these four tests, behavioral data was collected over four weeks (3 Early and 1 Mid).

In behavioral assays with synthetic compounds, each upwind arm received either the synthetic compound in an open microcentrifuge tube, or an empty microcentrifuge tube control (7, 8). Each synthetic compound was tested using 1 mg of neat material (Sigma Aldrich, St. Louis, MO), and all insects used were Early adults, except for an additional test of 1-nonanol using Mid adults. The frequency and direction of choice was compared using a Chi Square test, where significance at α=0.05 was reached when the G-statistic reached 3.841 or above (31, 32).

Results

Analysis of honeydew volatiles

GC-MS analyses of SLF honeydew volatiles revealed the presence of four ketones, six esters, and three alcohols, all of which existed in both sexes but at different ratios (Table 1). Two compounds in male honeydew occurred at ratios over 1.5 times

TABLE 1 A summary of the compounds found in the honeydew headspace volatiles collected from male and female spotted lanternflies, *Lycorma delicatula*, between 19 August and 27 September, 2019.

Compound	Relative percent ♂ ± SE (n=6)	Relative percent ♀ ± SE (n=7)	Ratios ♂ : ♀	Behaviorally Active[1]	Antennally Active	Retention index
isoamyl acetate	29.5 ± 5.9	17.1 ± 3.4	1.7: 1	–	M, F	875
2-heptanone	0.03 ± 0.2	0.1 ± 3.9	1: 3.6	Y	M, F	887
2-octanone	5.9 ± 5.3	4.8 ± 4.1	1.2: 1	Y	M, F	989
2-nonanone	3.0 ± 4.3	7.0 ± 5.3	1: 2.4	Y	M, F	1088
2-phenyl ethanol	12.2 ± 2.9	19.6 ± 13.3	1: 1.6	–	M, F	1108
2-ethylhexyl acetate	2.5 ± 1.6	4.5 ± 3.8	1: 1.8	–	M, F	1146
benzyl acetate	24.5 ± 12.3	22.8 ± 4.9	1.1: 1	Y	M, F	1150
1-nonanol	4.9 ± 5.4	5.9 ± 7.8	1: 1.2	Y	M, F	1170
2-undecanone	1.2 ± 1.8	5.0 ± 3.9	1: 4.1	–	M, F	1290
(*Z*)-3-nonenyl acetate	4.5 ± 1.0	4.3 ± 0.9	1: 1	–	M, F	1290
nonyl acetate	9.1 ± 2.8	5.4 ± 1.2	1.7: 1	–	M, F	1307
n-decyl acetate	2.7 ± 0.6	3.4 ± 0.7	1: 1.3	–	M, F	1407
1-dodecanol	1.7 ± 0.4	2.1 ± 0.5	1: 1.2	–	M, F	1470

[1]Behaviorally active components are indicated (Y). Minus signs "-" denote the compounds that were not tested in behavioral bioassays.
Antennal responses to synthetic compounds were recorded from both males (M) and females (F).

higher than in female honeydew: isoamyl acetate and nonyl acetate. Conversely, the ratios of five compounds were over 1.5 times higher in females than in males: 2-heptanone, 2-nonanone, 2-phenyl ethanol, 2-ethylhexyl acetate, and 2-undecanone. The ratios of 2-octanone, benzyl acetate, 1-nonanol, and (*Z*)-3-nonenyl acetate, *n*-decyl acetate, and 1-dodecanol were similar in the honeydew volatiles of both sexes (Table 1). In GC-EAD analyses, all of these produced antennal responses in both SLF males and females (Table 1). Due to limitations in time and insects, only the first five compounds found to have antennal activity in preliminary EAD recordings were tested in behavioral assays: 2-heptanone, 2-octanone, 2-nonanone, benzyl acetate, and 1-nonanol (Table 1).

Behavioral assays

Control sessions showed that alternating male and female SLF tested in the olfactometer had low response rates and no directional bias (Figure 2A). Honeydew volatiles from either male or female SLF produced different levels of attraction of males or females, compared to the control arm in the y-plate olfactometer. By testing 5 mm x 10 mm filter papers saturated with honeydew, potential unknown differences in amount of honeydew production between males (which are smaller) and females (which are larger) can be ruled out. Thus, differences in male and female attraction likely can be ascribed to differences in composition between the honeydew produced by males and females. Male SLF were significantly attracted to the volatiles of honeydew excreted by male SLF, with an overall response rate of 74% (G=13.72, α=0.001, df=1, n=35). Female SLF were not attracted to male honeydew volatiles. Neither sex was attracted significantly to female honeydew volatiles, however, both males and females showed a trend towards attraction to female honeydew volatiles that approached significance (Figure 2B).

In behavioral assays with synthetic compounds, benzyl acetate was significantly attractive to both sexes during Early, 2-heptanone was significantly attractive only to Early males, 2-octanone was significantly attractive to Early females but males trended towards it, and 2-nonanone was significantly attractive only to Early females. Conversely, 1-nonanol trended in the opposite direction for Early males and females, and had a significant repellent effect on Mid females but not Mid males (Figure 2C).

Discussion

In the current study we described the behavioral function and volatile profiles of honeydew derived from adult male and female SLF. Adult males, but not females, were significantly attracted to male honeydew volatiles. A trend of attraction by both male and female SLF to honeydew volatiles derived from females suggests that female honeydew volatiles may have shown attraction with more replication or with more material in the olfactometer.

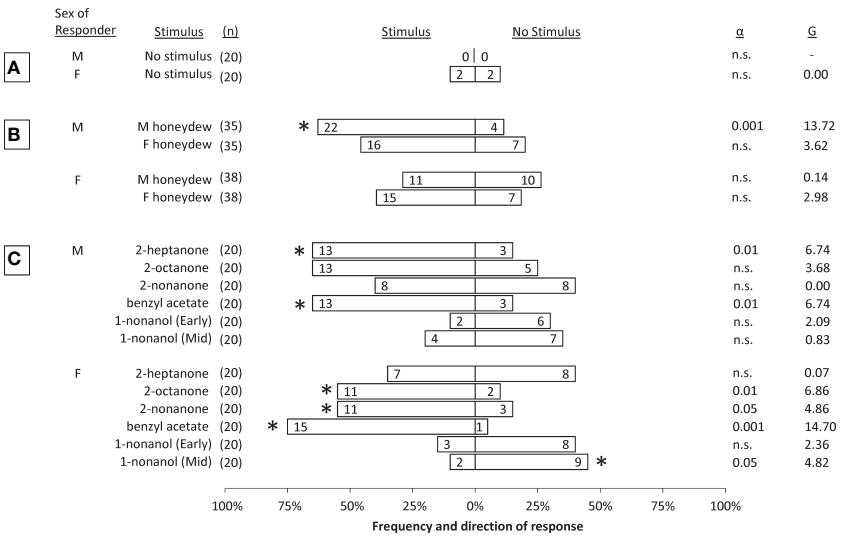

FIGURE 2
Choices made by male and female spotted lanternflies, *Lycorma delicatula*, in dual-choice bioassays comparing no stimulus to: **(A)** no stimulus (controls); **(B)** volatiles emitted from honeydew excreted either by male (M) or female (F) conspecifics, and **(C)** synthetic compounds (1 mg) found in honeydew volatiles. All tests were conducted using Early adults except where indicated. The numbers inside the bars indicate the numbers of insects that responded to the respective choice within 3 min. The number of insects tested (n) (including non-responders) are shown for each test. Asterisks represent a significant deviation from expected frequencies between two choices with critical α levels and G-statistics provided (Chi Square test). Alpha below 0.05 is not significant (n.s.).

Interestingly, the fact that male honeydew volatiles attracted only male SLF in bioassays aligns with field results found by Cooperband and Murman (12). In that study, wild male SLF were attracted to host trees with sleeves containing confined aggregations of males, resulting in a significantly male-skewed wild sex ratio on those trees. Conversely, significantly more female-biased wild SLF sex ratios occurred on trees that had confined female aggregations (12). Strongly skewed sex ratios with either male or female bias on different trees, or at different times in the season, have been documented in SLF (12, 33, 34). Thus, a potential mechanism for the observed phenomenon of extreme sex ratio bias in SLF field aggregations is presented here.

Although all 13 compounds described here from SLF honeydew headspace volatiles were eventually found to elicit antennal responses, technical challenges in initially developing EAD capabilities with adult SLF antennae hampered the beginning of this study. Limitations in time and insects led us to select only the first five compounds that were found to be antennally active to test for attraction. The issues were resolved in a subsequent year, and EAD was conducted again on all compounds, which were all found to elicit antennal responses from both male and female SLF. Unfortunately, conducting behavioral bioassays on the remaining compounds was not possible due to the time and logistical constraints when working with this univoltine insect.

Volatiles from SLF honeydew headspace were identified as ketones, esters, and alcohols. Similar chemical profiles were documented from the honeydew headspace volatiles of both sexes, but they occurred in different ratios. However, those ratios were not fixed over time. This study did not seek to evaluate seasonal changes in chemical ratios. Instead, we reported the average ratios taken over the season from Early, Mid, and Late adult SLF. Benzyl acetate attracted both Early males and females in the y-plate olfactometer, whereas 2-heptanone attracted only Early males, and conversely, 2-octanone and 2-nonanone attracted only Early females. One identified compound, 1-nonanol, showed a significant repellent effect on Mid females and no effect on Mid males. Preference differences between males and females for specific ratios of the same chemicals might explain why male SLF were attracted to honeydew derived from males, but females were not. The fact that SLF produce large quantities of honeydew that can be collected, and the sensitivity of the SPME fibers and the GC-MS, facilitated our ability to collect and detect the presence of minute quantities of volatile compounds. With an average peak containing about 8 ng of material, however, we cannot rule out the possibility of a sex-specific compound that may be present below our level of detection.

Studies in other hemipterans have demonstrated the importance of volatiles from honeydew in attracting natural enemies. Honeydew volatiles described for several species include hydrocarbons, disulfides, ketones, alcohols, aldehydes, carboxylic acids, a pyrazine, and a monoterpene (14, 18, 21). Most studies on honeydew were focused on carbohydrate contents as a food source for natural enemies and ants (35). Conspecific and sex-specific attraction to honeydew has been documented to occur in psyllids, in which only males were attracted to conspecific honeydew, but the responsible compounds were not characterized (36). To our knowledge, this is the first evidence of attraction to conspecific honeydew volatiles in a planthopper.

It is well documented that SLF honeydew accumulates and thickly coats the trunks and bases of *A. altissima* trees, and may become white and frothy over time when SLF densities are high (24) which can also produce a strong fermentation odor (MC, pers. obs.). The honeydew in this study accumulated for only two days on a clean foil surface. Although beyond the scope of the current study, we should not ignore the potential role of microbes dwelling in hemipteran honeydew as a source of volatiles which may act as semiochemicals (37). Several studies isolated bacteria from hemipteran honeydew (38, 39), the volatiles of which acted as kairomones for natural enemies (21, 37) or mosquitoes (21). A wide range of chemicals have been described from bacterial volatiles, including alcohols, aldehydes, carboxylic acids, esters, hydrocarbons, and ketones (40–42), but none were the same compounds we collected from SLF honeydew headspace.

All of the compounds found in SLF honeydew are known to occur in both plants (43–52) and insects (53–65). The five compounds tested for attraction all serve as pheromone components for species across multiple insect orders. For example, benzyl acetate was found in pheromones of bees (53) and bed bugs (54). The current study is the first report, to our knowledge, of a planthopper species attracted to benzyl acetate. In ants, 2-heptanone has been reported as part of an alarm pheromone (55). We found sexual differences in SLF attraction to 2-heptanone and 2-nonanone (Figure 2), and interestingly, such sexual differences are present in other insects as well (56, 66). For example, 2-octanone, one of numerous compounds found in the excreta of mixed sex groups of bedbugs *Cimex hemipterus*, produced a positive attraction index in only male bedbugs (56). The compound 2-nonanone has been reported as an aggregation pheromone (57), sex pheromone component (58), and alarm pheromone component in ants (55). In a fly, 1-nonanol was suggested as a female attractant (67).

There are numerous avenues one could pursue for additional research, for instance, investigating whether the host plant species being fed upon alters the volatile profile and attractiveness of honeydew (68). Volatile and sugar profiles of hemipteran honeydew may vary with different host plants (68). In the current study, SLF honeydew was collected while they were feeding on *A. altissima*. SLF have a wide range of host plants with different volatile profiles (1, 8), but their host range narrows as they develop, and adults accumulate on *A. altissima* (69, 70). Examining the volatile profiles and attractiveness of SLF honeydew produced while feeding on other host plants could be a revealing way to study their host plant relationships and may help narrow down important semiochemicals. Our bioassays used 1 mg lures, a dose previously used to test SLF attraction to host

plant volatiles, or kairomones (8), which typically occur in larger amounts than pheromones. Dose-response studies could reveal whether the compounds are behaviorally active at the nanogram range or lower, which is the range expected for a pheromone (71). In addition, synthetic blends of honeydew volatiles in sex-specific ratios should be tested for attraction of males and females.

In an effort to determine how SLF locate each other from a distance for purposes of mating or the formation of aggregations, this study evaluated SLF honeydew volatiles as a possible mechanism for conspecific attraction, and described the components of headspace volatiles from SLF honeydew. All honeydew compounds elicited antennal responses from male and female SLF adults, and the behavioral function for male and female SLF of five of those compounds individually was described. Our results introduce a potential new mechanism for SLF, and perhaps other honeydew producers, to locate conspecifics in response to semiochemical cues emitted from their own honeydew. This mechanism also may be involved in driving the male- or female- skewed SLF sex ratios observed to naturally occur on different trees at specific times in adult development (12, 33). Complete behavioral testing of each of the remaining compounds as well as synthetic blends would help to fully understand this system. In addition, dose response testing could improve our understanding of behavioral function, as some compounds may be attractive at low doses and repellent at high doses.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MC secured funding, conceived and designed experiments, analyzed data, and wrote the manuscript; HF analyzed GCMS files, conducted GCEAD, and wrote the manuscript; LM processed honeydew samples, collected volatiles, prepared and injected samples on GCMS, and conducted preliminary GCEAD and preliminary analysis of honeydew GCMS files; IC conducted dual-choice bioassays; KM collected honeydew and insects, oversaw all field components, and edited the manuscript; MW supervised personnel, facilitated research, and edited manuscript; DC supervised personnel, facilitated research, and edited manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2022.982965/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Photograph of a Y-plate.

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Impacts of short-term feeding by spotted lanternfly (*Lycorma delicatula)* on ecophysiology of young hardwood trees in a common garden

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Spotted lanternfly (SLF; *Lycorma delicatula* White; Hemiptera: Fulgoridae) invaded the US from Asia and was first detected in 2014; currently, populations have established in 14 states primarily in the Northeast and Mid-Atlantic. It feeds voraciously on phloem sap from a broad range of host plants, with a preference for tree of heaven (*Ailanthus altissima* [Sapindales: Simaroubaceae]), grapevines (*Vitis* spp. [Vitales: Vitaceae]), and several common hardwood tree species. We evaluated the impacts of fourth instars and adults confined to a single branch or whole trees on gas exchange attributes (carbon assimilation [photosynthetic rate], transpiration and stomatal conductance), selected nutrients, and diameter growth using young saplings of four host tree species planted in a common garden. In general, the effects of adults on trees were greater than nymphs, although there was variation depending on tree species, pest density, and time post-infestation. Nymphs on a single branch of red maple (*Acer rubrum* [Sapindales: Sapindaceae]), or silver maple (*Acer saccharinum* [Sapindales: Sapindaceae]) at three densities (0, 15, or 30) had no significant effects on gas exchange. In contrast, 40 adults confined to a single branch of red or silver maple rapidly suppressed gas exchange and reduced nitrogen concentration in leaves; soluble sugars in branch wood were reduced in the fall for silver maple and in the following spring for red maple. Fourth instars confined to whole silver maple trees reduced soluble sugars in leaves and branch wood, and reduced tree diameter growth by >50% during the next growing season. In contrast, fourth instars in whole tree enclosures had no effects on black walnut (*Juglans nigra* [Fagales: Juglandaceae]). SLF enclosed on tree of heaven at 80 adults per tree suppressed gas exchange after two weeks of feeding, but did not alter

non-structural carbohydrates, nitrogen concentrations, or tree growth. Results suggest that moderate to heavy feeding by SLF on young maple saplings may impair tree growth, which could have implications for production nurseries and forest managers.

KEYWORDS

red maple, black walnut, tree physiology, feeding damage, photosynthesis

Introduction

Plant responses to herbivory may include changes in both primary and secondary metabolism (1, 2). Chewing insects, such as caterpillars, are well known for causing extensive tissue damage and activating the jasmonic acid signaling pathway, leading to the production of a variety of plant defensive secondary metabolites (2), as well as having impacts on plant resource allocation (3). As damage from defoliation is more easily documented than the extent of feeding by piercing/sucking insects, impacts on plant primary metabolism by defoliators have received more attention than for sap-feeding herbivores, yet sap-feeders can also have pronounced effects on plant physiology (1, 4, 5). Sap-feeding herbivores consume carbohydrates and nutrients from phloem and/or xylem tissue, potentially reducing available energy and nutrients for above- and belowground growth of plants which can impact short- and long-term plant health. A meta-analysis conducted by Zvereva et al. (1) found that across studies from 52 papers, sap feeders usually reduced growth and photosynthesis, and that generalist herbivores had more negative impacts than specialists. However, changes in plant primary metabolism in response to sap-feeders can vary considerably among insect guilds and plant species (1, 3).

To our knowledge, other than the brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), no other planthoppers have been investigated for impacts on plant primary metabolism until recently (4, 5). This is likely because most planthoppers are not considered pests. *N. lugens* is a specialist on rice and a major economic pest throughout Asia (4). Feeding on susceptible rice plants reduces photosynthesis and interferes with translocation of nutrients, reducing plant growth (6, 7).

The invasive planthopper, spotted lanternfly (*Lycorma delicatula* White; Hemiptera: Fulgoridae; hereafter SLF), provides an opportunity to investigate the impacts of a generalist planthopper on host tree physiology. Unlike the specialist, *N. lugens*, SLF has a worldwide host range of 103 plant taxa, more than 56 of which have been reported as hosts in the US (8). It has a strong preference in its native and introduced ranges for tree-of-heaven (*Ailanthus altissima* [Sapindales: Simaroubaceae]) and wild and cultivated grape (e.g., *Vitis* spp.). Other frequent hosts in the US include several native deciduous trees including maples (*Acer* spp.), walnuts (*Juglans* spp.), birches (*Betula* spp.), and willows (*Salix* spp.). SLF is native to China and has spread to Vietnam, South Korea, Japan, and most recently the US (9). First detected in Berks County, Pennsylvania in 2014, SLF has established populations in 14 states in the US to date (10). SLF has proven to be a serious economic pest of several deciduous trees and agricultural crops, especially grapevines (11, 12).

SLF has four instars that develop from late spring to mid-summer before becoming adults in late July and early August (13). The adults feed on copious amounts of phloem sap to reach sexual maturity and begin laying eggs in late August or early September. Oviposition continues until the adults die from a hard freeze, usually in mid-November or early December. The long duration of the adult stage is particularly destructive to the health of cultivated grapes (11, 12). A recent study found that adult SLF feeding at increasing densities markedly reduced carbon assimilation (hereafter referred to as C assimilation) and late-season concentrations of non-structural carbohydrates and nitrogen, with the strongest impacts on belowground root carbon and nitrogen storage; reduced starch storage in the roots can lead to winter mortality of vines (5). C assimilation (photosynthetic rate) is the general term most widely used by plant ecophysiologists for fixing inorganic C (CO_2) into organic C (carbohydrates) (14, 15).

While SLF likely co-evolved with the preferred hosts of grape and tree of heaven in its native range and can compete with grapevine sinks for resources leading to whole-plant carbon limitation (5), effects on the health and physiology of tree hosts native to the US have not been investigated. In the US, SLF frequently utilizes important ornamental and/or forest trees such as silver maple (*Acer saccharinum* [Sapindales: Sapindaceae]), red maple (*Acer rubrum* [Sapindales: Sapindaceae]), weeping willow (*Salix babylonica* [Malpighiales: Salicaceae]), black walnut, and river birch (*Betula nigra* [Fagales: Betulaceae]). In Pennsylvania alone, the annual economic losses from SLF for the ornamentals and forest industries are estimated at $8 million and $16.7 million per year, respectively (16).

Due to voracious SLF feeding and the tendency for them to aggregate in high numbers on individual trees (17), we hypothesized that SLF can modify the allocation of resources for defense against herbivory at the expense of growth in its hardwood tree hosts. Other than tree of heaven, healthy mature ornamental and forest trees have rarely been killed by SLF, although canopy dieback and plant health decline has been observed, with occasional mortality of saplings of black walnut (9) and maples (18). SLF also produce copious amounts of honeydew, which promotes the growth of sooty mold on plants below feeding sites, impeding photosynthesis of affected plants (19).

In 2019 and 2020, we measured C assimilation, transpiration, and stomatal conductance in response to SLF feeding pressure. We also evaluated concentrations of non-structural carbohydrates and nitrogen, as well as tree growth at increasing densities for silver maple, red maple, black walnut, and tree-of-heaven using planted saplings in a common garden. We used multiple methods to expose trees to SLF feeding. We began by confining SLF to single branches of young trees, followed by whole-tree enclosures, which more closely resemble field conditions. We hypothesized that plants, in response to feeding pressure, may initially compensate with enhanced gas exchange attributes, but that over time these variables would decline. We also hypothesized that the plants' source/sink relationships would be altered as the insect competes for essential nutrients such as C and N. Besides competing directly with plant sinks, sap-feeding insects can change gene expression for the processes of N assimilation and translocation, such as in the brown planthopper on rice (4, 20) and SLF feeding on grapevines (21). We further hypothesized that tree growth would be reduced the following year.

It is important to note that in 2019 when we started this study, there was no published literature on the impacts of any fulgorid on its host plants to guide us, so in designing our experiments for which life stages to test on which host trees, we drew on our observations of the unusual tendency of frequent movement of this insect and what we know about factors that influence plant responses to herbivory. Based on 8 years of watching this fulgorid's behavior in the wild and results from a nymph dispersal study we conducted in 2019 (22), we have observed that nymphs tend to move as often as every few days among different host plants but will arrest for a few weeks on black walnut as late-stage nymphs (Walsh and Hoover, pers. obs.), while they will stay on tree of heaven for long periods of time at any life stage (23). Despite SLF's strong preference for tree of heaven (23–25), because its fitness is enhanced when it uses diet mixing (26), they tend to move off tree of heaven for periods of time to feed on other hosts and then move back (17) when they become late-stage adults. Adults then tend to move off trees of heaven when the trees begin to senesce, ending up on maples if they are available, or other hosts that have not yet entered dormancy (Walsh and Hoover, pers. obs).

Materials and methods

To determine the impact of SLF feeding pressure on hardwood tree physiological responses, we used saplings for our study, which allowed us to confine SLF to the same tree for the duration of the experiments. We used two different types of field experiments over two years (2019 and 2020) in which fourth instars or adults were confined to: 1) a single branch in a sleeve cage or 2) whole-single-tree enclosures in a common garden in Berks County, PA. The methods used for measuring physiological plant responses and collecting tissue samples for total non-structural carbohydrate (TNC) and nutrient analyses were the same for both types of experiments. All insects used in the experiment were field collected. For gas exchange measurements in the whole-tree studies, we randomly selected a branch for each measurement date using young, fully expanded leaves each time. We did not use the same leaf at different time points because for some trees (tree of heaven and black walnut) the compound leaves made it difficult to use the same leaflet more than once, due to the compression applied to the leaf by the cuvette in the gas exchange instrument during the measurement process. For all experiments, the number of SLF that died each day (or nymphs that molted to adult in experiments with fourth instars) were recorded and replaced as needed for the duration of each experiment. Also, the number of replicates for each experiment was limited by the time required to take gas exchange measurements with the LI-COR instrument during the window of time solar radiation was equally available to all experimental and control trees (see detailed methods for measuring gas exchange below).

In planning the duration of our experiments, we considered several factors that influence tree primary metabolism in response to herbivory, including insect density and life stage, tree age and size, and the duration of feeding (14). We have found in the wild and in confined rearing of SLF that the combination of smaller and younger trees with the older insects produces greater effects on the plants (Walsh and Hoover, pers. obs). For example, in previous studies when rearing confined SLF on potted trees, we had to swap out tree of heaven, black walnut, and maples for fresh trees weekly or insect mortality was high and development was delayed. Also, as mentioned above, other than tree of heaven, most of the trees that are killed by SLF are saplings or trees of heaven (of any size) following heavy, prolonged feeding, especially by adults (18). Thus, due to the small size of the trees in this study, experiments were limited in duration by issues with increasing SLF mortality. Short-term studies of herbivores on primary plant metabolism are not uncommon in the literature in both herbaceous plants (27, 28) and forest trees (29).

Experimental setup

In fall 2018, a 0.8-ha common garden was established in Blandon, PA consisting of four blocks of trees (hereafter referred

to as Blocks 1-4). Each block contained an equal number of black walnuts, red maples, silver maples, and trees-of-heaven. Block 1 was planted in a completely randomized design with 25 trees per species for a total of 100 trees. These silver and red maples were planted from 26.5-L containers (2.5 and 3.5 years old, respectively), and black walnut (18 months old) from 7.6-L containers (Octoraro Native Plant Nursery, Kirkwood, PA). Tree-of-heaven plants were grown from seed in a greenhouse and transplanted when trees were 1.2-1.5 m tall. The larger trees in Block 1 were of sufficient size to use in the first year of this study (2019).

Blocks 2-4 were arranged in a randomized split-plot design and each plot contained an equal number of black walnuts, silver maples, red maples, and tree of heaven. Within each block there were four plots, one plot per tree species, and plots were randomized within each block. Within each plot there were 48 trees of the same species, and these plots were replicated in each of the 3 blocks. Thus, there were 144 trees per species for a total of 576 trees. Red and silver maple trees were transplanted from 2 to 3-year-old, 1-1.2 m tall, 3.8-L potted trees planted the year before from bareroot stock (Cold Springs Nursery, Doylestown, PA) and maintained in a greenhouse for one year before being transplanted. Black walnut trees (18 months old) were transplanted from 7.6-L pots (Octoraro Native Plant Nursery), and tree of heaven were transplanted from 1.2-1.5 tall, 3.8-L potted plants grown from seed in a greenhouse for one year. All trees were spaced 3 m apart and each block was spaced 3.5 m apart. A single row of buffer trees was planted to surround the entire garden using the same tree species as its neighbor to minimize edge effects. Drip irrigation and deer fencing were installed, while weed management, rodent control and winter pruning were performed by a commercial landscaper. Trees were fertilized in the fall of each year by Bosold Landscaping.

For each experiment we aimed to select trees of different species and within species that were similar in size, especially in caliper measurements (diameter at breast height, DBH). We avoided using the same trees for different experiments within the same year. Details for each experiment are shown in Supplemental Table S1.

Impacts of SLF confined to a single branch using sleeve cages

To determine the impact of SLF feeding pressure on a single branch, two experiments were conducted at the common garden in 2019, one with fourth instar nymphs in July and one with adults in August using varying densities. The larger sizes of fourth instars and adults, as well as the damage incurred to vineyards by the influx of SLF late in the season, strongly suggest that these are the most impactful life stages (11). On July 18, 2019, 18 silver maple and 18 red maple trees were selected in Block 1 and randomly assigned to one of three treatments: control (0 insects), light density (15 nymphs/sleeve), or moderate density (30 nymphs/sleeve). Mean DBH of the maples in this block was 26.2 ± 0.69 mm (SEM) for red and 30.0 ± 0.84 mm for silver. There were 6 replicates per treatment combination (2 tree species x 3 density treatments) for a total of 36 experimental trees. Also, in this first year of experiments at the common garden in 2019, the only trees that were established and growing well enough to use were the red and silver maples in Block 1.

Sleeve cages were sewn from mesh fabric (Joann Fabrics, State College, PA) (68 cm x 28 cm) in the shape of a bag and closed at the proximal end of the branch with a cable tie to contain nymphs on healthy branches of experimental trees. A branch with no sleeve cage was flagged on each tree (controls and treatments) to determine if the sleeve alone impacted plant responses. Branches were approximately 0.8 cm in diameter. Fourth instars were field collected and introduced into the sleeves on July 21, 2019. Sleeves were checked for mortality and dead nymphs were replaced daily to maintain treatment densities. For each tree, one leaf from the branch in each sleeve cage and the flagged branch with no sleeve cage were used to measure gas exchange just before the introduction of nymphs (July 18) and again on the day the experiment was terminated (August 2) when nymphs were all molting to adults overnight and mortality was increasing (12-day experiment). Samples for carbohydrate concentrations were not taken for branches exposed to nymphs in sleeve cages due to no detectable effects of SLF on gas exchange (see Suppl. Table 3B results).

To determine if adult feeding pressure on a single branch impacts tree physiology, 20 red and 20 silver maple trees were randomly selected in Block 1 and assigned to one of two SLF densities, 0 or 40 adults/branch. Given that we had seen no effects of nymph feeding pressure on gas exchange measurements, we used a single high density of SLF to determine if we would see any effects of SLF on maples. We set up 10 replicates per treatment combination (10 replicates x 2 tree species x 2 treatment densities) for a total of 40 experimental trees. Adults were collected on September 24, 2019 and introduced into a sleeve cage on each experimental tree, while empty sleeve cages were placed on control trees. This study was terminated on October 4, 2019 when decline in photosynthetic rates of controls indicated trees were entering dormancy, confounding the variables we were measuring (11-day experiment).

Gas exchange measurements were made as described below on September 24 and 27 and October 1 and 4 using one leaf from the branch in each sleeve cage and one leaf from a flagged branch on each tree without a sleeve cage to determine if the cage alone affected results. For non-structural carbohydrate assays, branch samples were collected on the same dates as gas exchange attributes were measured. Tree roots were collected the following spring as soon as the ground thawed (February 11, 2020) and processed as described below.

Impacts of SLF on trees using whole-tree enclosures

During the second year of this study when trees were more established, we investigated the effect of fourth instar and adult feeding pressure on plant physiology using whole-tree enclosures. This permitted insects to move around on the tree as they do in the wild. Specifically, it allowed them to freely select feeding locations, regulate body temperature, and minimize weather exposure, which helped to reduce insect mortality (see Results section below for details).

Enclosures for whole trees were constructed from the same mesh as the sleeve cages by sewing together a four-sided tube approximately 132 cm wide x 3.5 m tall with two sleeve openings on opposite sides that were closed with a cable tie for easy access to the foliage inside the cages. Enclosures were attached at the top of the trunk with a cable tie to a 3.3-m-tall steel pipe sunk into the ground 30-40 cm deep and distanced 60 cm from the trunk (to avoid damaging the roots). This was necessary to prevent the enclosures from falling over during windy periods. The enclosure covered the entire tree and was attached with a cable tie to the trunk just below the lowest branches to prevent escapes.

Black walnut trees in Block 1 and silver maple trees in Block 2 were randomly selected to be used for fourth instars at densities of 0, 40, 80, or 120 per tree. While fourth instars will feed on silver maple readily (20), they can complete their entire life cycle on mature black walnut (26); SLF are often observed in large numbers on single branches of black walnut as third and fourth instars, moving to maples when walnuts begin to senesce (15). Trunk DBH was 18.6 ± 0.55 mm for black walnut and 14.4 ± 1.20 mm for silver maple. There were five replicates per treatment combination (2 species x 4 densities x 5 replications) for a total of 40 experimental trees. On July 22, 2020, fourth instar nymphs were introduced into the enclosures. Leaf gas exchange was measured as described below prior to insect introduction (July 22) and on three additional dates following insect introduction (July 26, 29 and 31). Enclosures were checked daily for fourth instars that had died or molted overnight to adults and replaced with new fourth instars. After 10 days, the experiment was terminated when all fourth instars were molting to adult overnight. To quantify non-structural carbohydrates, branch and leaf stem samples were collected on July 22 and 31, 2020.

For the adult study, feeding pressure was evaluated on silver maple and tree of heaven that were randomly selected and assigned to treatments in Block 1; we used these larger trees to ensure adequate food for adults. On August 6, 2020, adults were introduced into whole-tree enclosures at densities of 0, 40, 80, and 120 per tree with 5 replicates per treatment for each tree species for a total of 40 trees. Tree diameters were 35.05 ± 1.39 mm for silver maple and 30.0 ± 1.33 mm for tree of heaven. Leaf gas exchange was measured as described below prior to insect introduction on August 6 and August 9, 12, 17, 20 and 25 (20-day experiment). To quantify non-structural carbohydrates and nutrients, branch and leaf stem samples were collected on August 6 and 25 and the experiment was terminated on August 25, 2020. Roots were sampled on April 7, 2021 to determine effects of prior year SLF feeding on belowground carbon stores after overwintering.

Tree gas exchange measurements.

Attributes of gas exchange (C assimilation, transpiration, and stomatal conductance) were measured on one leaf per treated branch and for controls on the dates described above from 09:00 to 13:00 under saturating light conditions (1500 μmol m^{-2} s^{-1}) using a LI-6400 Portable Photosynthesis System (LI-COR Biosciences; NE, US).

Non-structural carbohydrate and nutrient analyses

Stem and root samples were taken from trees infested with SLF as described above. Samples were bagged, immediately frozen in dry ice, and transported to the laboratory for storage at -80°C for later processing. Samples were lyophilized at -50° C and 0.01 mbar negative pressure, then ground using a Wiley model 1 mill with a 2-mm-mesh screen. TNC concentrations were extracted from samples using a modified Somogyi-Nelson reducing sugar determination method (30, 31). This method uses enzymatic digestion to rapidly quantify reducing sugars (e.g., glucose) and starch concentrations and is a cost-effective approach to analyze multiple samples. Briefly, two, 5 mg subsamples of freeze-dried tissue from each shoot, leaf, and root sample were weighed into 2-mL microcentrifuge tubes for soluble sugar extraction and starch digestion. One mL of deionized water was added slowly to each tube to wet the sample material and tubes were boiled in a hot water bath for 20 minutes. Tubes were then removed from the hot water bath and immediately placed in an ice bath until cool. For the soluble sugar extraction, 100 μL of 0.5 M sodium acetate was added to each subsample. For starch digestion, 100 μL of 0.5 M sodium acetate containing 5 units of amyloglucosidase (E.C.3.2.1.3.) and 2.5 units of α-amylase (E.C.3.2.1.1.) was added to each subsample. Tubes were incubated for 24 h at 30°C. Digestion was stopped by boiling samples for 5 min. Tubes were immediately placed in an ice bath until cool.

For the colorimetric analysis, 500 μL of Nelson's reagent A was added to soluble sugar and starch extracts in 5-mL plastic tubes. Extracts were diluted as necessary to ensure that sugar samples were within the range of the standard curve (0-120 μg

glucose mL^{-1}). Tubes were boiled for 10 min and immediately placed in an ice bath to cool. Once cool, 500 µL of Nelson's reagent B was added to each tube followed by 3.5 mL of water. Samples were vortexed and placed in the dark for 30 min. Sample absorbance was read at 520 nm using a UV-1600PC Spectrophotometer (VWR, Radnor, PA). The difference in absorbance between the soluble sugar and starch determination tubes was used to calculate starch concentration. Note that this method does not detect sucrose because it is not a reducing sugar.

Stem, leaf, and root samples were submitted to The Pennsylvania State University Agricultural Analytical Services Laboratory for quantification of N by combustion (32) for some but not all experiments. We did not send samples for N combustion for experiments where no changes in gas exchange attributes or carbohydrates concentrations were detected in response to SLF feeding pressure.

Statistical analyses

Statistical analyses were carried out using JMP Pro 16.1 (SAS Institute Inc., Cary, NC, USA). Linear mixed models were used to determine if SLF treatments had effects on various aspects of tree physiology (gas exchange, tissue carbohydrate and nitrogen concentrations, and tree growth) and daily SLF mortality. Given the non-normal distribution of response variables, which were evaluated with a Shapiro-Wilk W test, data were natural log-transformed ([ln X] +1) before mixed model analysis; the explanatory variables were not transformed. In cases when the log transformation was not sufficient to achieve the normality of distribution due to considerable skewness of the response variable, we used generalized linear mixed models (GLMM) with a gamma distribution and a log link function on untransformed data, which do not require normal distributions. The underlying assumptions of the models were tested, including the distribution and homoscedasticity of residuals, as well as homogeneity of variance across the SLF treatment categories (Leven's test).

A range of explanatory variables was considered in mixed models as fixed effects, including the SLF treatment type (expressed as different SLF densities per tree enclosure), tree species, number of days since SLF were first released on the plant, and the interaction between them. Tree number was modeled as a random effect to evaluate the degree to which between-subject variability of individual trees influenced experimental outcomes. A forward selection procedure was used for choosing a model that best fit the data by adding explanatory variables to a base model one at a time. Overall model fit and parsimony were assessed based on *p*-values, the normality of distribution of model residuals, Akaike's Information Criteria (AIC), and Bayesian Information Criterion (BIC). Statistical significance of differences between groups of categorical variables were evaluated with Tukey's Honest Significant Difference (HSD) test on least square means, but for datasets with unequal variances we used the Games-Howell *post-hoc* test. The Games-Howell *post-hoc* test is used to compare all possible combinations of group differences when the assumption of homogeneity of variances is violated (33). This *post hoc* test is based on Welch's degrees of freedom correction; it uses Tukey's studentized range distribution for computing the p-values and compares the difference between each pair of means with appropriate adjustment for the multiple testing.

We used $p < 0.05$ to designate statistically significant differences between treatments, and $p < 0.1$ to designate marginally significant differences. This is an approach that has been used by others when sample size is limited in field experiments (5, 34), including for a study of impacts of SLF feeding on grapevines (5). It also avoids committing a Type II error (failure to detect a difference when there is one). Exact *p*-values are reported to facilitate data interpretation and transparency.

Results

Gas exchange in sleeve cages.

In 2019, fourth instar nymphs confined in sleeve cages at high density (30 SLF), while not significantly different from the controls, marginally enhanced photosynthetic rates of both silver and red maple leaves, while the low density (15 SLF) marginally suppressed rates at the end of the 12-day experiment (August 2, 2019) (Supplemental Table S2, Figure S1). Feeding of nymphs had no effects on stomatal conductance or transpiration.

In contrast to nymphs, sleeve cages containing 40 adults confined to a single branch significantly suppressed C assimilation by 4- to 20-fold compared to controls by day 3 ($p<0.0001$, Figure 1). The full mixed model with measurement date, tree species, SLF treatment type (branch with or without SLF) and interaction terms of these explanatory variables showed that the degree of influence differed by day of the experiment (Table S3A). C assimilation for all trees started at about the same rate at the beginning of the experiment, but at day 3 ($p = 0.0252$), 7 ($p = 0.0050$), and 10 ($p = 0.0349$), SLF feeding significantly suppressed C assimilation compared to controls. Sleeve cages alone had no significant effect, as evidenced by comparing C assimilation measured on branches with sleeves and no SLF to those without sleeves on both treatment and control trees (Supplemental Table S3B).

Adult feeding also altered stomatal conductance and transpiration. Mean conductance decreased by 51% for red maple ($p = 0.0007$) and 65% for silver maple ($p<0.0001$) on branches fed on by SLF. Feeding also affected the temporal

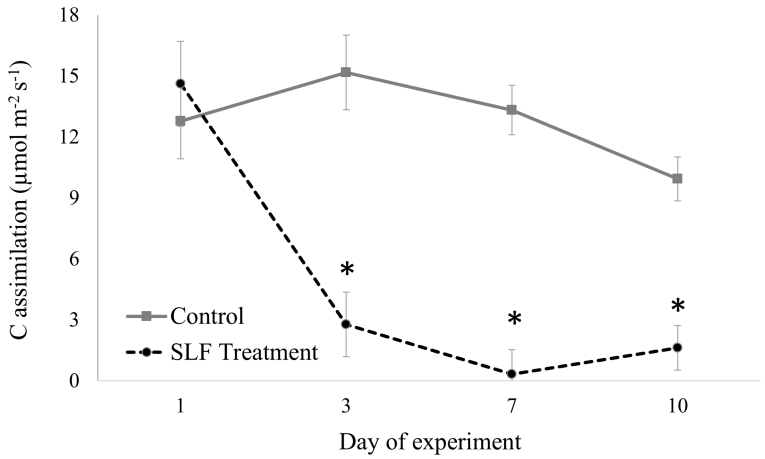

FIGURE 1
C assimilation over time measured on leaves in sleeve cages at the common garden in 2019. SLF adult densities were 0 or 40 per sleeve cage. Measurements were made on Sept. 24 and 27, and Oct. 1 and 4, 2019. Asterisks denote dates when rates on treated trees were significantly different from controls ($p < 0.05$).

patterns of conductance in a similar manner for both tree species (Figure 2); trees with no SLF infestation showed peak conductance in the middle of the experiment, whereas for infested trees, conductance steadily declined. The response of transpiration (Figure 3) to feeding did not differ between tree species, but rather depended on the day of the experiment ($p<0.0001$), declining sharply on infested branches, increasing on control tree branches by Day 3, then gradually declining on all branches towards the end of experiment (mean decline was 47% for the whole experiment).

Gas exchange in whole-tree enclosures

In 2020 using whole-tree enclosures, we documented effects of feeding by fourth instar nymphs on gas exchange attributes; in general, these metrics were higher for black walnut than silver maple across SLF densities. C assimilation tended to increase slightly over time for all treatments (40, 80, and 120 per tree) for black walnut compared to controls and tended to decrease slightly over time for silver maple except for the last date (Supplemental Figure S2); however, these effects were not statistically significant for the 10-day duration of the experiment (Supplemental Table S4A). There were also no significant effects on stomatal conductance or transpiration at any nymph densities.

For adults confined to whole trees for 20 days, the overall model revealed that responses in gas exchange to different densities of adult feeding pressure were dependent on tree species and time post-infestation (Supplemental Table S4B). For both tree species, moderate (80 adults/tree) and high-density (120 adults/tree) feeding pressure significantly suppressed C assimilation ($p = 0.0001$ and $p = 0.0433$, respectively) compared to controls. However, the timing of the responses differed between tree species. For tree of heaven (Figure 4A), C assimilation by trees exposed to adults with moderate density was marginally lower than for controls on Day 7 ($p = 0.0680$) and declined on Day 9 for all trees including controls exposed to adults. On later dates, however, C assimilation for control trees recovered to near previous levels then gradually decreased, while there was no recovery for treated trees; instead, C assimilation continued to decline sharply. On day 15, C assimilation was significantly suppressed at moderate adult SLF density by 54% compared to controls ($p = 0.0349$) and by 52% compared to low SLF density ($p = 0.0356$). On the last day of the experiment (Day 20), C assimilation for moderate SLF density was significantly lower than controls ($p = 0.0320$), but not significantly different from the low-density treatment ($p = 0.1669$).

For silver maple (Figure 4B), at 7 days post-infestation, the moderate density of SLF marginally suppressed C assimilation by 65% compared to control trees ($p = 0.0509$, Tukey *post-hoc* test). These differences were not significant for the remainder of the experiment.

Temporal patterns and the magnitude of changes in conductance and transpiration responses to adult SLF feeding largely mimicked those of C assimilation, gradually declining with increasing pest pressure (Supplemental Figures S3, 4; Supplemental Table S4B). Transpiration and stomatal conductance for tree of heaven declined significantly for trees with low SLF density ($p = 0.0062$ and $p = 0.0234$, respectively) compared to control trees on Day 4 (Supplemental Table S4B). On the last measurement date, transpiration and conductance for control trees or trees with low and high density SLF were not

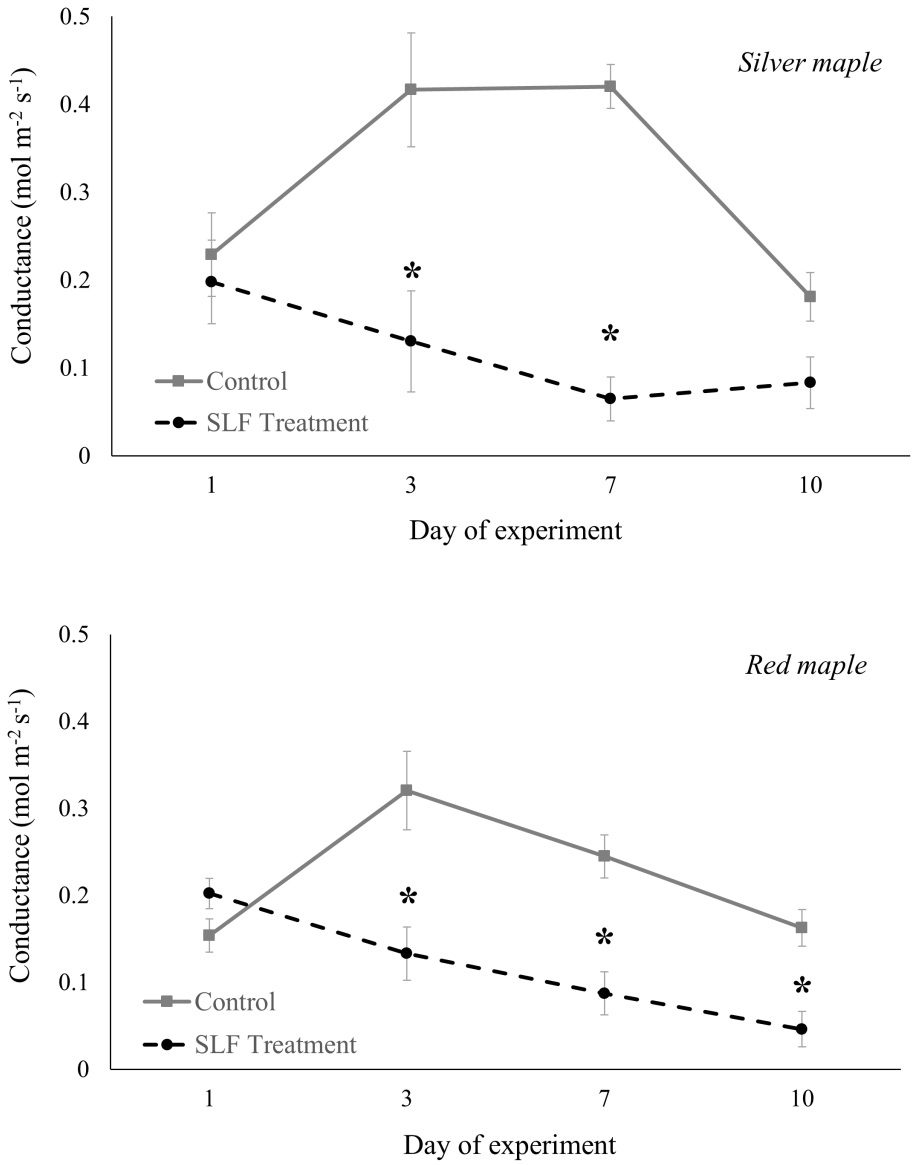

FIGURE 2
Stomatal conductance of silver maple and red maple by SLF treatment measured on leaves in sleeve cages at the common garden in 2019 on Sept. 24 and 27, and Oct. 1 and 4. Spotted lanternfly adult densities were 0 or 40 per sleeve cage. Asterisks above the standard error bars denote dates when conductance on treatment trees were significantly different from controls ($p < 0.05$).

different from each other, but the trees with moderate densities of SLF had lower levels than the controls (significantly lower on day 20 for transpiration $p = 0.0031$ and conductance $p = 0.0053$). Transpiration and conductance in silver maple declined for trees with moderate and high-density adult infestations, but trees with light infestation had similar transpiration rates to the control. The difference between moderate density and controls were marginally significant on Day 4 (transpiration lower than controls by 48% and conductance by 42%). However, on Day 7 transpiration was significantly reduced by 67% and conductance by 63%. For high density, these variables were significant only on Day 7 (transpiration lower by 64%, conductance by 63%).

Non-structural carbohydrates in sleeve cages

SLF feeding significantly reduced the concentrations of soluble sugars in wood tissue of branches (mostly xylem), and

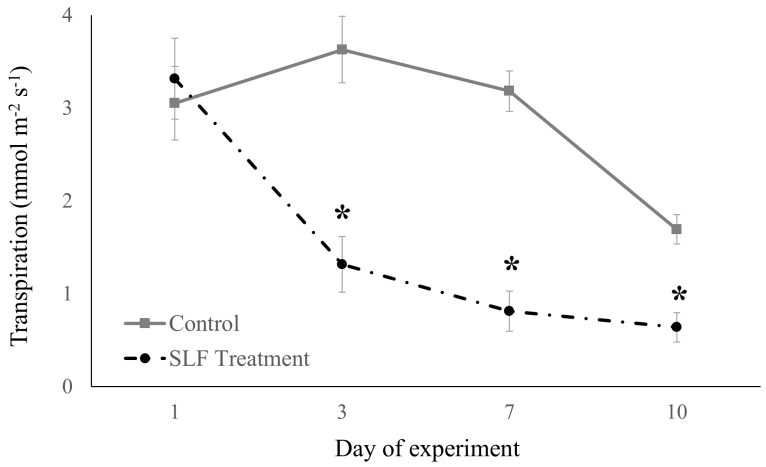

FIGURE 3
Transpiration measured in sleeve cages at the common garden in 2019 on Sept. 24 and 27, and Oct. 1 and 4 in response to 40 SLF adults per cage compared to controls. Asterisks above the standard error bars denote dates when transpiration rates on treatment trees were significantly different from controls ($p < 0.1$).

this response differed by tree species and season of when samples were taken (Table S5A). For wood tissues in branches sampled in the fall immediately after the end of the experiment, soluble sugar concentration was reduced by 65% in silver maple branches treated with SLF compared to controls ($p = 0.0059$, Tukey test), but not in red maples (Figure 5A). However, there were no significant differences in TNC, soluble sugars (glucose equivalents), or starch concentrations in roots or bark of silver or red maple trees resulting from exposure to adult SLF in sleeve cages in the 2019 experiment (Supplemental Tables S5A, B).

Branches fed on by SLF and sampled again the following spring (2020) after overwintering had soluble sugar concentrations in woody tissue that were 62% higher in silver maple ($p<0.0001$) and 40% lower in red maple ($p = 0.043$), compared to controls (Figure 5B).

Non-structural carbohydrates in whole-tree enclosures 2020

SLF treatment with the moderate density of fourth instars (80 per tree) significantly reduced the fraction of soluble sugars to TNC by 23-29% in leaves of silver maples compared to control trees ($p = 0.0433$, Games-Howell *post-hoc* test) and compared to high SLF density ($p = 0.0170$) (Supplemental Tables S6A, B, Figure 6). Carbohydrate concentrations in branch woody tissue for silver maple declined in a density-dependent manner in response to nymphs; soluble sugar concentrations were 53% lower on average in silver maples exposed to high-density nymph feeding pressure ($p = 0.0029$) compared to controls, and 33% lower compared to trees with low nymph density ($p = 0.0138$) (Supplement Table S6B, Figure 6). TNC concentrations also decreased significantly in silver maple branches by 40% in trees exposed to high SLF density compared to controls ($p = 0.0211$), and by 41% compared to low SLF density ($p = 0.0185$). Carbohydrate concentrations for black walnut were unaffected by 4th instar feeding pressure at any density (Supplement Table S6A).

Adults in whole-tree enclosures had no significant effects on carbohydrates for any of the tissue types (roots, branch, leaves) collected from silver maple or tree of heaven at any density (Supplement Table S7).

Effects of SLF feeding on nitrogen

Independent of tree species, leaves from trees with adult SLF confined to a branch in sleeve cages had 20% lower N concentrations compared to controls ($p = 0.0205$) and a 25% higher ratio of carbon to nitrogen ($p = 0.0447$) in leaves compared to trees without SLF (Table S8). We did not detect any significant effects of adults on nitrogen in roots or leaves collected from silver maple or tree of heaven in whole-tree enclosures at any SLF density (Supplement Table S9).

Tree growth

Tree diameters (at breast height, 1.4 m) were measured before and after the whole-tree enclosure experiments to determine if SLF feeding impacted tree growth; we did not document diameter growth of trees exposed to SLF in sleeve cages because we did not expect effects on whole-tree growth from feeding a short time on a single branch. Silver maples exposed to fourth instars in whole tree enclosures showed

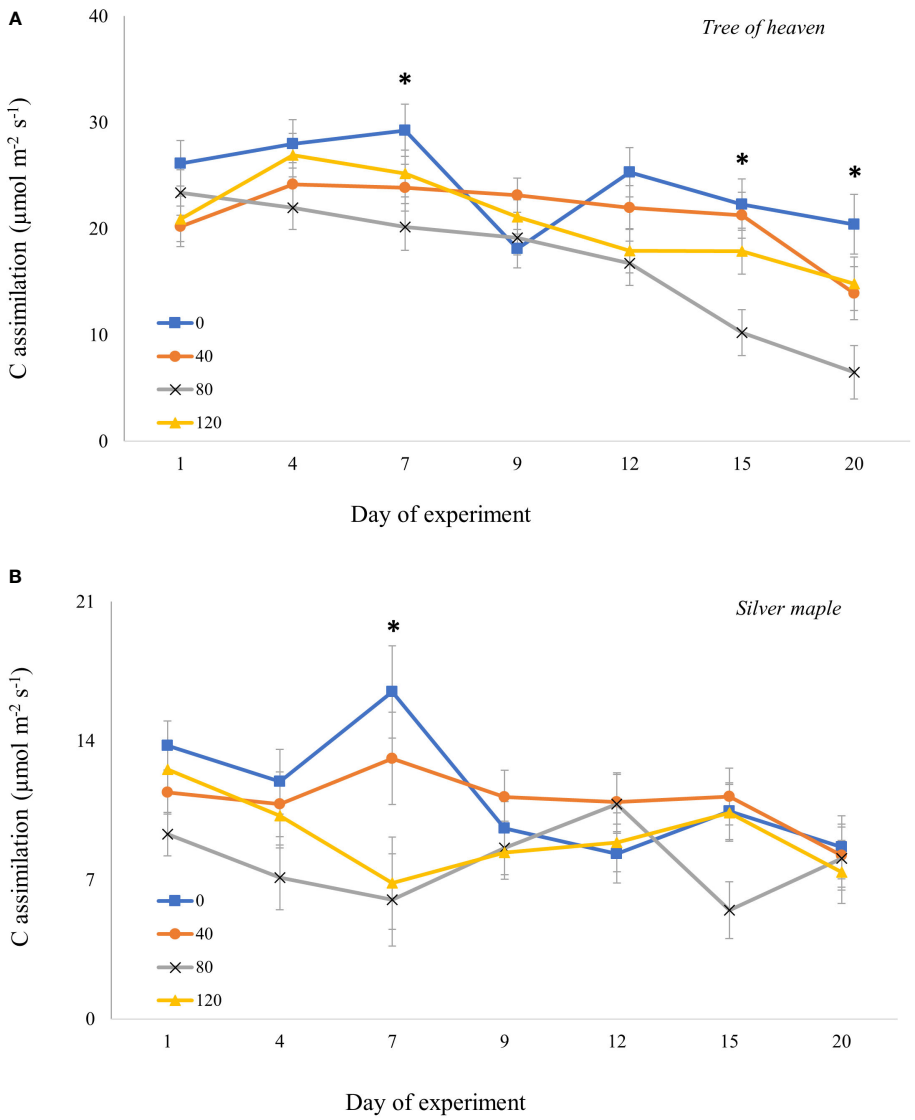

FIGURE 4
Carbon assimilation (photosynthetic rate) over time for tree of heaven **(A)** and silver maple **(B)** at the common garden. Spotted lanternfly adult densities were 0, 40, 80, and 120 per whole-tree enclosure. Note the differences in photosynthesis rates on the y-axes. Asterisks above the standard error bars denote dates when C assimilation rates on treatment trees were significantly different from controls ($p < 0.05$).

significantly stunted diameter growth during the next growing season (2021), and the decline was proportional to SLF density. Compared to controls, treatments with the highest SLF density reduced diameter growth by 55% ($p = 0.0005$), by 42% for moderate density ($p = 0.0139$), and by 38% for low density ($p = 0.0359$) (Figure 7). There were no significant effects from fourth instars on growth of black walnut trees in the same experiment. There were also no effects on diameter growth of silver maple or tree of heaven in response to feeding by SLF adults in whole-tree enclosures for three weeks during the same season (2020) or when measured the following growing season (2021).

SLF mortality during experiments

For all experiments, enclosures were checked daily for mortality and dead insects were replaced to maintain constant feeding pressure by the same life stage present at the start of the experiment. In 2019, mortality of fourth instars in sleeve cages depended on tree species ($p = 0.0355$) and day of the experiment ($p<0.0001$); more nymphs died on red maple (mean of 18 ± 1.1% per day) than on silver maple (mean of 11 ± 1.1% per day), and the proportion of insects that died increased towards the end of experiment for both tree species. Tree number as a random effect

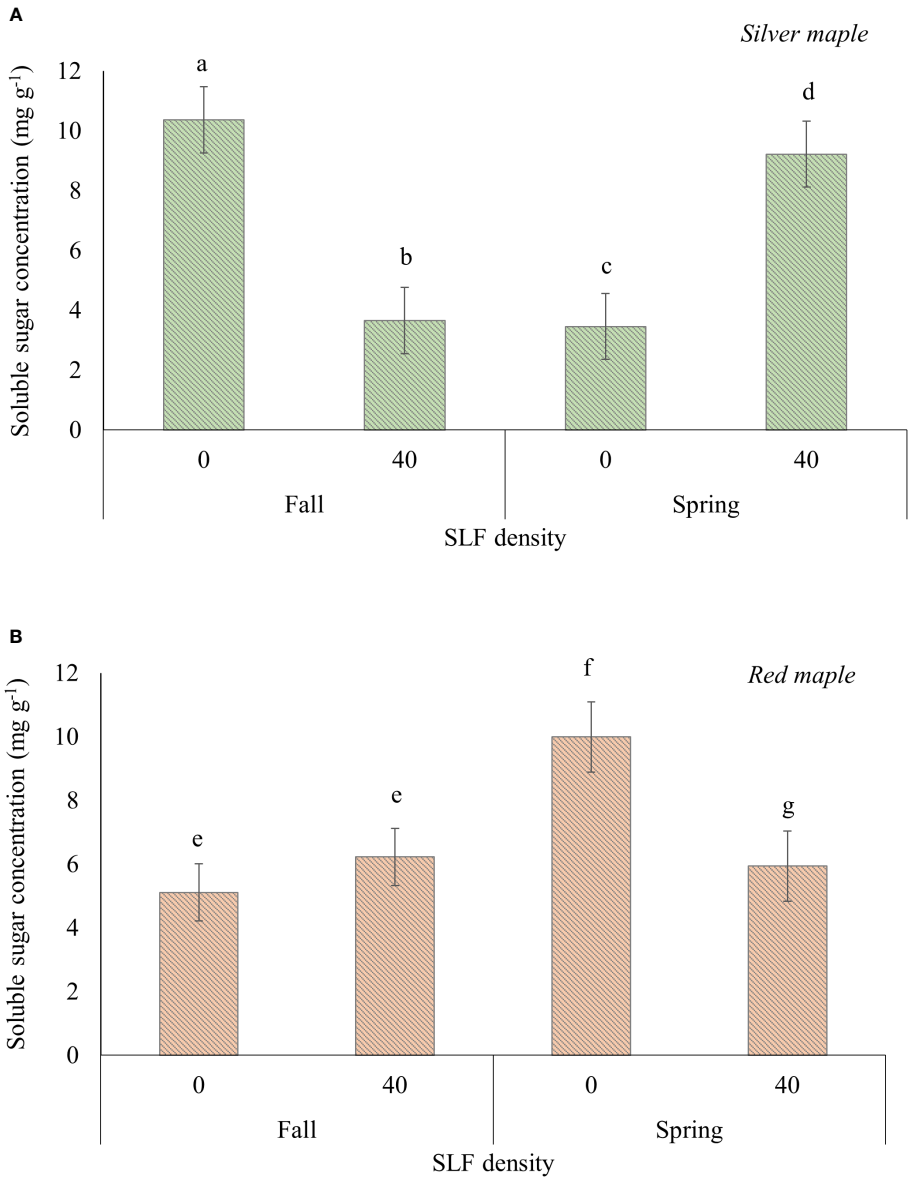

FIGURE 5
Average (least square means) concentrations of soluble sugars in branch woody tissue of silver **(A)** and red maple **(B)** trees exposed to SLF adults in sleeve cages. Branches were collected on October 4, 2019 (fall sampling), and March 27, 2020 (spring sampling). Carbohydrate concentrations are given in mg of glucose equivalents per g of dry tissue. Significant differences ($p < 0.05$) in soluble sugar concentrations between treatments for a given tree species within a sampling season are shown as different letters above the standard error bars.

was also significant ($p = 0.0021$), indicating high variability in percentage mortality of SLF among trees within treatment. There was no difference in the percentage of insects that died as a function of SLF density (15 or 30 fourth instars/sleeve).

Mortality of adults in sleeve cages in 2019 was greater than for nymphs and differed by tree species ($p<0.0001$) and day of the experiment ($p < 0.0001$). A higher percentage of SLF died in sleeves on red maple (43.6 ± 3.1% per day) than on silver maple (30.9 ± 3.1% per day), increasing towards the end of the experiment for both tree species. Tree number as a random effect was not significant ($p = 0.2424$).

In 2020, fourth instar mortality in whole-tree enclosures depended on tree species ($p < 0.0001$), day of the experiment ($p =0.0005$), and SLF density ($p = 0.0156$). There was a higher mean percentage of dead nymphs on silver maple (12.7 ± 0.64% per day) than on black walnut (5.9 ± 0.64% per day). Mean daily mortality decreased gradually until Day 6 for black walnut and Day 4 for silver maple, then went up towards the end of the experiment for

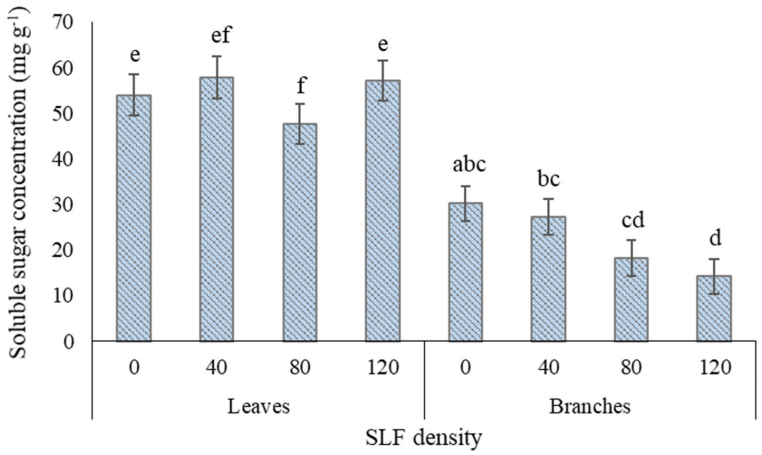

FIGURE 6
Average (least square means) concentrations of soluble sugars in leaf and branch tissue of silver maple exposed to varying densities of fourth instar SLF nymphs during the whole-tree enclosures experiment. Leaves were collected on July 22 and 31, 2020, and branches were collected on July 31, 2020. Carbohydrate concentrations are given in mg of glucose equivalents per gram of dry tissue weight. Significant differences ($p < 0.05$) in fraction of soluble sugars for leaves and in soluble sugar concentrations for branches between treatments within a tissue type are shown as different letters above the standard error bars; standard errors are for each mean total non-structural carbohydrate concentration.

both tree species. On Days 4 ($p = 0.0333$), 5 ($p = 0.0029$), and 6 ($p = 0.0011$) there were significantly lower percentages of daily mortality than on the first 3 days of the experiment. Interestingly, daily mortality decreased with increasing number of SLF per enclosure; at low density daily mortality was the highest (12%, $p = 0.0227$), and was significantly greater than in the enclosures with high SLF density (7%), while the moderate SLF density was intermediate (8%), which were not significantly different from the other treatments (Figure 8). Tree number as a random effect was significant ($p = 0.0143$).

Not surprisingly, adult mortality was markedly lower in whole-tree enclosures than in sleeve cages with daily mortality depending on tree species ($p < 0.0001$) and day of the experiment ($p < 0.0001$). Overall, adult SLF mortality on whole trees was generally low; mortality on silver maple was 5% per day and on tree of heaven it was 2% per day. For both tree species, mortality increased slightly until Day 4, followed by a decrease towards Day 8, then leveling off towards the end of experiment. Tree number as a random effect was significant ($p = 0.0043$), indicating high variability in percentage mortality of SLF among trees within treatment. There was a similar trend in mortality as a function of SLF density for adults as for nymphs, with higher mortality in enclosures with the lowest density, but these differences were not statistically significant ($p = 0.2700$).

Discussion

Plants use a variety of strategies to tolerate and defend against herbivory; they may shift rates of photosynthesis and alter allocation of carbon and nitrogen resources to growth (3, 5), or to induced plant defenses (1, 19). Some plants respond by reducing carbohydrate reserves available for overwintering (5) and for growth the next year (26). In young grapevines, adult SLF feeding was shown to have dramatic effects on C assimilation and belowground starch reserves and these effects were density dependent (5).

We found that SLF adults confined to a single branch, in contrast to nymphs in sleeve cages, rapidly suppressed C assimilation, transpiration, and stomatal conductance for both red and silver maples, which was visible by 3 days post-infestation and continued for the duration of the experiment. Stomatal conductance was slightly more reduced for silver maple relative to red maple (65% vs. 51%, respectively, compared to controls), but it's important to note that daily percentage insect mortality was also lower on branches of silver maple than on red maple. In addition, nymphs in sleeve cages suppressed nitrogen concentrations in leaves of both maple species, but this did not occur in response to adult feeding. These findings may explain why we rarely see heavy feeding by fourth instars on maples in the wild; they tend to move to maples in mid-September as adults, so it's possible that the phenology of this host plant-insect interaction coincides with the timing during which SLF can more successfully tolerate tree defenses and/or obtain sufficient nutrients.

Soluble sugars in the wood of branches and nitrogen concentrations of both maple species in response to fourth instars declined markedly by the end of the experiment in the fall but recovered and was at higher concentrations for silver maples than controls the following spring, but not for red maple. This suggests that SLF can manipulate carbon allocation in these

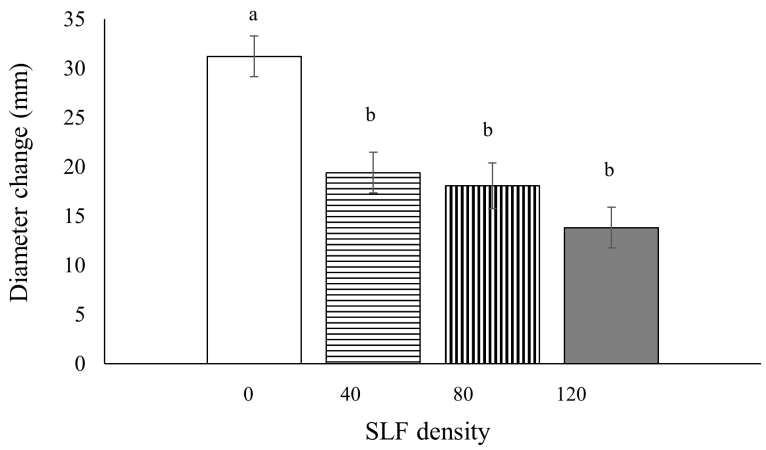

FIGURE 7
Mean tree diameter growth (at breast height) for silver maple during the growing season following the 2020 experiment as affected by SLF fourth instars feeding at different densities. Significant differences ($p < 0.05$) between treatments shown as different letters above the error bars. Tree diameters were measured on April 7, 2021 and March 15, 2022.

trees, but at different times points; soluble sugar concentrations in silver maple recovered by the next season but reduced soluble sugars in red maple were not evident until the trees had overwintered.

We expected fourth instars to reduce gas exchange attributes, TNC, and growth in black walnut trees given that this is a preferred host for late-stage nymphs (27) and that dieback is often observed when SLF congregate on mature black walnut branches in forests (28), parks and residential neighborhoods (Walsh and Hoover, pers. obs.). Instead, fourth instars had no effect on any variables we measured when given access to black walnut in whole-tree enclosures. In the field in July and early August, it is common to see overlapping life stages of SLF on trees and, thus, branches of black walnut may be heavily fed upon for several weeks as new third instars arrive and molt to fourth instars, prolonging the time late-stage nymphs feed on single black walnut branches. This pattern of movement is especially noticeable on mature black walnuts where fourth instars may also remain on branches after molting to adult until black walnuts begin to senesce. We also did not observe any

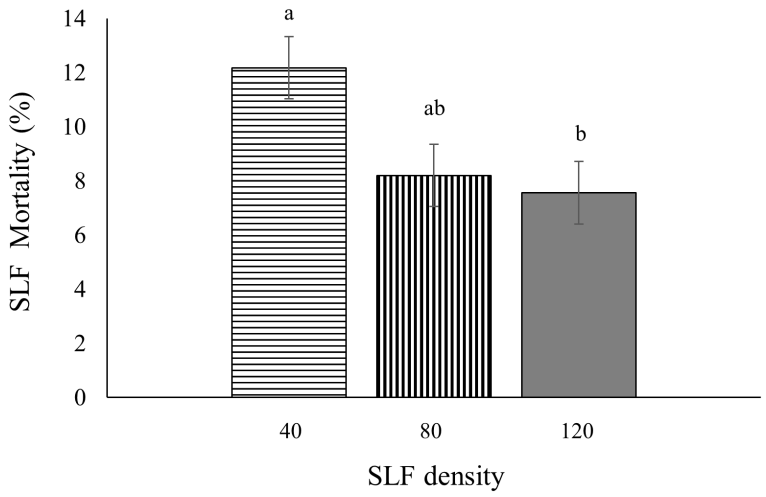

FIGURE 8
Mean percentage mortality per day for fourth instars in 2020 was influenced by SLF density in whole tree enclosures. Significant differences ($p < 0.05$) between treatments shown as different letters above the standard error bars.

wilting or branch dieback on these trees, so we suspect that our experiment on black walnut was too short (10 days) to reproduce the physiological impacts that can occur in the field with heavy late stage feeding pressure. However, because our aim was to examine the effects of fourth instars and not adults on black walnut, we terminated the experiment when every fourth instar was molting to adult overnight and mortality on this host was increasing, producing confounding factors.

While we did not find significant reductions in C assimilation by fourth instars in whole-tree enclosures in 2020 for silver maple, the soluble sugar (glucose equivalents) to TNC ratios were reduced in leaves in response to moderate feeding pressure compared to controls and compared to the other nymph densities. Also, soluble sugars in branch wood were reduced by half for silver maples fed on by the high density of nymphs compared to controls and by one-third compared to trees fed on by the low density of nymphs, indicating that SLF was able to manipulate carbohydrates in a density-dependent manner. Interestingly, the reductions in soluble sugars in leaves and branch wood of silver maples co-occurred with reductions in tree diameter growth in a density-dependent manner. Growth was reduced by more than half at the high SLF density, and reductions in growth gradually declined as SLF density decreased. This finding may have economic implications for maple saplings in production nurseries or regenerative growth in forests where slower growth in response to high SLF populations could be costly.

An unexpected finding was that the percentage mortality of nymphs in whole-tree enclosures was inversely related to SLF density; a greater percentage of nymphs died daily at the lowest SLF density than at higher densities for both silver maple and black walnut. It is possible that larger numbers of SLF were better able to manipulate resource allocation, as was shown for SLF adults on grapevines as the density of SLF increased (5). This may occur through a greater volume of salivary enzymes injected into the phloem during feeding at higher SLF densities, reducing the ability of the plant to limit sap flow by callose formation. This would be consistent with a previous report that the aphid *Megoura viciae* can prevent sieve tube plugging in the phloem using salivary proteins during feeding, which provides aphids with access to a continuous flow of phloem sap (29). Note that a similar trend in higher proportional mortality at lower SLF densities was also observed for adults on tree of heaven and silver maple, but these results were not statistically significant, perhaps due to the high variability among trees within treatment (tree number was a significant random effect for every experiment except for adults in sleeve cages).

The effects of adults when given access to the whole tree, and especially to the trunk where adults frequently feed, were more subtle than results of adults confined in sleeve cages. Suppression of gas exchange was greater and more consistent for tree of heaven than for silver maple, becoming evident after 2 weeks of feeding pressure. Adult feeding on whole trees also did not affect carbohydrate concentrations or tree diameter growth. For silver maple, we suspect that fourth instars may have had a stronger effect than adults due to differences in tree size, tree age, and feeding location. Silver maples exposed to fourth instars in our experiments were 2 years younger and less than half the diameter of the silver maples used for adults. Herbivores tend to have a greater impact on younger, smaller trees (14), and high SLF populations can kill saplings, whereas this rarely happens to larger trees, with the exception of tree of heaven that have been fed on heavily for several months, especially if they are attacked multiple years in a row (9, 15). In addition, since fourth instars tend to feed on branches rather than the trunk, their feeding activity is physically closer to where gas exchange, carbohydrates, and nitrogen were measured.

Our findings were similar, but not as consistent, as responses documented from heavy adult SLF feeding on young grapevines. In a recent study, intensive, continuous, late-season feeding by large adult SLF population densities (70 to 200 SLF per vine) significantly reduced gas exchange attributes in young grapevines (5). Adult SLF were found to compete with grapevine sinks for resources, leading to whole-plant carbon and nitrogen limitation, especially in the roots. In the US, the greatest economic impact from SLF introduction has been damage to vineyards. In Pennsylvania, it is not uncommon to find >100 SLF adults feeding on a single vine (12), and this heavy, repeated phloem-feeding can strongly reduce grape yields (up to 90%), fruit quality, and, in some instances, kill vines (11). In contrast, SLF rarely kill trees in the field other than occasional young saplings, and tree of heaven of any size, in response to long-term heavy feeding (18). In the field, tree of heaven is the preferred host for every life stage; thus, feeding pressure, especially by adults, can be very heavy for several months.

A potential explanation for failure to detect significant differences in plant physiology in all experiments may be the high degree of variability in our explanatory variables among trees within treatment. Tree number was a significant random effect in most experiments, except for adults confined to a single branch where impacts from SLF were overwhelming, suggesting that in most cases there was considerable variability from tree to tree within treatment, including in the ability of each tree to support the same number of SLF individuals. This lends credence to the term "hot" tree used by SLF researchers to describe high densities of SLF (nymphs or adults) on one tree when it's surrounded by others of the same species, size, and apparent health with few SLF, suggesting that some trees are better hosts than others (17). Larger sample sizes may have mitigated high variability, but sample size was limited by the time required to take measurements with the LiCor instrument during peak solar radiation. In some cases, the duration of the experiments may have been too short to impact the plant metrics we measured, but it was not possible to maintain SLF at the same life stage and density beyond the duration of our experiments, introducing a confounding factor we could not control.

We suggest that impacts of SLF in the wild are greater when trees are also stressed by other biotic or abiotic factors; under these circumstances SLF is another stressor and effects can be

cumulative. In 2019, we observed poor health and structural damage of red maples with cankers at the union of main branches after being heavily fed on during the 2018 growing season when rainfall was 150% of normal in Pennsylvania (35). Cultures were taken from these cankers, but only opportunistic, endemic fungi were detected (*Botryosphaeria* and *Nectria* spp.), which are usually benign, but spores can invade trees through wounds when splashed around by rain (36).

In summary, our results suggest that SLF late-stage nymphs feeding at high densities for relatively short durations of time on young maples may have only minor effects on gas exchange attributes, but could significantly reduce nutrient concentrations such as carbohydrates and nitrogen, which in turn may reduce diameter growth. Tree of heaven was more affected by adults than silver maple trees of similar size, suggesting that higher numbers may overwhelm the defenses of tree of heaven, which co-evolved with SLF in its native range (9). This finding also helps explain our observations of eventual death of saplings and mature tree of heaven. At the same time, declines in C assimilation in response to adult feeding in tree of heaven were not reflected in altered nutrient concentrations in roots or leaves, in contrast to the marked impacts on below-ground C and N content in young grapevines in response to heavy adult feeding pressure (5).

Based on our results, we recommend that production nurseries, forest managers, and homeowners continue to protect young maple and black walnut saplings, especially once SLF become adults, and minimize plant stressors to mitigate cumulative impacts. In the wild, we observe that SLF are more likely to feed on larger trees as they develop into later life stages, and more mature trees may not experience significant harm from even high populations, although late season feeding can occur for a month or more on maples for multiple years (17). Moreover, if trees are stressed, we cannot rule out that even larger trees may suffer reduced health and growth given that no long-term studies have been done on mature trees in response to SLF feeding.

Data availability statement

The original datasets used for this study can be found in Penn State University's Scholar Sphere at https://scholarsphere.psu.edu/resources/c0418d6c-6b38-407d-b879-469fffc6d442/ and in the Supplementary Materials. Further inquiries can be directed to the corresponding author.

Author contributions

DE and KH funded the study and planned the experimental design with EL. EL conducted most of the experiments, assisted by EP, OU, JH, and LI. LI conducted the data analyses and prepared the tables and figures. LI and KH wrote the manuscript. BW designed and planted the common garden and consulted on planning experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2022.1080124/full#supplementary-material

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The impact of host plant species on instar duration and body weight of nymphal *Lycorma delicatula*

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The spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is an invasive species of planthopper that was introduced to North America and is a threat to multiple industries. Nymphs and egg masses were collected to assess each instar's rate of development at a constant temperature of 25°C on the following hosts: *Ailanthus altissima* (Miller) (Sapindales: Simaroubaceae), *Vitis labrusca* (L.) (Vitales: Vitaceae), *Salix babylonica* (L.) (Malpighiales: Salicaceae), *Acer rubrum* (L.) (Sapindales: Sapindaceae), *Celastrus orbiculata* (Thunberg) (Celastrales: Celastraceae), *Ocimum basilicum* (L.) (Lamiales: Lamiaceae), and *Rosa multiflora* (Thunberg) (Rosales: Rosaceae). Host plant species was found to have a significant effect on developmental time for nymphs in the first through third instars, as well as on nymphal survival. Nymphs failed to develop through the second instar on *O. basilicum* and the third and fourth instars on *A. rubrum*. Host plant species also had a significant effect on the mean weight of nymphs in the first, second, and fourth instars (but not in the third instar), and on the hind tibia length and forewing width of adult nymphs. This variability in *L. delicatula* developmental time by host plant species can potentially impact phenology models, which should be updated to reflect these new insights. Rearing practices should also be refined to account for host plant influences on the physiology of *L. delicatula*.

KEYWORDS

phenology, survival, development, hostplant, lanternfly

Introduction

The spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is an invasive species of planthopper that was first detected in the United States in the summer of 2014. *L. delicatula* is native to China, Vietnam, and India; the United States is one of three countries invaded by this species, together with Japan and South Korea (1). Since its initial detection in Pennsylvania, *L. delicatula* has spread across the northeastern region of the United States and is now established in multiple states (1).

L. delicatula has four instars. The first-instar nymphs start to emerge from eggs in late April in North America (2). Nymphs of the first three instars are black and white, and fourth-instar nymphs are black, white, and red in color. Adults appear around mid-July and lay eggs from early September until temperatures are low enough to kill them. The eggs are deposited in grayish to tan-colored egg masses on various substrates, such as bark, stone, wood fences, and brick, on which the egg masses overwinter until the following spring.

L. delicatula has a broad host range consisting of 103 plant species (2). Despite this, *L. delicatula* has a preferred host, which is the tree of heaven, *Ailanthus altissima* (Miller) (Sapindales: Simarobaceae) (3). Recently, it was found that *L. delicatula* does not require *A. altissima* to complete its lifecycle, but that the removal of *A. altissima* from its diet is associated with reduced fitness (4). However, despite being widespread and commonly found in disturbed sites, *A. altissima* is not always available as a host for *L. delicatula*.

External temperature has a major influence on the development and growth of insects; however, other factors can also influence their growth. Previous research has shown that the host plant can affect an insect's phenology and should be considered in phenology models (5, 6). For example, the larvae of the Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), develop more quickly when feeding on *Prunus persica* (L.) (Rosales: Rosaceae) than when feeding on *Malus domestica* (Borkhausen) (Rosales: Rosaceae) (7). Likewise, nymphs of the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), from the second instar onward were found to develop more quickly when reared on *P. persica* than when reared on *M. domestica* (8).

The phenology of *L. delicatula* has been previously determined on *A. altissima* and *Parthenocissus quinquefolia* (L.) (Vitales: Vitaceae) (9, 10). In the latter study, when *L. delicatula* was reared on *P. quinquefolia* at room temperature (assumed to be slightly above 20°C), it was found that the developmental duration of the first-, second-, third-, and fourth-instar nymphs was 18.8, 20.9, 20.8, and 22.2 days, respectively (10). When *L. delicatula* was reared on *A. altissima* at that temperature, it was found that the duration of the first-, second-, and third-instar nymphs was 23.4, 24.0, and 40.4 days, respectively (9). The data for the fourth-instar nymphs were separated by sex, with male and female nymphs completing that instar within 39.5 and 50.1 days, respectively. These differences in results, with *L. delicatula* taking less time to develop on *P. quinquefolia* than on *A. altissima* at 20°C, suggest that host plant species also influences their development. In addition, fourth-instar nymphs were found to take fewer days to develop at 25°C when they were reared on fox grape, *Vitis labrusca* (L.) (Vitales: Vitaceae), than on *A. altissima* in an unpublished study (8), a finding which further stresses the importance of determining the developmental rate of *L. delicatula* on different host plants.

To further understand the effect of host plant species on the development of *L. delicatula*, it is important to rear nymphs on a variety of different host plants. In this study, the survival and development of nymphs and the weight and size of *L. delicatula* adult insects were examined using one of the following plants as a host: tree of heaven (*A. altissima*), fox grape (*V. labrusca*), weeping willow [*Salix babylonica* (L.) (Malpighiales: Salicaceae)], red maple [*Acer rubrum* (L.) (Sapindales: Sapindaceae)], Oriental bittersweet [*Celastrus orbiculata* (Thunberg) (Celastrales: Celastraceae)], basil [*Ocimum basilicum* (L.) (Lamiales: Lamiaceae)], and multiflora rose [*Rosa multiflora* (Thunberg) (Rosales: Rosaceae)]. The results from this study will help to further advance phenology models for this insect.

Methods

Source populations

On 17 June 2020, *L. delicatula* first-instar (n = 140) and second-instar (n = 63) nymphs were collected at a site in Hunterdon County, New Jersey, USA (Riegelsville, NJ). The site had *Vitis* spp., *Rosa* spp., *C. orbiculata*, *A. altissima*, *Celtis occidentalis* (L.) (Rosales: Cannabaceae), and *Juglans nigra* (Fagales: Juglandaceae), as well as other assorted unidentified shrubbery. The nymphs were found mostly in the shade, and egg masses were observed on site. The nymphs were transferred to a quarantine facility located in Ansonia, Connecticut, USA, as per the terms of the US Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) permits, in containers containing a single 50- to 70-cm-long sprig of wild grape, *Vitis volpina* L. (Vitales: Vitaceae), to sustain them for the trip. At the quarantine facility, the nymphs were sorted by instar and placed into a large mesh cage (60 cm × 60 cm × 120 cm; BugDorm 6S620, MegaView Science Co., Ltd, Taichung, Taiwan) with two or three 100-cm-tall potted *A. altissima* plants and a single *V. labrusca* plant and kept at 25°C with a photoperiod of 16 h : 8 h (L : D) and a relative humidity between 60% and 80%. Once the nymphs began molting to the next instar, 10 that molted on the same day were taken and set up in a smaller 32.5 cm × 32.5 cm × 77.0 cm mesh cage (BugDorm 4S3074, MegaView Science Co., Ltd, Taichung, Taiwan) containing two host plants of the same species for use in experiments. These smaller cages were kept at the same photoperiod, temperature, and humidity as the other larger cages. Any additional nymphs that molted were transferred to the large BugDorm cages and allowed to develop into later instars.

Plant rearing

The host plants that were used were selected for a variety of reasons. *Ailanthus altissima* was selected because it is the preferred host for *L. delicatula*, making it a good reference for comparison with other host plants. As *L. delicatula* poses a significant threat to wine grapes, it is important to determine if being reared on *V. labrusca* influences its developmental rate. *Salix babylonica* was selected because it is a common landscape tree and was one of the trees used in the study that showed that *L. delicatula* could complete development without *A. altissima* (4). *Celastrus orbiculata* was selected based on previous literature findings indicating that *L. delicatula*, in the early instars, commonly used it as a host. *Ocimum basilicum* is a common garden plant and *R. multiflora* is a common forest plant, and it has been found that *L. delicatula* feeds on both plants.

A. altissima was grown from seeds collected in Wallingford, Connecticut, USA, in October 2019. Seeds were initially planted in Jiffy Plugs and then transferred to tree pots measuring 7.6 cm × 7.6 cm × 20.3 cm (CN-SS-TP-308, Greenhouse

Megastore, Danville, IL, USA) filled with soil (Premier BK25, Promix M, Premier Horticultural Inc., Quakertown, PA, USA) after sprouting. The *A. altissima* seedlings were provided with 5–10 g (the amount was dependent on the size of the pot) of Osmocote fertilizer (ICL Specialty Fertilizers, Summerville, SC, USA) when they were first put into the tree pots, and monthly thereafter. Otherwise, the *A. altissima* seedlings were reared as described in Kreitman et al. (9).

Celastrus orbiculate was grown from cuttings from multiple plants obtained from the towns of Wallingford and Ansonia (CT, USA). *Rosa multiflora* was grown from cuttings obtained from multiple plants from Wallingford, Connecticut, USA, and from at least 10 individual plants from Ansonia, Connecticut, USA. Both Celastrus orbiculate and Rosa multiflora were obtained during the summer of 2020. *Ocimum basilicum* plants were grown from "hybrid herb, basil Prospera organic" seeds purchased from Seedway, LLC (Hall, New York, NY, USA) using the same method as for the *A. altissima* plants. *Celastrus orbiculate*, *R. multiflora*, and *O. basilicum* were all grown in the same soil and tree pots as the *A. altissima* plants.

The *A. rubrum* and *S. babylonica* plants were purchased from Cold Stream Farm LLC (Freesoil, MI, USA) in late March 2020. The *V. labrusca* bare-root plants were purchased from Double A Vineyard (Fredonia, NY, USA) and were shipped in the spring of 2020.

The effect of different host plant species on the development of nymphal *Lycorma delicatula*

Nymphal rearing in 2020

For this first year, the host plants used were *A. altissima*, *V. labrusca*, *S. babylonica*, and *A. rubrum*. Three cages of each host plant treatment were set up, with 10 second-instar nymphs or 10 third-instar nymphs per cage, both sourced from the rearing cages containing the field-collected nymphs. For the fourth-instar nymphs, five nymphs that molted on the same day were placed in a small cage for each host with 10 replicates of each over a period of 12 days. Each cage started with two plants, and new plants of the same host were added to the cages every 7 days for the second- through third-instar nymphs, and every 4 days for the fourth-instar nymphs. Nymphs were monitored daily for survival and molting, which was confirmed by a cast skin. Any newly molted nymphs were removed from the cages, weighed, and then preserved by freezing for later sexing. The second-instar nymphs were preserved in ethanol, and, therefore, we were unable to determine their sex. Measurements of forewing length, forewing width, and hind tibia length were taken for all frozen adult nymphs using a dissecting microscope.

Nymphal rearing in 2021

In 2021, we evaluated only the first- and second-instar nymphs of the hosts. The nymphs were hatched (first instars) or reared (second instars) from field-collected egg masses. The egg masses were collected by removing both the egg mass and the bark substrate that it was on using a chisel and hammer, from two sites in Pennsylvania and one site in New Jersey, on 20 October 2020 (Table 1). These egg masses were held individually in 60 mm × 15 mm Petri dishes (Falcon 351007, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 15°C until they hatched. On hatching, 20 nymphs that hatched from egg masses on the same day, from the same collection site, were placed in a small BugDorm cage (32.5 cm × 32.5 cm × 77.0 cm) containing two plants of the same species. The species of plants used were *A. altissima*, *V. labrusca*, *S. babylonica*, *A. rubrum*, *C. orbiculata*, *O. basilicum*, and *R. multiflora*. The range of host plant species was expanded in 2021 because of the promising preliminary results in 2020. Additional hatch from those egg masses was placed in the larger BugDorm cages (60 cm × 60 cm × 120 cm) with two or three 100-cm-tall-potted *A. altissima* plants and a single *V. labrusca* plant, and kept at a temperature of 25°C for rearing to be used as second-instar nymphs. For both the first- and second-instar nymphs, two cages were set up with nymphs from the New Jersey site, and one cage was set up with nymphs from each Pennsylvania site, for a total of four cages for each host. Voucher specimens were preserved in a freezer for reference, in addition to the voucher specimens of adult *L. delicatula* that were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, Connecticut, USA.

Statistical analyses

Statistical analyses were performed using SAS 9.4 (11). Data did not fit assumptions of normality per the Shapiro–Wilk and Anderson–Darling tests. PROC UNIVARIATE was then used to assess the fit of the data to a gamma distribution. Each model was fitted to a gamma distribution with a log-link function because the response variables had long right tails. PROC GLIMMIX was used to evaluate the fixed effect of host on the duration and body mass of each instar. If the sex was known, the fixed effects of sex and the interaction of sex and host plant species were added to the model. The state(s) in which the egg masses were collected, and of the cages, were treated as random effects. PROC GLIMMIX with a beta distribution and logit

TABLE 1 Approximate locations (latitude and longitude), collection date, and hosts from which the egg masses of *Lycorma delicatula* used in this study were obtained.

Collection location	Collection date	Host (number of egg masses)	Latitude	Longitude
Spruce Run Reservoir, Clinton, NJ, USA	10 October 2020	*Betula pendula* Roth (93) and dead trees (23)	40°39′ 47.03″N	74°55′36.02″ W
The Woodlands, Philadelphia, PA, USA	10 October 2020	*Prunus* spp. (110), *Broussonentia papyrifera* (L. Vent.) (Rosales: Moraceae) (8), *Acer platanoides* (L.) (Sapindales: Sapindaceae) (7), and *Crataegus* spp. (Rosales: Rosaceae) (10)	39°5′ 45.86″N	75°12′19.37″ W
Neshaminy State Park, Bensalem, PA, USA	10 October 2020	*Betula nigra* (L.) (Fagales: Betulaceae) (33), *Betula lenta* (L.) (Fagales: Betulaceae) (18), *Acer rubrum* (25), *Prunus* spp. (18), and *Pinus strobus* (L.) (Pinales: Pinaceae) (24)	40°4′ 31.87″N	75°55′0.59″ W

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link function was used to evaluate the effects of host on the overall survival for each instar. Percentage survival was calculated for each cage. Values of 1 were replaced with 0.9999, and values of 0 were replaced with 0.0001, because the beta curve allows only values between 1 and 0. Differences among means were determined using Tukey–Kramer *post hoc* analysis and a α value equal to 0.05. Statistical comparisons of nymphal survival curves between host plants for each instar were carried out using a Peto–Wilcoxon test in Statistix 10.0 (12). For this analysis, any nymphs that molted or were inadvertently killed were censored.

Results

Survival

Overall, host plant species had an impact on nymphal survival. There was no significant difference in the survival curves (χ^2 = 4.98, d.f. = 3, p = 0.1730), but there was a significant difference in overall survival ($F_{3,9}$ = 5.03; p = 0.0256), between host plant species for the second-instar nymphs in 2020. In 2020, the overall percentage survival of second-instar nymphs was lowest on *A. rubrum* among all host plant species (Figure 1). The survival curves (χ^2 = 114.44, d.f. = 3, p < 0.0001 for third-instar nymphs and χ^2 = 100.48, d.f. = 3, p < 0.0001 for fourth-instar nymphs) and overall survival percentages ($F_{3,16}$ = 5.12; p = 0.0113 for third instar nymphs, and $F_{3,36}$ = 9.48; p < 0.0001 for fourth-instar nymphs) were significantly different between host plant species for both third- and fourth-instar nymphs in 2020. Third- and fourth-instar nymphs reared on *A. rubrum* and *S. babylonica* had the numerically lowest percentage survival. There was a significant difference in survival between host plant species for both the first- and second-instar nymphs in 2021 (χ^2 = 87.12, d.f. = 6, p < 0.0001, for first-instar nymphs and χ^2 = 55.00, d.f. = 6, p < 0.0001, for second-instar nymphs). There was no significant difference in overall survival by host for first-instar nymphs ($F_{6,21}$ = 1.66; p = 0.1811), but there was a significant difference for second-instar nymphs ($F_{6,21}$ = 3.44; p = 0.0159). In 2021, overall survival was numerically highest for first-instar nymphs reared on *C. orbiculate*, *A. altissima*, and *A. rubrum*, and lowest for those reared on *O. basilicum* and *S. babylonica*, but there were substantial differences between the individual cages in percentage survival (Figure 2). Second-instar nymphs had the highest overall percentage survival when reared on *C. orbiculate, A. altissima, S. babylonica*, and *V. labrusca*, and the lowest overall percentage survival when reared on *O. basilicum*.

Nymphal development

2020 development

Host plant species did not have a significant effect on the mean time spent in the second instar in 2020 ($F_{3,78}$ = 2.49; p = 0.0660)

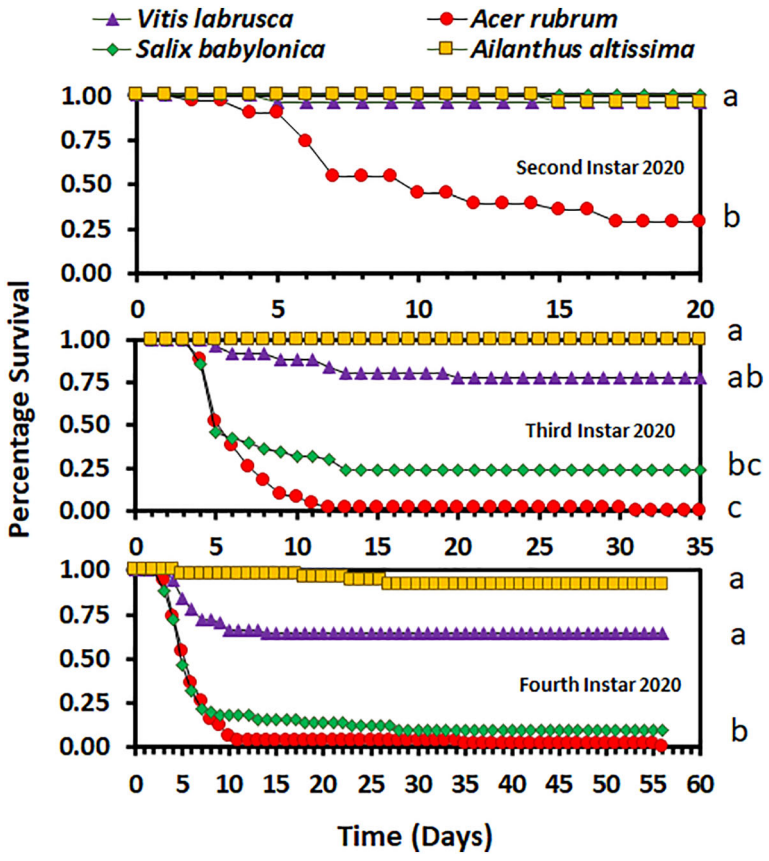

FIGURE 1
Survivorship curves for *Lycorma delicatula* nymphs reared in 2020 by instar and host plant species. Different letters to the right of the graphs indicate differences between the overall survival for the host plant species.

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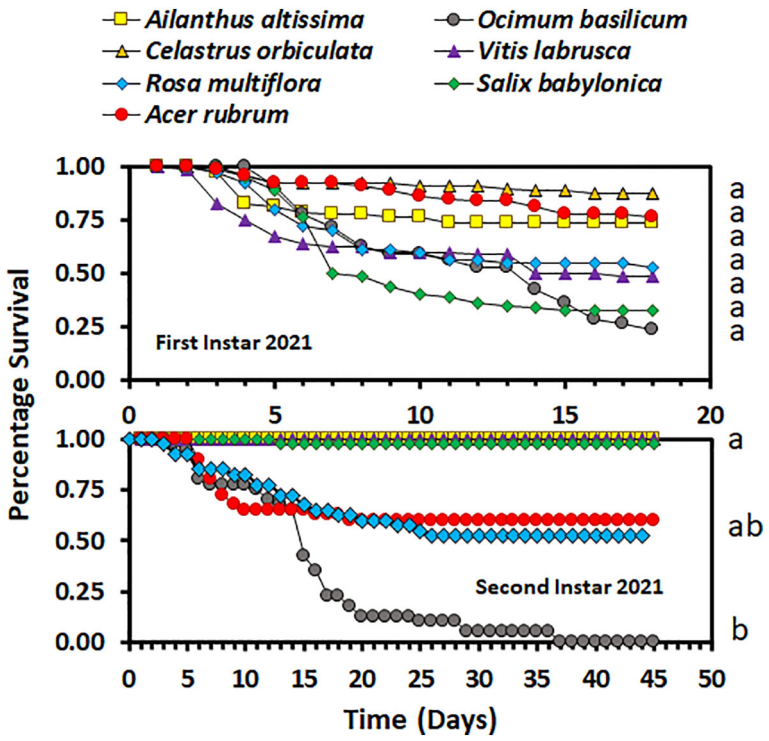

FIGURE 2
Survivorship curves for *Lycorma delicatula* nymphs reared in 2021 by instar and host plant species. Different letters to the right of the graphs indicate differences between the overall survival for the host plant species.

(Figure 3). Second-instar nymphs reared on *A. rubrum* spent significantly more time in that instar than nymphs reared on *V. labrusca*. In addition, host plant species did not have a significant effect on the weight of nymphs on completing the second instar ($F_{3,78}$ = 0.91; p = 0.4400).

For third-instar nymphs, the mean development time was significantly affected by sex ($F_{1,186}$ = 30.87; p > 0.0001), with female nymphs having longer development time (Figure 3). Host plant species had a significant effect on the mean development time in the third instar ($F_{2,186}$ = 8.98; p = 0.0003); however, there was no significant difference for nymphs reared on *A. altissima* and *V. labrusca*. There was no significant interaction of host plant species and sex for third-instar nymphs ($F_{2,186}$ = 0.35; p = 0.7029). Female nymphs reared on *S. babylonica* spent significantly longer in the third instar than all other nymphs. None of the nymphs reared on *A. rubrum* were able to complete the third instar.

Sex had a significant effect on the mean weight of nymphs that completed the third instar ($F_{1,186}$ = 126.8; p < 0.0001) (Figure 3). For each host plant species, female nymphs weighed significantly more than male nymphs reared on the same host plant species. Neither host plant species ($F_{2,186}$ = 1.68; p = 0.193) nor the interaction of host plant species and sex ($F_{2,186}$ = 1.99; p = 0.143) had a significant effect on the mean weight of nymphs that completed the third instar. No significant difference was found in the mean weight of male or female nymphs reared on any of these hosts. In addition, female nymphs that were reared on *A. altissima* weighed significantly more than male nymphs that were reared on either *S. babylonica* or *V. labrusca*. Female nymphs reared on *V. labrusca* also weighed significantly more than male nymphs that were reared on *S. babylonica*.

Sex had a significant effect on the mean development time spent in the fourth instar ($F_{1,59}$ = 7.26; p = 0.009) (Figure 3); however, host plant species did not have a significant effect on the mean time spent in the fourth instar ($F_{2,59}$ = 0.88; p = 0.4195). Likewise, the interaction of host and sex also did not have a significant effect on the mean time spent in the fourth instar ($F_{2,59}$ = 0.6; p = 0.5526). Female nymphs reared on *A. altissima* had a significantly longer developmental time than male nymphs that were reared on the same host plant. None of the fourth-instar nymphs reared on *A. rubrum* were able to complete the fourth instar.

2021 development

For first-instar nymphs, host plant species had a significant effect on developmental time ($F_{6,262}$ = 24.21; p < 0.0001) (Figure 4), with nymphs reared on *A. rubrum* having a significantly longer developmental time than those reared on all other host plant species, except for *O. basilicum*. There was no significant difference in mean nymphal development time in first instar between those reared on *A. altissima*, *V. labrusca*, and *C. orbiculata*. First-instar nymphs also spent significantly less time in the first instar when reared on *A. altissima* than those reared on *R. multiflora*, *S. babylonica*, *A. rubrum*, and *O. basilicum*. The weights of first-instar nymphs were also significantly affected by the host plant species ($F_{6,271}$ = 22.41; p > 0.0001), although no significant differences were observed in the weights of first-instar nymphs reared on *A. altissima*, *V. labrusca*, *R. multiflora*, and *S. babylonica*. Nymphs reared on *C. orbiculata* weighed significantly less than those reared on *V. labrusca* or *A. altissima*; however, no significant difference was seen when their weights were compared with those reared on *R. multiflora* or *S.*

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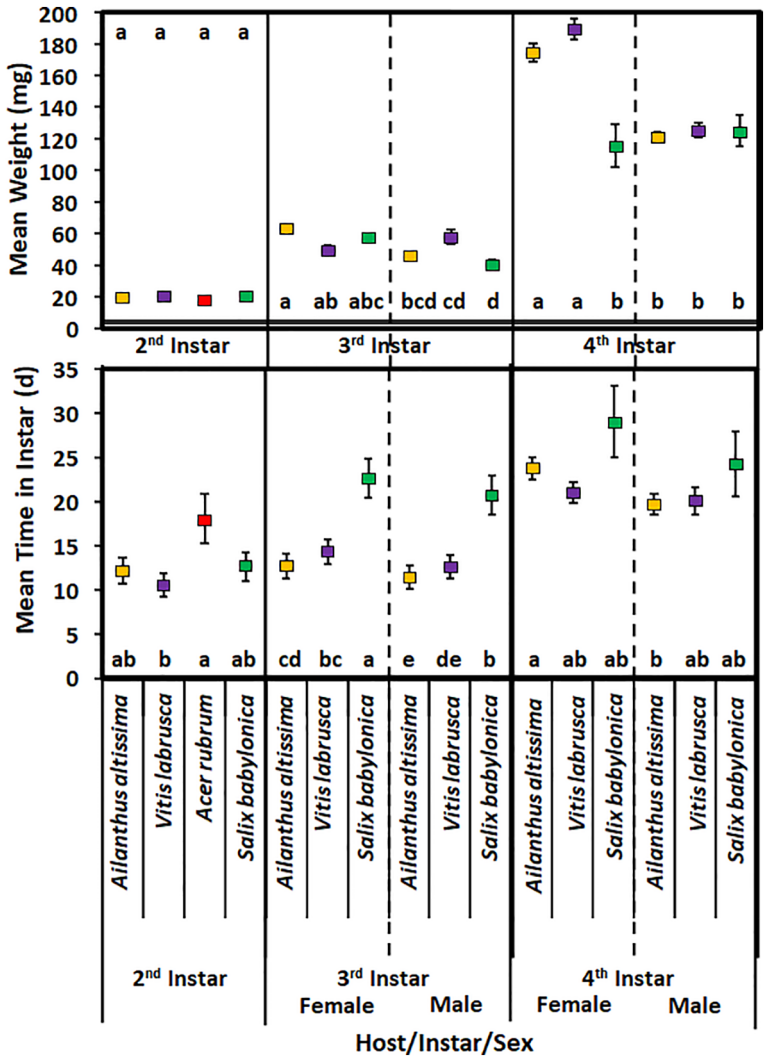

FIGURE 3
Mean time (days) spent in instar and weight (mg) of *Lycorma delicatula* nymphs reared in 2020 by host plant species, instar, and sex. Means with a different letter are significantly different from each other at a *p*-value < 0.05 using Tukey–Kramer grouping.

babylonica. Nymphs reared on *O. basilicum* and *A. rubrum* weighed significantly less than nymphs reared on all other hosts.

For second-instar nymphs, both host plant species ($F_{5,186}$ = 9.25; $p < 0.0001$) (Figure 4) and the interaction of host plant species and sex ($F_{5,186}$ = 3.25; p = 0.008) had significant effects on the mean development time in the second instar. Sex alone did not have a significant effect ($F_{1,186}$ = 0.62; p = 0.432) on the mean development time in the second instar. When reared on *A. rubrum*, males spent significantly longer in that instar than females. In addition, male nymphs reared on *A. rubrum* took significantly longer than second-instar nymphs reared on any other host, except for *R. multiflora*. None of the nymphs reared on *O. basilicum* were able to complete the second instar.

For the second-instar nymphs, sex had a significant effect on their mean weight ($F_{1,186}$ = 113.27; $p < 0.0001$) (Figure 4), with female nymphs weighing more than male nymphs. Host plant species also significantly impacted the mean weight of nymphs ($F_{1,186}$ = 22.25; $p < 0.0001$); however, the interaction of host plant species and sex was not significant ($F_{1,186}$ = 0.22; p = 0.956). Female nymphs reared on *S. babylonica* weighed significantly more than female nymphs reared on *C. orbiculate*; however, no significant difference was seen when their weights were compared with the weights of female nymphs reared on *V. labrusca*, *A. altissima*, and *S. babylonica*. Female nymphs reared on these hosts weighed significantly more than female nymphs reared on *C. orbiculata*, *A. rubrum*, or *R. multiflora*. No significant difference in weight was found between male nymphs reared on *V. labrusca*, *C. orbiculata*, *A. altissima*, and *S. babylonica*; however, male nymphs reared on either *V. labrusca*, *A. altissima*, or *S. babylonica* weighed more than male nymphs reared on either *R. multiflora* or *A. rubrum*.

2020 adult mass and morphometrics

Host plant species ($F_{2,59}$ = 3.97; p = 0.024), sex ($F_{1,59}$ = 32.42; $p < 0.0001$), and the interaction of host plant species and sex ($F_{2,59}$ = 8.02; p = 0.001), had a significant effect on the mean weight of adults that completed the fourth instar in 2020 (Table 2). Adult female nymphs that completed the fourth instar, and which had been

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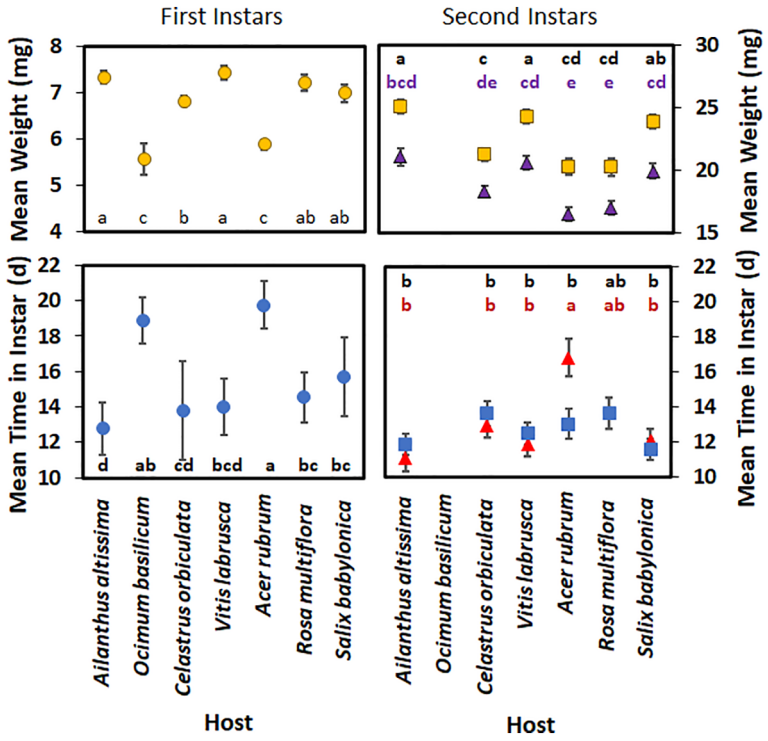

FIGURE 4
Mean time (days) spent in instar and weight (mg) of *Lycorma delicatula* nymphs reared in 2021 by host plant species and instar. Triangles represent males, whereas squares represent females for second instar nymphs. Means with a different letter are significantly different from each other at a *p*-value < 0.05 using Tukey–Kramer grouping.

reared on either *A. altissima* or *V. labrusca*, weighed significantly more than male adult nymphs that completed the fourth instar, and which had been reared on *A. altissima*, *V. labrusca*, or *S. babylonica*, as well as female adult nymphs that completed the fourth instar and had been reared on *S. babylonica*. Likewise, there was no significant difference in the mean weight of male and female adult nymphs that completed the fourth instar and which had been reared on either *A. altissima* or *V. labrusca*. There was also no significant difference observed in the mean weight of adult male nymphs that completed the fourth instar and were reared on *A. altissima*, *V. labrusca*, and *S. babylonica*, or female adults that completed the fourth instar and were reared on *S. babylonica*.

Sex had a significant effect on adult forewing length ($F_{1,64.92}$ = 28.16; p < 0.0001), whereas host plant species ($F_{2,20.14}$ = 2.81; p = 0.084) and the interaction of host plant species and sex ($F_{2,68.73}$ = 2.42, p = 0.097) did not (Table 2). Both host plant species ($F_{2,75}$ = 3.71; p = 0.029) and sex ($F_{1,75}$ = 13.32; p = 0.001) had a significant effect on the forewing width of adult nymphs; however, the interaction of host plant species and sex ($F_{2,75}$ = 2.01; p = 0.142) did not. Female adult nymphs reared on either *A. altissima* or *V. labrusca*

TABLE 2 Mean [± SE (*n*)] adult *Lycorma delicatula* body weight (g), forewing length (mm) and width (mm), and hind tibia length (mm) at different combinations of host plant species and sex in *L. delicatula* reared on three host plants in 2020.

Measure	Host plant species and sex[a]						Statistics		
	Ailanthus altissima		*Vitis labrusca*		*Salix babylonica*				
	Male	Female	Male	Female	Male	Female	*F*	d.f.	*p*-value
Weight (g)	0.120 ± 0.003b (32)	0.175 ± 0.006a (14)	0.124 ± 0.004b (12)	0.187 ± 0.006a (20)	0.124 ± 0.01b (4)	0.115b (1)	8.02	2,59	0.001
Forewing length (mm)	17.43 ± 0.22b (31)	21.43 ± 0.39a (13)	18.01 ± 0.35b (12)	21.55 ± 0.33a (20)	17.65 ± 0.59b (4)	18.39ab (1)	2.42	2,68.73	0.097
Forewing width (mm)	7.683 ± 0.11b (31)	9.18 ± 0.19a (13)	7.852 ± 0.18b (12)	9.361 ± 0.11a (20)	7.585 ± 0.30b (4)	7.56ab (1)	2.01	2,75	0.142
Hind tibia length (mm)	9.875 ± 0.10b (31)	11.05 ± 0.16a (13)	10.08 ± 0.15b (12)	11.15 ± 0.14a (20)	9.79 ± 0.28b (4)	9.58ab (1)	2.84	2,69.31	0.066

[a]Means followed by the same letter are not significantly different at a p-value ≤ 0.05 using Tukey–Kramer grouping. Sample size (N) is the number of survivors.
d.f., degrees of freedom.

had significantly wider and longer forewings than male nymphs reared on these hosts. Likewise, host plant species ($F_{2,19.75} = 3.93$; $p = 0.037$) and sex ($F_{1,64.45} = 10.49$; $p = 0.002$) had a significant effect on adult hind tibia length, whereas the interaction of host plant species and sex ($F_{2,69.31} = 2.84$; $p = 0.066$) had no significant effect. The hind tibia length of female adult nymphs that completed development on either *A. altissima* or *V. labrusca* was significantly longer than the hind tibia length of adult male nymphs reared on these two hosts.

Discussion

Host plant species had an effect on nymphal survival. Nymphs reared on *A. altissima* and *V. labrusca* survived equally well, but survival was decreased for those reared on *R. multiflora*, *A. rubrum*, and *O. basilicum*. Nymphs failed to develop through the second instar on *O. basilicum* and through the third and fourth instars on *A. rubrum*. Host plant species was found to have a significant effect on the development time of *L. delicatula* nymphs in the first through third instars. Host plant species was also found to have a significant effect on the mean weight of nymphs in the first, second, and fourth, but not in the third, instars. Host plant species had a significant effect on adult hind tibia length and forewing width. These findings should be incorporated into phenology models for *L. delicatula* to account for host effects.

First-instar nymphs reared on *A. rubrum* and *O. basilicum* took longer to develop and later, as second-instar nymphs, had the lowest weights. The inability of second-instar nymphs reared on *O. basilicum* to complete the second instar, and of third- and fourth-instar nymphs reared on *A. rubrum* to complete development, suggests that the performance of earlier instars is indicative of host viability for later instars. Declining host viability as nymphal development progresses was also seen in the percentage survival of second- through fourth-instar nymphs in 2020; specifically, second-instar nymphs reared on *S. babylonica* had a similar percentage survival to those reared on either *A. altissima* or *V. labrusca*. This trend of reduced viability as development progresses can also be seen in previous research, in which a shift away from *R. multiflora* as the dominant host was observed in the *L. delicatula* third instar (13). In addition, this trend of reduced survival on hosts where nymphs take longer to develop in earlier instars is also seen in *H. halys* nymphs (8). Another study found that the number of host plants on which *L. delicatula* nymphs could complete the first instar was higher than the number on which it could complete the second instar (14). In addition, in that study, second-instar nymphs reared on *A. rubrum* failed to complete that instar, and third-instar nymphs reared on *S. babylonica* failed to complete that instar, supporting the results seen for those hosts with later-instar nymphs in this study. Interestingly, *L. delicatula* has consistently good survival during all instars on its preferred host, *A. altissima*. Furthermore, life stages where host plant species had a significant effect on the mean time spent in each instar, *A. altissima* was one of the hosts that nymphs spent the least amount of time feeding on during each instar, as inferred from the slow-growth, high-mortality hypothesis (15). In many cases, there was no significant difference in mean development time in instar between nymphs reared on *V. labrusca* and those reared on *A. altissima*, thus suggesting that *V. labrusca* is comparable to *A. altissima* as a host for *L. delicatula* nymphs. The interaction of host and sex had a significant effect on the mean time spent in instar only for second-instar nymphs; however, that is most likely a result of the fact that male second-instar nymphs reared on *A. rubrum* spent significantly longer in that instar than nymphs reared on other hosts, excluding *R. multiflora*.

Host plant species also had a significant effect on the mean weight of *L. delicatula* nymphs in the first and second instars. This difference in weight is more likely explained by nutritional differences in the host plant, rather than by differences in plant defensive compounds, as *L. delicatula* is known to sequester defensive compounds (16). Mean weight was also significantly affected by sex in the second, third, and fourth instars. The significant effect on mean weight of the interaction of host and sex in fourth-instar nymphs may limit the use of weight for sexing *L. delicatula* nymphs. The differences between sexes in development time and weight were reflective of each other, as the lighter males took less time to develop than the heavier females. Lower weights in fourth-instar nymphs were also associated with less optimal temperatures in previous research, which further hints at *S. babylonica* being a less optimal host than either *A. altissima* or *V. labrusca* for female fourth-instar nymphs (9). The general similarities in the mean time spent in an instar for first- and second-instar nymphs among different host plant species suggest that weight might be a better indicator of host suitability for those instars. For first-instar nymphs, longer developmental times also resulted in nymphs with lower weights. Growth rate affects the size of an individual, but the final size is determined by factors that terminate growth and lead to a molt. Many insects have a critical weight they must achieve before they molt and, if this weight is not reached, they do not survive. The critical weight has been determined for *Manduca sexta* L. (Lepidoptera: Sphingidae), and molting frequency is associated with growth rate (17). In addition, slower growth rate has been seen in *M. sexta* in response to suboptimal temperatures or nutrition, which matches the trend shown in this study for *L. delicatula*. Thus, it may be the case that, on suboptimal hosts, reach the critical weights for each instar only just before molting.

Adult morphometrics differed by sex, further suggesting that there is size-based sexual dimorphism in *L. delicatula* adults. Host plant species had a significant effect on the hind tibia length and forewing width of *L. delicatula* adults. These factors are more indicative of nymph size than forewing length, which has been shown to affect the flight capabilities of *L. delicatula* (18). In *L. delicatula* adults, weight appears to be a proxy for sex and nourishment level. Nourishment level could have an impact on nymph flight capabilities, and this is particularly important in the context of dispersal, as extra nourishment could be used to sustain longer flights (19). Heavier weights can also allow adult to persist longer without food sources, as seen with other hemipterans, and thus increase the odds of individual nymphs surviving human-mediated dispersal events, such as those occurring on planes or cargo ships (20). Landscape-level decisions, in terms of host quality for *L. delicatula*, could also play a role in dispersal through shipping hubs, ports, and airfields.

The results of this study have implications for phenology models for *L. delicatula* because phenology is affected by the host plant that individual nymphs feed on. Dynamic models accounting for host preference by instar are needed moving forward, so that accurate predictions of phenology can be made. As the mean development time did not differ significantly between nymphs reared on either *A. altissima* or *V. labrusca*, the degree-day requirements from Kreitman et al. (9) should be viable for degree-day modeling for monitoring the growth of *L. delicatula* in vineyards, where the development of *L. delicatula* on grape plants takes 12.6–12.77 days to complete. Regardless of which host the second-instar nymphs were reared on, the time spent in that instar was

shorter than the time spent in the second instar at 25°C in the previously mentioned study. However, as the previous study did not account for sex in that instar, it could potentially not be a true comparison. Furthermore, the use of plastic tubes in that study and the use of the BugDorm cages in this one makes it harder to make comparisons because the cages could hold more, and larger, host plants. This same trend was also observed for nearly every host plant in the case of third-instar nymphs, with the exception of female third-instar nymphs reared on *S. babylonica*. The same trend was apparent with fourth-instar nymphs, which in both studies accounted for their sex. This difference in developmental rates between the two studies only confirms the disadvantages of using plastic tubes over other containers for rearing nymphs. This is different from previous research that found that both first- and second-instar nymphs took longer to develop on *Vitis rotundifolia* var. Carlos (Michaux) than on *A. altissima* (21). This suggests that *L. delicatula* nymphs perform differently depending on the variant and species of *Vitis* that they are reared on. Further studies that look at different host plant species and use a combination of host plant species similar to that found in forest and landscape environments are needed to get a better idea of how different host plant species influence the development of *L. delicatula* nymphs.

Overall, this study shows that the development of *L. delicatula* can be influenced by host plant species. Moving forward, it is important to consider potential host options when developing management strategies for *L. delicatula*. Furthermore, this research can be extrapolated to identify what nutrients *L. delicatula* require to complete development based on their host utilization. The differences in development time by host plant species indicate a potential issue regarding the use of phenology models to predict the current life stage. Sampling for field data to use for validating phenology models is important and might be affected at a site level by the host plants that are present. As being reared on certain host plants results in nymphs having a lower weight, this study can also inform host plant choice when mass rearing *L. delicatula* for potential parasitoid use. The risk of *L. delicatula* damage to *O. basilicum* in homeowner gardens seems to be minimal, as *O. basilicum* does not appear to be a viable host for later instar nymphs. It is important for further research evaluating *L. delicatula* nymphal utilization of other species of *Vitis* to be undertaken, as its presence is a major threat to grape production.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Part of DK's M.Sc. thesis. Authors jointly conceived the study and got the funding for it. MK and DK collected and analyzed the data. MK prepared the figures. DK wrote the first draft of the paper, and all authors edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The potential climatic range of spotted lanternfly may be broader than previously predicted

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Spotted lanternfly (*Lycorma delicatula* White) is an invasive planthopper that was introduced to the United States from Asia and readily spreads *via* human aided means. Three geographically separated populations in the United States (NJ, PA, and WV) were collected and used to assess the effects of fluctuating thermal regimes that included temperatures above or below the upper (T_{max}) and lower (T_{min}) developmental thresholds, respectively, on nymphal survival and development, and to determine if there was within- and among-population variation in hatch timing and temperature responses of nymphs. Nymphs exposed to temperatures > T_{max} and <T_{min} were able to develop when those temperatures were part of an alternating regime, even though development took longer, and the average survival was lower than that of the corresponding constant temperature. When individuals from different geographically separated populations were exposed to the same temperature regimes, there was intra- and inter-population variation in time to hatch, instar duration, and estimated T_{min} values. The NJ population on average hatched earlier than the PA populations. There was 1-4°C difference in estimates of the T_{min} for the first through third instars for individuals from different populations. In addition, the time in instar estimates for constant 15 and 25°C from this study were 26 and 7 days faster, respectively, than estimates from previous studies. The variability in thermal responses documented in this study is large enough to have impacts on predicted phenology and potential risk of establishment especially in areas previously considered too cold to be at risk. This new information should be incorporated into phenology and risk models to improve their predictive ability.

KEYWORDS

phenology, survival, development, climate, temperature

1 Introduction

Extreme temperatures, close to or exceeding thermal thresholds, increase mortality and limit development along the climatic edges of a species geographic niche and can, in part, determine the potential distribution of invasive species in novel habitats. Invasive insect species with broader geographic ranges generally are assumed to have a wider thermal tolerance and/or more variation in performance tolerances among populations (1). The

variation in thermal tolerances among populations can have a genetic basis, with differential selection occurring in local environments. Variation can also be the result of phenotypic plasticity, or the consequence of maternal or epigenetic effects, or a combination of these factors (1).

Inadvertent human-aided introduction (or spread) of species can rapidly create disconnected populations that are exposed to widely varying thermal environments. These environments can vary in the timing and severity of temperature extremes, number of days temperatures exceed the lower developmental threshold and variation around the mean annual temperature (2). Populations introduced to these novel environments may rely on thermal response variation present in the founding populations to allow establishment, and selective pressures may result in genetic divergence. For example, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and some cereal aphids, are invasive insects that have rapidly responded to thermal selection for heat tolerance which has allowed them to invade even tropical environments (3, 4). There are also cases of invasive insects developing higher tolerances to extreme cold and exhibiting variation among populations in cold tolerance, even a parthenogenetic species, *Adelges tsugae* Annand (Hemiptera: Adelgidae) (5). Other experiments using *Lymantria dispar* L. (Lepidoptera: Erebidae) have done simulated reciprocal transplants to assess the ability of different populations to deal with the northern and southern temperature extremes of the insect's range, finding some populations outperform others in the new environments (6). Studying geographically distinct populations of an invasive species that is readily spread by human activity provides unique opportunities to assess its ability to utilize novel environments, look for population level variation in thermal responses, and assess where the species may be able to establish.

The spotted lanternfly (SLF) (*Lycorma delicatula* White [Hemiptera: Fulgoridae]) is an invasive planthopper that was introduced to the United States from Asia that readily spreads *via* human activity. It was first detected in Berks County, PA in 2014 and has since spread and established across the eastern United States (7, 8). In the first five years since its introduction, long distance dispersal events of up to 92 km have been documented (9). Primarily these long-distance movements occur when eggs are laid on vehicles (trains, cars, etc.) or materials that are stored outdoors and moved (8). This insect is a phloem feeder that utilizes over 100 hosts plants and causes direct or indirect damage to some economically important hosts as well as causing nuisance problems (10, 11). If unmanaged it is expected to spread throughout the United States to all suitable habitats by 2037 (12).

Using the modelling program MAXENT to estimate suitability in the U.S. from relationships of environmental variables at known occurrences in the native range, one study concluded the northernmost areas of New England, and the far southeastern US would be unsuitable for SLF establishment (13). Using a process-based modeling approach to determine spread probability over time, Jones et al. (12) showed that similar areas of both the northern and southern US may be at a low risk for spread and establishment. Whether SLF will be able to establish and spread as predicted by these models will be validated or disproven as the insect continues to invade new areas.

Previous studies focusing on the temperature responses of SLF have shown that this species can survive and develop at constant temperatures between 15 and 30°C (14). At constant temperatures outside this range, the species can survive for some time (2-35 days depending on temperature) but is unable to complete development (14). Lower developmental thresholds are estimated to be about 13°C for first and second instar nymphs and 6-8°C for third and fourth instar nymphs (14). Upper developmental thresholds are estimated to be 43°C for first instar nymphs and close to 35°C for all other instars (14). All this previous work has been done at constant temperatures in a laboratory using populations of SLF collected in Pennsylvania near the original introduction site and no work has been done to quantify the variation in temperature responses of the species throughout the entire range it now occupies or assess the actual thermal performance of the species in a more ecologically relevant way. In addition, a modeling effort to map the life-history of SLF in occupied and uninvaded ranges using all available laboratory temperature response data (15) had to adjust the developmental rate estimates by Kreitman et al. (14) to better match those in the field. This indicates there is a need for additional work on temperature responses of this species. Also, further work utilizing SLF thermal responses to model potential range in the invaded areas is needed.

No prior studies have assessed the effects of fluctuating temperature regimes on egg hatch nor survival or development of spotted lanternfly nymphs. Fluctuating temperatures within the permissive range (upper developmental threshold [T_{max}] ≥ x ≥ lower developmental threshold [T_{min}]) can improve insect performance, and in regimes that include stressful temperatures the permissive temperature portions can allow the insect to recover from the harmful effects of thermal extremes (temperatures outside the permissive range) (16). As a consequence, fluctuating regimes could allow development outside the permissive range, although development may be delayed, and high fluctuation amplitudes can cause more severe negative effects (16). Temperatures exceeding the estimated developmental thresholds of SLF nymphs occur in more northern and southern parts of the eastern US and at higher elevations where they may end up due to human aided transport. Understanding how extreme temperatures may affect this insect when part of normal daily fluctuations in temperature would improve phenology models and predictions of potential range.

There were two goals of this study. First, we assessed the effects of alternating regimes on SLF nymphal survival and development, using temperature exposures above and below the known developmental thresholds. Second, we determined if there was variation in hatch timing and temperature responses of nymphs from different SLF populations. Then we discuss how this information could impact and be incorporated into estimates of the SLF's potential geographic range and phenology models.

2 Materials and methods

2.1 Source populations

One hundred and sixteen egg masses were collected October 20, 2020 from *Betula* sp. or dead trees along the bird watching path at Spruce Run Reservoir in Clinton, NJ (40° 39'47.03"N, 74°

55'36.02"W), which is in the USDA Plant Hardiness Zone (https://planthardiness.ars.usda.gov/) 6A. On October 22, 2020, 135 egg mases were collected from trees (*Prunus serotina* Ehrhart [Rosales: Rosaceae], *Acer platanoides* L. [Sapindales: Sapindaceae], *Morus papyrifera* L. [Rosales: Moraceae] or *Crataegus monogyna* Jacquin [Rosales: Rosaceae]) in The Woodlands cemetery in Philadelphia, PA (PA1: 39° 56'45.86"N, 75°12'19.37"W) and 118 egg masses from trees (*Betula nigra* L. [Fagales: Betulaceae], *B. lenta* L. [Fagales: Betulaceae], *Acer rubrum* L. [Sapindales: Sapindaceae], *P. serotina*, or *Pinus strobus* L. [Pinales: Pinaceae]) in the Neshaminy State Park, Bensalem, PA (PA2: 40° 4'31.87"N, 75°55'0.59"W) which are both in the 7B plant hardiness zone. On January 15, 2021, 73 egg masses were collected from the bark of dead *Ailanthus altissima* (Miller) Swingle (tree of heaven [TOH]) (Sapindales: Simaroubaceae) trees in forest strips surrounding an industrial area in Winchester, VA (39° 12'35.6"N, 78°11'18.7"W). To collect egg masses, we carefully chiseled through the bark around the egg mass and then lifted the bark off the tree without bending it.

2.2 Egg mass preparation and temperature treatments

Eggs collected in Winchester, VA were stored in a nearby barn (39°12'06.2"N 78°09'12.5"W, 6B plant hardiness zone) from the date of collection until March 10, 2021, when they were shipped over-night to the Forest Service Quarantine Laboratory in Ansonia, Connecticut. The Clinton, NJ and two PA populations were collected within a few weeks of being laid and brought directly back to the quarantine facility in CT. The two PA populations were combined (i.e., put together in cages) for all but the first instar treatments so the populations are referred to by the two-letter state for all nymphal data. The egg masses were brought into the quarantine laboratory and put on screens under a laminar flow hood for 30 minutes to remove excess moisture before being placed individually in 60 × 15 mm petri dishes (Corning Inc., Falcon product #351007). The petri dishes with egg masses were then held in clear plastic boxes (60-100 petri-dishes per box) and placed in either a chamber set to a constant 15°C, 65% RH and a 14:10 L:D cycle, or placed in a chamber in which the temperature cycled between the mean high and low temperatures for the specific week of the year (following a sign wave shape). The high and low temperatures, humidity, and light cycle was changed weekly to mimic the average weekly parameters in Napa, CA from 2010-2020 as reported by the National Oceanic and Atmospheric Administration (https://www.noaa.gov/). Napa was chosen for use as a validation data set for another study that is developing the phenology model and since it is a major grape growing region that is concerned about SLF establishment. In this study the Napa regime only served as a fluctuating regime that was closer to natural for hatching part of the egg masses. The 15°C regime was used because previous work has shown that the eggs will progress to hatch without lower temperatures, and this could provide information about how eggs would respond in areas where winters are mild. Forty-five egg masses each from the NJ and the two PA sites were place in the alternating regime; all the other egg masses were held at 15°C. The two temperature regimes (constant and variable) provided staggered hatch times which allowed greater repetition of cages in smaller growth chambers. Hatch was checked daily, and nymphs were removed for use in the study. Cumulative percent hatch for both populations, and for individual egg masses was tracked for the NJ population and two PA populations for eggs held at 15°C. The VA population could not be directly compared because it was overwintered at naturally occurring temperatures. All SLF egg masses were transported to Ansonia, CT where the Forest Service quarantine laboratory is located under Animal Plant Health Inspection and Pennsylvania State permits. Voucher specimens were deposited at the Yale Peabody Museum of Natural History, New Haven, CT.

2.3 Hosts

Spotted lanternfly nymphs were reared in caged enclosures containing both *TOH* and *Vitis labrusca* L. (Vitales: Vitaceae) (concord grape [grape]) vines as food sources, with one exception (see 1.5 for details). Mixed hosts were used since pervious work has shown they develop and survive better than when only offered single hosts (17).

Ailanthus altissima seedlings were grown in a greenhouse from locally (southern Connecticut) sourced seeds that had been stratified for more than one year at 4°C. Seeds were sprouted in Jiffy peat plugs (4 cm diameter), then potted in tree pots measuring 7.6 × 7.6 × 20.3 cm (CN-SS-TP-308, Greenhouse Megastore, Danville, IL). As trees grew, they were repotted into 16.5 (diam) x 17.8 (tall) cm black pots and then 22.2 (diam) x 27.3 (tall) cm black pots to support larger trees for use in the larger cages used for group rearing nymphs to specific instars (see section 2.3 for details). Through the rest of the paper the pots will be referred to as "tree pots" (7.6 cm a side) and "black pots" for the bigger pots (16.5 cm diam). Trees used for the larger group rearing cages were ~120 cm tall (including the pot) and had stems that were ≥ 1 cm diameter at the base. Trees used in treatment cages were ~77 cm tall (including the tree pot). The shorter trees were placed in two groups based on diameter, with 4-6 mm trees used in cages with first and second instars, and trees with diameters between 7-10 mm used in cages with third and fourth instars.

Grape vines were purchased from Double A Vineyard (Fredonia, NY) as bare root stock and received in Spring 2020. The vines were planted either one to a tree pot or 3-4 in the larger black pots, the same type of pots as used for the TOH seedlings. Single vines in tree pots with a minimum of one cane that was 1 m long were used in the treatment cages and for rearing first and second instar nymphs in rearing cages. The black pots with multiple vines were used in the larger rearing cages for third instar nymphs.

Prior to the addition of SLF nymphs, the soil in each pot was covered with a white paper towel, which was cut to allow the stems to pass through. Paper towels allowed water to pass to the soil and allowed gas exchange while preventing the insects from accessing the soil. The pots containing the trees and vines were fertilized monthly using Osmocote fertilizer (ICL Specialty Fertilizers, Summerville, SC) and watered daily or as needed to maintain soil moisture.

2.4 Rearing spotted lanternfly nymphs for instar-specific experiments

SLF nymphs were either placed directly into treatments right after hatch (first instars) or reared to the beginning of an instar (second – fourth) and then exposed to the treatments until the molt to the next instar (or death) occurred. Nymphs from each population and instar combination were reared in separate cages. Two sizes of cages were used: small 32.5 × 32.5 × 77.0 cm (BugDorm 4S3074) and large 60 x 60 x 120 cm (BugDorm 6S620) mesh cages with clear front and back (MegaView Science Co., Ltd, Taichung, Taiwan). The majority of the rearing cages were held in chambers set at 25°C, 65% RH and a 14:10 Light : Dark cycle but a few were held at room temperature (20-22°C) to slow development when necessary because of chamber space limitations. Initially one grape and two TOH pots were placed in each rearing cage, sized appropriately for the instar being reared (i.e., smaller plants for younger instars). First instars were reared in groups of 50-100 in small cages or 300-500 in large cages. Fifty to seventy second instars were reared in small cages and in groups of 150-350 in large cages. All third instars were reared in groups of 35-240 (higher numbers when held at room temperature) in large cages. Fresh plants were added weekly or more often if needed. Cages were checked daily and all new molts, molt skins, and dead nymphs were removed. Nymphs from different rearing cages from the same population and instar were combined to make the cohorts that were placed in the treatment cages.

2.5 Study treatments

2.5.1 First and second instar treatments

First and second instar nymphs were exposed to three temperature treatments: constant 15°C, 8 hours at 5°C and 16 hours at 20°C, and constant 25°C. The first two treatments both provided an average daily temperature of 15°C, are consistent with temperatures that nymphs may experience in April and could be directly compared to assess the effects of the alternating regime. The temperature in the alternating regime was instantaneously switched by either modifying the temperature in the same chamber where the rearing cages were located (with the temperature being reached within<5 minutes, or by moving the cages containing the nymphs to another chamber already set with the next temperature. The 5°C portion of the alternating cycle exposed the nymphs to a temperature below the estimated developmental threshold (13°C for firsts and 12° C for seconds) (14) and the average low monthly temperature at the NJ site in April (https://www.worldweatheronline.com). This would be similar to a rapid drop in temperature during a spring cold wave that first and second instar nymphs might be exposed to. The two constant temperature treatments (constant 15 and 25°C) provided two points on a temperature response curve for each instar that could be used to see if different geographic populations responded the same way over this temperature range. Two cages with 30 first instar nymphs from each population (one cage from each location in PA) were set up in the first two treatments but only 20 per cage were used in the 25°C treatment cages (Table 1). The first instar nymphs in each cage came from multiple egg masses (6-16 egg masses for the PA and NJ populations, and 3-5 egg masses for the VA population). Three cages of second instars were set up for the two constant temperatures, 20 nymphs per 15°C cage and 10 nymphs per 25°C cage. A total of four cages of 20 second instar nymphs were set up in the alternating regime, 3 cages from the VA and 1 cage form the NJ population. This was because the chamber space for the alternating regime was limited and the timing of the seconds from the PA populations did not coincide with when space was available. Daily counts were made of the dead and newly molted nymphs, which were removed from the

TABLE 1 Summary of experimental design for nymphal (cages/number of nymphs in each cage) work.

Temperature Treatment (°C)	Population	Instar			
		First	Second	Third	Fourth
15	NJ	2 cages/30 nymphs	3 cages/20 nymphs	3 cages/15 nymphs	
	PA	2 cages/30 nymphs	3 cages/20 nymphs	3 cages/15 nymphs	
	VA	2 cages/30 nymphs	3 cages/20 nymphs	4 cages/15 nymphs	
20/5	NJ	2 cages/30 nymphs	1 cages/20 nymphs		
	PA	2 cages/30 nymphs			
	VA	2 cages/30 nymphs	3 cages/20 nymphs		
25	NJ	2 cages/20 nymphs	3 cages/10 nymphs	4 cages/15 nymphs	5 cages/10 nymphs
	PA	2 cages/20 nymphs	3 cages/10 nymphs	5 cages/15 nymphs	4 cages/10 nymphs
	VA	2 cages/20 nymphs	3 cages/10 nymphs	5 cages/15 nymphs	2 cages/10 nymphs
35/20	NJ			4 cages/15 nymphs	3 cages/10 nymphs
	PA			5 cages/15 nymphs	3 cages/10 nymphs
	VA			4 cages/15 nymphs	2 cages/10 nymphs
40/20	PA			2 cages/15 nymphs	
	VA			2 cages/15 nymphs	

Temperature treatments: 20/5 = 8 hours at 5°C and 16 hours at 20°C, 35/20 = 35°C for 8 hours and 20°C for 16 hours, and 40/20 = 40°C and 20°C for 6 and 18 hours.

cages. All newly molted nymphs were weighed and frozen. New third instars were sexed by looking at the terminal ventral segments (Figure 1 shown for fourth instar nymphs but thirds are the same just smaller). Males have a black heavily sclerotized band at the anterior end of the genital opening that is absent in the female. Time in instar was calculated for each nymph. Any nymphs that drowned, were consumed by a spider (spiders were occasionally found due to greenhouse exposure to host plants) or were otherwise accidently killed were censored from the data before percentage mortality was calculated.

2.5.2 Third and forth instar treatments

Third instar nymphs were exposed to four temperature treatments: Two constant temperature regimes (15°C and 25°C) and two fluctuating regimes. One fluctuating regime exposed the nymphs to 35°C for 8 hours and 20°C for 16 hours (for an average temperature exposure of 25 degrees C), and 40°C and 20°C for 6 and 18 hours, respectively, yielding an average temperature of 25 degrees. Fourth instars were exposed to only the 25°C constant and the 8 hours at 35°C and 16 hours at 20°C treatments because of insufficient chamber space and limited numbers of surviving fourth instar nymphs for use in the study. Temperature changes were handled here the same way they were handled in the first and second-instar nymph studies described above. The two alternating temperature treatments provided an average daily temperature of 25°C which is the average monthly temperature in July for many areas where SLF is found. 35°C represents the estimated upper developmental threshold for the third and fourth instars and the 40°C part is above the threshold (14). These treatments are meant to approximate what third and fourth instars might be exposed to during a short heat wave during June or July in areas where the SLF is found or could potentially disperse into. The two constant temperatures also allowed between population comparisons of responses to temperatures. A total of 7 cages at 15°C (3 NJ, 3 PA, and 4 VA), 14 cages at 25°C (4 NJ, 5 PA, and 5 VA), 13 cages at 35/20°C (4 NJ, 5 PA, and 4 VA), and 4 cages at 4/20°C (2 PA, and 2 VA) of third instar nymphs were setup with an average of 15 nymphs per cage (range 6-20) (Table 1). Fewer fourth instar cages averaging 10 per cage (range 6-11) were setup as follows: 11 cages at 25°C (5 NJ, 4 PA, and 2 VA), 8 cages at 35/20°C (3 NJ, 3 PA, and 2 VA). All newly molted individuals were weighed, frozen, and sexed. Time in instar was calculated for each nymph. Nymphs were censored from the data in the same way as detailed for first and second instars before percentage mortality was calculated.

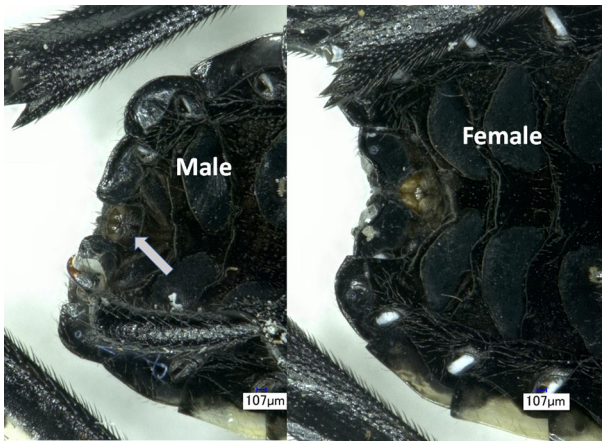

FIGURE 1
Differences between male (left) and female (right) SLF fourth instar nymph. Terminal ventral segments are shown with an arrow indicating the black heavily sclerotized band at the anterior end of the genital opening in the male, that is absent in the female.

2.5.3 Cages and hosts

All treatments for all instars were conducted in cages which were 32.5 × 32.5 × 77.0 cm (BugDorm 4S3074). Initially, each cage had one TOH tree and one grape vine placed in them. An exception to this was applied to the first and second instar cages held at a constant 25°C, which included only TOH (no vines in 2 first and 3 second instar PA cages and 2 first and 1 second NJ cages) as these cages were used simultaneously in a related study on host plants (Kreitman et al. in press this journal) that only used one host per cage. Overlap in the studies was necessary since nymphs and space were both limited and the imbalance in sample structure is addressed in the methods and results.

The TOH plants were replaced every 21, 21, 7, 4, and 4 days in the constant 15°C, alternating 5/20°C, constant 25°C, alternating 35/20°C, and alternating 40/20°C trials. The differences in the rotation time for plants allowed for the maintenance of host quality under conditions where nymphs were developing and depleting hosts more rapidly. Grape vines were not replaced.

2.6 Statistical analysis

Kaplan-Meier product limit estimates of the survival functions were used to calculate the days with 95% confidence intervals that 90, 75 and 50 percentiles of nymphs survived when exposed to different temperature treatments (18). This method of estimating survival functions was used because it can handle the censored data (individuals that survive and molt) and does not require any assumptions about the shape of the function. Nymphs that drowned, were consumed by spiders, or accidentally killed were removed from the analysis since their time of death was unnatural. A Mantel-Haenzel test was used to compare the survival between two temperature treatments so that an adjustment could be made for the potentially confounding factor of differences between populations in survival (18).

The normality of the data was checked using a Shapiro-Wilk test and when the data was not normally distributed was right-skewed, a PROC UNIVARITE was used to assess the fit of a gamma distribution (19). Time (days) to hatch at 15°C, time (days) in instar and newly molted nymphal weights (mg) were analyzed using PROC GLIMMIX (19). The time in instar data was fit to a gamma distribution with a log link, and the hatch and weight data were fitted to a normal distribution. Residuals analyses using Levene's test indicated that variances were equal. Models that compared multiple temperature treatments accounted for population differences by including population as a random effect, and models that compared populations within a single temperature treatment included cage as a random effect. Models for data obtained for first instars (i.e. time spent as a first instar and weight of newly molted second instars) had temperature treatment as a fixed effect and all other models (i.e. those involving instars 2-4 for time and 3-4 for weight) had sex and the interaction between temperature treatment and sex added as fixed

effects. The model for time to hatch just had population as a fixed effect. Differences between means were assessed using the Tukey-Kramer test with an $\alpha = 0.05$ (19).

Rough estimates (based on only two temperatures) of the lower threshold for development (T_{min}) were calculated using the constant temperature data (individual values) for each instar and population. First the relationship between temperature (t) and developmental rate (y = 1/time in instar (days)) was fit to a linear model using Excel (Microsoft Corporation software) following:

$$y = bt + a$$

Then, the intercept (a) was divided by the slope (b) to calculate T_{min} (the x-intercept). Estimates for *a* and *b* were calculated by using least squares regression (18). The population regression lines were then compared for equality of variance (Bartlett's test (20)), if variances were equal then slopes were compared, and if slopes were also equal then the y-intercepts of the lines were compared to determine if the lines were the same or not (18). The standard error on the T_{min} estimate was calculated using the method developed by Campbell et al. (21).

3 Results

3.1 Nymphal survival

Percentage survival curves for each temperature treatment and instar are provided in Figure 2. About half of the decline in first instar survival for the 15°C and 20/5°C treatments occurred in the first 14 days and then losses of nymphs in both treatments dropped to a rate of about 1% every five days until about day 77. Survival in the 20/5°C treatment was almost half that of the 15°C treatment despite the average temperature being the same. Most of the declines in survival for the 25°C treatment occurred in the first 14 days and only a few individuals that did not molt survived until about 70 days then died.

Second instar nymph survival declined at a constant rate of about 5% every 5 days for the first 77 days in the 20/5°C treatment while survival of nymphs at 15°C was high during the first 28 days and then declined at a rate of 1% every three days for the next 28 days. Ninety-six percent of second instar nymphs at 25°C survived and all the declines in survival occurred in the first 19 days.

Declines in survival of third instar nymphs began at about 7 days in the 25, 35/20, and 40/20°C treatments but did not start until about 35 days in the 15°C treatment. No declines in survival occurred after 28, 35, 56, and 72 days for the 35/20, 25, 40/20, and 15°C treatments respectively. The nymphal survival in the 40/20°C treatment was less than half that in the 25°C treatment which was less than half that of the 35/20°C treatment. Fourth instar survival was low in both temperature treatments, but lower in the 35/20°C treatment than the 25°C treatment. The sharpest declines in survival occurred in the first 42 days in both treatments.

Estimates of the days that 90, 75 and 50 percentiles of nymphs survived in each treatment and instar combination are provided in Table 2. These estimates predicted that 90% of nymphs will survive 7-15 long cold snaps and 6-to-24-day heat waves, depending on the instar and intensity of the temperature extreme. The observed

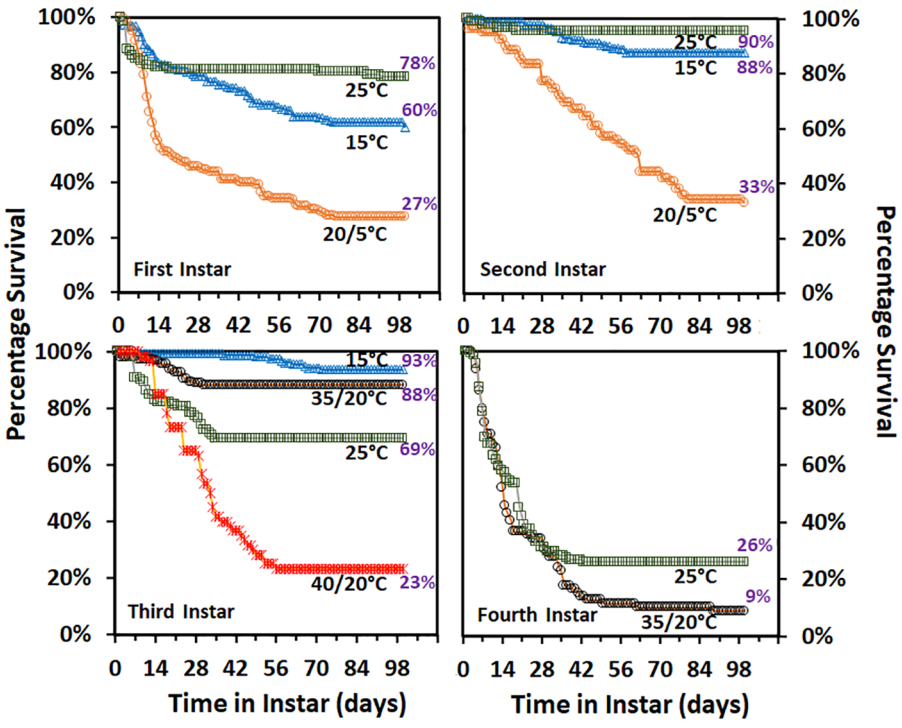

FIGURE 2
Percentage survival of nymphal SLF held in different temperature treatments by instar. Total percentage nymphs that molted is shown at 100 d. Temperature treatments: 20/5 = 8 hours at 5°C and 16 hours at 20°C, 35/20 = 35°C for 8 hours and 20°C for 16 hours, and 40/20 = 40°C and 20°C for 6 and 18 hours.

nymphal survival was different for all within instar temperature treatment comparisons, adjusted for population differences, where treatments had the same average daily temperature (Table 3).

3.2 Impact of extreme temperature alternations on instar duration

The first instar was significantly longer (6 d) when nymphs were exposed to the 20/5°C treatment than the constant 15°C treatment (F = 21.08, df = 1. 15, p<0.0001, Table 4). Duration of the second instar did not differ by temperature treatment (F = 0.99; df = 1, 166; p =0.32), sex (F = 0.63; df = 1, 166; p =0.43) or the interaction between the two (F = 0.04; df = 1, 166; p =0.84). The time in the third instar differed between temperature treatments (F = 79.62; df = 2, 302; p =0.00) and with sex (F = 11.83; df = 1, 302; p =0.00), but not the interaction between the two (F = 1.13; df = 2, 302; p =0.32). The third instar was the shortest (male 17d and female 19 d) when nymphs were exposed to constant 25°C, longer (male 20 d and female 23 d) when exposed to 35/20°C, and longest (male 28d and female 33 d) when exposed to 40/20°C. Duration of the fourth instar varied with temperature (F = 8.56; df = 1, 35; p =0.01) but not with sex (F = 3.15; df = 1, 35; p =0.08), or the interaction between the two (F = 0.76; df = 1, 35; p =0.39). As with the third instars the fourth instar duration was longer (6 d in females and 10 d in males) for nymphs exposed to 35/20°C than those exposed to constant 25°C. All these comparisons

TABLE 2 The 90, 75 and 50 percentile estimates of the number of days (95% confidence intervals) SLF nymphs in each instar survived in each temperature treatment.

Temperature Treatment (°C)	Estimated Survival Percentile	Instar			
		First	Second	Third	Fourth
15	**90**	9.0 (8-13)	43.6 (33-56)	65.0 (54-72)	
	75	37.8 (18-47)	57.1 (50-NA)	71.7 (62-NA)	
	50	60.9 (53-74)	NA	NA	
20/5	**90**	7.0 (5-8)	15.0 (2-22)		
	75	10.0 (9-11)	29.5 (21-43)		
	50	15.0 (13-25)	55.7 (43-63)		
25	**90**	3.0 (3-8)	18.1 (12-NA)	8.2 (7-11)	6.0 (6-7)
	75	16.9 (10-87)	NA	25.5 (14-28)	8.0 (7-9)
	50	NA	NA	29.0 (26-31)	20.0 (14-24)
35/20	**90**			24.0 (19-28)	6.0 (5-7)
	75			30.3 (24-NA)	8.0 (7-12)
	50			NA	15.0 (13-18)
40/20	**90**			14.0 (12-18)	
	75			19.0 (14-29)	
	50			32.5 (29-37)	

Estimates were obtained using the Kaplan-Meier product limit estimates of the survival functions (18). Percentiles that could not be calculated because mortality was low in that treatment are denoted as NA (not available). Cells in the table that are greyed out are treatment and instar combinations that were not done. Temperature treatments: 20/5 = 8 hours at 5°C and 16 hours at 20°C, 35/20 = 35°C for 8 hours and 20°C for 16 hours, and 40/20 = 40°C and 20°C for 6 and 18 hours.

TABLE 3 Comparisons of the survival between pairs of temperature treatments for nymphs of SLF reared using the same average daily temperature exposure, adjusted for population differences in survival.

Instar	Treatment 1	Treatment 2	Statistics		
			Chi Squared	Degrees of freedom	P value
First	**15°C**	**20/5°C**	37.2	1	< 0.0001
Second	**15°C**	**20/5°C**	54.9	1	< 0.0001
Third	**25°C**	**35/20°C**	20.0	1	< 0.0001
Third	**25°C**	**40/20°C**	43.5	1	< 0.0001
Third	**35/20°C**	**40/20°C**	75.2	1	< 0.0001
Fourth	**25°C**	**35/20°C**	9.3	1	0.0023

Statistics are for a Mantel-Haenzel test with population (from NJ, PA, and VA) as a random effect (18). Temperature treatments: 20/5 = 8 hours at 5°C and 16 hours at 20°C, 35/20 = 35°C for 8 hours and 20°C for 16 hours, and 40/20 = 40°C and 20°C for 6 and 18 hours.

TABLE 4 Developmental time (days) and newly molted weights (mg) for different nymphal instars of SLF exposed to different temperature treatments (mean + SE (*n*)).

Instar	Sex	Temperature Treatment	Time in Instar (Days)[a]	Newly Molted Weight (mg)[a]
First	**U**	**15°C**	45.30 ± 3.02a (110)	6.98 ± 0.07a (110)
First	**U**	**20/5°C**	51.31 ± 3.53b (48)	6.68 ± 0.10b (48)
Second	**F**	**15°C**	43.66 ± 1.79a (66)	21.91 ± 0.62b (66)
Second	**F**	**20/5°C**	41.76 ± 3.01a (10)	28.15 ± 1.85a (10)
Second	**M**	**15°C**	42.34 ± 1.66a (90)	19.11 ± 0.48c (90)
Second	**M**	**20/5°C**	39.65 ± 3.47a (6)	18.45 ± 1.53bc (6)
Third	**F**	**25°C**	18.62 ± 2.11d (62)	55.11 ± 2.89ab (62)
Third	**F**	**35/20°C**	23.41 ± 2.65b (70)	55.70 ± 2.90a (70)
Third	**F**	**40/20°C**	32.63 ± 4.60a (4)	46.37 ± 3.61bc (4)
Third	**M**	**25°C**	17.14 ± 1.94d (78)	43.85 ± 2.27c (78)
Third	**M**	**35/20°C**	20.32 ± 2.29c (86)	43.80 ± 2.26c (86)
Third	**M**	**40/20°C**	27.93 ± 3.47a (10)	40.91 ± 2.57c (10)
Fourth	**F**	**25°C**	29.72 ± 2.05a (14)	142.30 ± 10.98a (14)
Fourth	**F**	**35/20°C**	35.80 ± 4.68a (20)	138.50 ± 13.12a (20)
Fourth	**M**	**25°C**	23.40 ± 1.42b (3)	103.90 ± 7.82b (3)
Fourth	**M**	**35/20°C**	33.05 ± 3.75a (4)	90.87 ± 8.06b (4)

[a]Within instars (across all temperature treatments and sexes), means followed by the same letter are not significantly different based on a Tukey test with $\alpha = 0.05$ (19). Temperature treatments: 20/5 = 8 hours at 5°C and 16 hours at 20°C, 35/20 = 35°C for 8 hours and 20°C for 16 hours, and 40/20 = 40°C and 20°C for 6 and 18 hours.

treated population as a random effect to account for between population variation.

3.3 Newly molted nymphal weights

Nymphs that molted to the second instar in the 20/5°C treatment weighed 0.3 mg more than those in the constant 15°C treatment (F = 5.81; df = 1, 154; p =0.02; Table 4). The weight of newly molted third instars did not differ by temperature treatment (F = 3.8; df = 1, 166; p =0.05), but did by sex (F = 27.52; df = 1, 166; p =0.00) and the interaction between the two (F = 7.17; df = 1, 166; p =0.01). Females that molted to the third instar in the 20/5°C treatment weighed 6.2 mg more than those in the 15°C treatment. The weight of nymphs that molted to the fourth instar differed between temperature treatments (F = 5.92; df = 2, 302; p =0.00) and with sex (F = 60.69; df = 1, 302; p =0.00), but not the interaction between the two (F = 1.25; df = 2, 302; p =0.29). Fourth instar females weighed the least when exposed to the 40/20°C treatment and weighed about the same as males in all three temperature treatments. Weights of individuals that molted to the adult only differed by sex (F = 69.44; df = 1, 35; p =0.00) and not temperature (F = 3.23; df = 1, 35; p =0.08) or the interaction between the two (F = 1.4; df = 1, 35; p =0.24). New adult females weighted more than males in both temperature treatments.

3.4 Between population variation in hatch timing and time in instars

Time to hatch varied by population (F = 781.72; df = 2, 2205; p =0.00). The NJ eggs hatched faster (86.0 ± 0.3 days) than PA1 eggs (92.9 ± 0.3 days) which hatched faster than the PA2 eggs (102.2 ± 0.3 days) when held at 15°C. Although the average time to hatch differed between populations, there was substantial overlap in the hatch timing (Figure 3). The PA2 population appears to have three modes, one small mode roughly corresponding to the distribution of the NJ population (75-95 days), a large mode with a mean about 20 days later than the NJ mean (95-115 days) and a final smaller mode with a mean close to 120 days. The PA1 population hatch appears to span most of the range of the other two populations with possibly two modes falling at the mean of the NJ population and corresponding to the largest mode of the PA2 population. These modes resulted from differences between egg masses in mean time to hatch and the size of the mode corresponded to the percentage of egg masses with that hatch timing (Table 5). There was also evidence that the timing of when the first NJ individuals hatched was missed, most of the eggs that hatched for two eggs masses were found on day 76 when hatch for most egg masses was spread out over multiple days (duration of hatch averaged NJ 7.8 ± 2.7, PA1 7.7 ± 5.0, and PA2 9.3 ± 5.2 days).

Time in the first instar varied by population when nymphs were held at 15°C (F = 3.22; df = 2, 104; p =0.04) but not when held at 25°C (F = 0.3; df = 2, 88; p =0.74) (Figure 4). Nymphs from the NJ population completed the instar faster at 15°C than did those from the PA population, whereas the VA nymphs completed the instar with an intermediate number of days compared to the other populations. Time in the second instar for nymphs held at 15°C did not differ between populations (F = 0.26; df = 2, 144; p =0.77), by sex (F = 0.41; df = 1, 144; p =0.52), and there was no interaction between the two (F = 0.54; df = 2, 144; p =0.59). Nymphs of both sexes from the NJ population tended to complete the instar faster than those from the other populations which might impact phenology despite not being

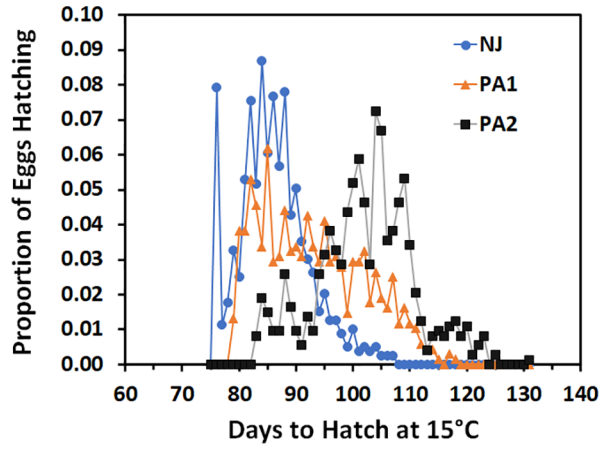

FIGURE 3
Proportion of SLF eggs from three different populations held at 15°C that hatched over time (days).

statistically significant. However, when second instars were held at 25° C the time in instar differed by population (F = 15.72; df = 2, 72; p =0.00) and the interaction between population and sex of the resulting third instars (F = 6.85; df = 2, 72; p =0.00), but not by sex (F = 1.32; df = 1, 72; p =0.26) (Figure 5). Male second instars from the PA population completed the instar faster at 25°C than those from the NJ population which completed the instar faster than those from the VA population. Female second instars from the PA population completed the instar faster than those from the VA population and those from the NJ population took an intermediate number of days to complete the instar at 25°C. The PA population had the only difference between sexes in the number of days in the second instar, with females completing it faster than males.

Time in the third instar for nymphs held at 15°C did not differ between populations (F = 0.26; df = 2, 144; p =0.77), by sex (F = 0.41; df = 1, 144; p =0.52), or the interaction between the two (F = 0.54; df = 2, 144; p =0.59) (Figure 6). However, the trend was the same as seen at 15°C for the first instars; nymphs from the NJ population completed the instar faster than those from the PA population and the VA nymphs completed the instar in an intermediate number of days. When third instars were held at 25°C the time in instar differed by population (F = 12.32; df = 2, 124; p =0.00) and sex (F = 15.91; df = 1, 124; p =0.00), but not by the interaction between the two (F = 1.89; df = 2, 124; p =0.16). The time in third instar for both sexes for nymphs from the PA population was shorter than that of nymphs from the VA population and the NJ nymph time in instar was intermediate to that of the other two populations. Female nymphs from both the PA and VA populations spent longer in the third instar than did males from the same population.

3.5 Between population variation in estimated lower developmental thresholds

The parameters for the linear regressions for developmental rate verses temperature and the estimated T_{min} for each population and instar combination are given in Table 6. The T_{min} for the first instars varied by 1-2°C between populations with NJ (10.04°C) being the lowest and PA (11.59°C) being the highest. For second and third instar nymphs, the estimated T_{min} of the NJ and VA populations were similar while the PA population had a 1-4°C higher estimated value. The slopes of the first instar and second instar lies were significantly different. The assumption of equal variances was not valid for the third instar lines so comparisons of slope and Y-intercepts could not be done. Third instar variation increased with temperature.

4 Discussion

Several findings from this study could have an impact on the projected potential range of SLF and its ability to utilize novel habitats where human-aided transport takes it. Nymphs exposed to temperatures > T_{max} and <T_{min} were able to develop when those temperatures were part of an alternating regime with a favourable temperature, even though development was slower, and survival was lower than at the average corresponding constant temperature (Table 4). Additionally, when individuals from geographically distant populations were exposed to the same temperature regimes there was intra- and inter-population variation in time to hatch, instar duration, and estimated T_{min} values (Tables 5, 6).

When insects are exposed to temperatures near their critical thermal minimums (the temperature at which locomotion stops, different from the T_{min}), they enter a state called a chill-coma that is reversable, where coordinated movement does not occur (22). For the first few days when first instar nymphs were first moved to 5°C during the 20/5°C alternating regime they would fall off the plants and lay upside down as if dead for a few minutes them get up and return to the plants. This suggests that 5°C was cold enough to cause cold stress but that the insects were able to acclimate to it. Cold stress causes oxidative damage, decreased potential in neuromuscular membranes and disruption of the ion/water homeostasis across cell membranes, but exposure to warmer, favourable temperatures provide an opportunity for cells to effect repairs (23, 24). The cumulative effects of the stress still had negative effects on survival and delayed

TABLE 5 Proportions of SLF egg masses with mean time to hatch in each of three groups for three populations of spotted lanternfly.

	Percentage of Egg Masses with Mean Time to Hatch (days)		
Population	76-94	95-114	115-125
NJ	82.4	17.6	0.0
PA1	56.3	43.8	0.0
PA2	10.6	78.7	10.1

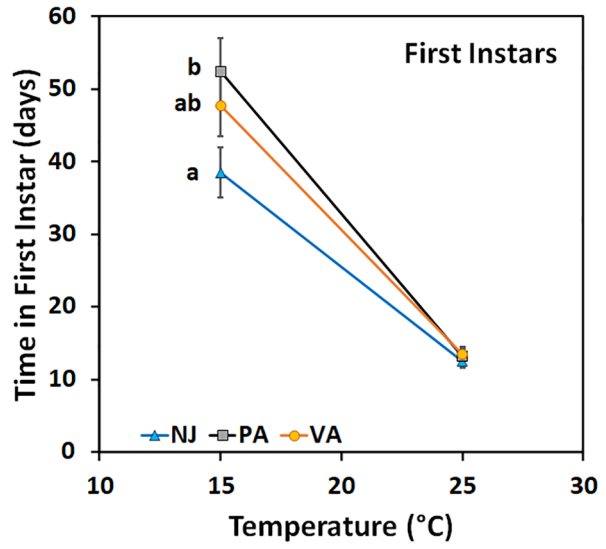

FIGURE 4
Time in instar (days ± SE) for first instar SLF from three populations held at two constant temperatures. Values within a constant temperature across both sexes followed by a different letter are statistically different (Tukey $\alpha < 0.05$) and if no letter are shown there were no differences between populations.

development. However, the negative effects were not as pronounced in the second instar, indicating that it may have a slightly different temperature tolerance. Sensitivity to temperature has been shown to vary independently across stages (25). In the SLF the first instar nymphs are the most likely to experience the cold temperatures, and they have a broader range of temperatures that they tolerate than do the second instars. The delay in development in the first instar was also only 6 days (Table 4) which indicates that some development must have occurred at 5°C. The first instar's ability to survive in an alternating regime that includes 5°C suggests the estimated T_{min} reported in Kreitman et al. (14) may be inaccurate. So, it is likely that SLF would be able to survive and develop in colder environments than previously thought.

When insects are exposed to temperatures near T_{max} many potentially irreversible changes occur in insects that can lead to death or deleterious effects on biology and morphology: altered cellular pH and ion concentrations, changes in macromolecules (e.g. proteins, DNA, RNA, lipids), and alterations in cell structures (26). In addition, small increases in temperature can have increasingly stronger effects until abruptly hitting the lethal temperature (27). This fits with what was observed in this study. When third and fourth instar SLF were exposed to 35 or 40°C temperatures as part of an alternating regime with exposure to 20°C, development was delayed compared to the average constant temperature (25°C, Table 4). The increased time spent in the instar, especially in the 40/20°C regime, suggests that the nymphs were not able to develop or developed at a much slower rate during the extreme temperature part of the regime. Survival however declined dramatically for third instar nymphs as the amplitude of the difference between the temperatures increased: <20% mortality in 35/20°C and close to 80% in the 40/20°C regime (Figure 2). Fourth instar mortality was high in general due to the limitations of the laboratory rearing environment, but survival in the alternating regime was higher than the constant temperature regime. Consequently, SLF can develop when temperatures above T_{max} (estimated to be 35°C for third and fourth instar nymphs (14)) are part of an alternating regime just as has been observed for *Drosophila melanogaster* Meigen (Diptera : Drosophilidae) (16). Greater detrimental effects not only increase as the temperature increases but become more profound when the amplitude of the difference

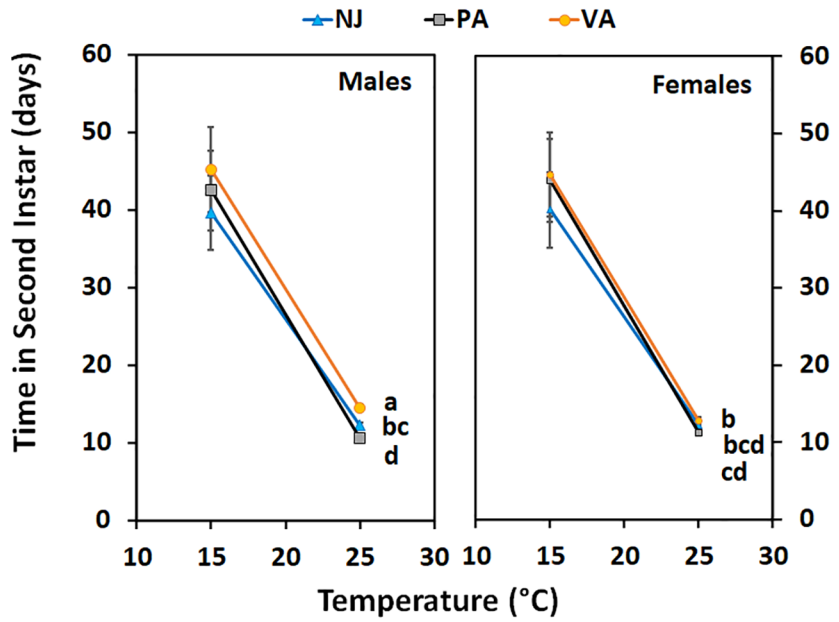

FIGURE 5
Time in instar (days ± SE) for second instar SLF of each sex from three populations held at two constant temperatures. Values within a constant temperature across both sexes followed by a different letter are statistically (Tukey $\alpha < 0.05$) different and if no letter are shown there were no differences between populations.

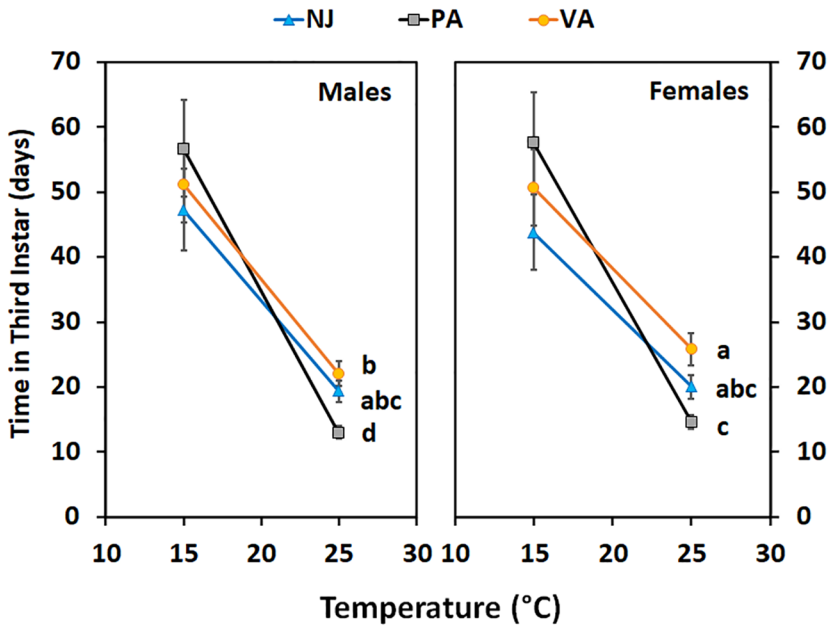

FIGURE 6
Time in instar (days ± SE) for third instar SLF of each sex from three populations held at two constant temperatures. Values within a constant temperature across both sexes followed by a different letter are statistically (Tukey $\alpha < 0.05$) different and if no letter are shown there were no differences between populations.

between high and low temperatures increases because of increasing energy demands. The percentage deviation between constant and alternating temperatures is generally smaller if the amplitude of the fluctuations is <7°C and larger if >7°C (28). If this holds true for SLF then as it moves south or into warmer regions it may reach thermal conditions that may limit its range but that the natural diurnal alternation of temperatures may buffer it somewhat from the deleterious effects. The benefits of alternating temperatures may however be minimal when close to the upper thermal limit, only extending the tolerable temperature range by ≤ 1°C (29). Care should

TABLE 6 Parameters (± SE) for developmental rate verses temperature regressions and estimated lower developmental thresholds based on the 15 and 25°C data for each SLF population and nymphal instar combination.

Instar	Population	Slope	Intercept	Adj. r^2	Estimated T_{min}	Comparison of population lines[a]
First	**NJ**	0.00533 ± 0.00025	-0.05323 ± 0.00245	0.97	10.04 ± 0.21	Equal variances: χ^2 = 4.24, df 2, p=0.1199 Equal slopes: F = 4.59; df 2, 198; p=**0.0112** Slopes are different
	PA	0.00587 ± 0.00153	-0.06841 ± 0.00305	0.96	11.59 ± 0.21	
	VA	0.00536 ± 0.00145	-0.05912 ± 0.00306	0.95	10.94± 0.26	
Second	**NJ**	0.00566 ± 0.00125	-0.05907 ± 0.00239	0.96	10.37± 0.18	Equal variances: χ^2 = 5.82, df 2, p=0.0545 Equal slopes: F = 33.82; df 2, 236; p<**0.0001** Slopes are different
	PA	0.00667 ± 0.00132	-0.07628 ± 0.00257	0.97	11.39± 0.15	
	VA	0.00503 ± 0.00163	-0.05240 ± 0.00308	0.92	10.48± 0.27	
Third	**NJ**	0.00533 ± 0.00025	-0.05323 ± 0.00245	0.97	7.31± 0.68	Equal variances: χ^2 = 8.06, df 2, **p=0.0178** Variances are different, assumptions for further comparisons not valid
	PA	0.00533 ± 0.00025	-0.05323 ± 0.00245	0.97	11.88± 0.34	
	VA	0.00533 ± 0.00025	-0.05323 ± 0.00245	0.97	8.07± 0.67	

Analyses were done using Statistix (18) and the sexes were combined.
The Bartlett's test statistics on the comparison of the lines is provided.
In the comparison of population lines, the bold is the significant p-values.

be taken in extrapolating these results to the field, since the study used instantaneous changes in temperature whereas temperature in the field generally changes more gradually.

The average time spent in each instar when reared at 15 and 25°C was shorter in this study than reported by Kreitman et al. (14). First instars completed development at 15°C an average of 26 days faster and third and fourth instars completed development at 25°C an average of 7 days faster than previously reported. These differences are substantial and could affect the predicted phenology of SLF when used in a model. One SLF model that attempted to use the previously reported developmental rates had to adjust them to match developmental rates with those in the field (15) and reported for other hosts in the laboratory (30). The adjustments made for the modelling effort were to speed the developmental rate up for each instar, especially for the third and fourth instars (2.13 and 2.62 times respectively), which is in line with the faster development seen in this study. In Addition, the percentage mortality of nymphs in this study is lower than in the previous study (14) and mortality rate also had to be lowered in the modelling effort to make the model predictions match field observations (15). These differences are likely explained by the methods used in the two studies. The cages used in the current study allowed larger more robust TOH plants to be used than the tubes used in the previous study (14), which the authors of the previous study acknowledged were not ideal, especially for the larger nymphs. This is also consistent with the documented effects that the host used in the study can have on SLF nymphal development (17). In addition, the higher humidity and condensation present in the tubes could have trapped the nymphs and prevented them from feeding normally.

Exposure to extreme temperatures also had effects on the weights of newly-molted SLF nymphs. First and third instar (female) nymphs exposed to extreme temperatures as part of alternating regimes had lower weights compared to nymphs in the comparable average constant temperature (Table 4). The lower weights were probably due to energy being diverted from development to recovery from thermal stress or production costs of protectants against further thermal stress (31). For higher temperatures exposures, another possible explanation is that developmental rate would increase with increasing temperature, which can result in smaller body sizes (32). The exception was that second instar females that developed in the 20/5°C regime weighed more than those that developed in the 15°C constant temperature. One possible explanation is that the larger nymphs present in the 20/5°C were the only ones that were able to survive, since mortality was very high. The lack of weight differences between treatments in the fourth instars probably has a lot to do with the small sample size. Evaluating fourth instars in the laboratory is difficult because they have very high host demands which necessitate frequent plant changes and much reduced numbers per cage. Thus, results obtained for fourth instars should not be used in predicting what may happen in the field or used in models.

There was variation in the timing of hatch among egg masses both within and among populations when held at 15°C (Table 5). There are many possible reasons for this variation: historical factors like local adaptation, temperatures experienced before collection, and maternal effects, genetic variation, oviposition time, or individual plastic variation (33–35). For example, the exact temperatures the eggs were exposed to before collection and when each egg mass was laid are not known and that could have affected hatch timing. There is likely a consistent hatching stage but variation in hatch timing for SLF. Embryos may develop at different rates until they reach hatching competence after completing diapause but stay in a reduced metabolic state while they wait for another cue to hatch (which could differ between populations). If the cue to hatch never comes the embryos may die when energy reserves are exhausted as was seen in earlier work with SLF eggs (36). There is also evidence that the resumption of embryonic development is controlled by the expression of a heat shock protein and that a chill period is required to start that expression (37). The variation in hatch timing will buffer populations from mass mortality if they hatch too early or late in highly variable environments. In areas where the growing season is shorter there could be a major advantage to hatching as early as conditions become favourable, thus allowing sufficient time to reach the adult stage and oviposit before conditions become unfavourable again. If the first laid eggs also tend to hatch first and many females at a particular location are killed by cold before they can lay, selection for faster nymphal development and earlier hatch could occur. The earlier hatch in the NJ population compared to the PA ones would be advantageous since the average monthly highs are 2°C warmer and the monthly lows are 1°C warmer (based on data obtained from https://www.worldweatheronline.com) than at the NJ site, effectively resulting in a shorter growing season. The SLF populations have not been present at these sites that many years so selection may not have occurred yet. Another possible scenario that could explain hatch differences is that the preferred hosts at a site may decline in quality and nymphs may have to use alternate hosts, both of which could affect maternal provisioning of the eggs and timing of oviposition (can grow slower on less preferred hosts). Either egg provisioning or oviposition timing could in turn affect hatch timing. The SLF has been in the Philadelphia area longer than either the NJ or VA sites used in this study. Further work to determine exactly when eggs enter and exit diapause and how temperature effects that is needed to be able to determine the underlying reasons for the differences in hatch timing.

Variation in time in instar at the two constant temperatures also varied between populations and by instar. Insect populations exhibit local adaptation or plasticity in their developmental responses to temperature and this can vary between life stages (38). These differences can be the result of changes in the developmental thresholds and/or thermal requirements to complete development (38). The rough estimates (based on only 2 temperatures) of T_{min} for the first and second instars suggested that there may be 1-2°C variation between populations. When compared to previous estimates of T_{min} (13°C for firsts and 12°C for seconds) that variation could be up to 3°C (14). There is up to 4°C difference between the third instar T_{min} from this study and what was previously reported (14). There is also evidence of phenotypic plasticity across all instars since the slopes of the reaction norms (thermal response lines) of the populations are not equal (Figures 4–6) and there is evidence of a genotype by environment interaction since the lines cross for the second and third instars (Figures 5, 6; Table 6). When lines cross it indicates that the phenotypic responses of the genotypes present in the populations differ based on the temperatures they are exposed to; the PA population grew the slowest at 15°C and the fastest at 25°C. The

relative responses of the populations are consistent with the USDA plant hardiness zones they come from; first instars from the NJ population from the coldest zone grew the fastest at cooler temperatures than the other populations. This is also consistent with the temperature that the first instar nymphs would be exposed to at these sites in April when they hatch: NJ high 15°C and low 5°C, PA high 18°C and low 6°C (data from https://www.worldweatheronline.com). The patterns are inconsistent with the predicted decrease in T_{min} and increase in thermal requirements as latitude increases (38, 39). But there are two other factors that may play a role, elevation and heat islands of big cities. Just as ambient temperatures decrease by 7°C per 10° latitude, they also decrease by 6°C per km increase of altitude (40). Urban heat islands in the Northeast average 7-9°C warmer than surrounding rural areas (41). The PA populations were from lower elevations than the other two populations and from Philadelphia, PA area where there is an urban heat island. A broader survey of populations from across the SLF current range would be needed to assess the full variation in thermal responses. However, even the variability documented in this study is large enough to have impacts on predicted phenology and potential risk of establishment especially in areas colder areas than previously considered at risk.

Accurate phenology models based on SLF's thermal responses are necessary for predicting when monitoring needs to occur, when the right stage is present for application of control methods, and for estimating the risk of establishment across the US. The new information from this study on variation present within and among populations in thermal requirements for hatch and development, as well as ability of nymphs to develop when exposed to alternating periods of temperatures above and below developmental thresholds and favourable temperatures should be integrated into the existing phenology models that rely on the older data (14) and used when new models are developed. Further work assessing more populations from a broader range of geographic locations and climates is still needed to better refine regional phenology predictions, but the present data will at least provide a starting point for the needed refinements to the models.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MK and DK conceived the study. MK and GH got the funding for the study. MK and DK collected the data. MK analysed the data, prepared the figures and tables, and wrote the paper. All authors edited the manuscript. MK revised the paper based on reviewer input. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Spotted! Computer-aided individual photo-identification allows for mark-recapture of invasive spotted lanternfly (*Lycorma delicatula*)

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The spotted lanternfly is an invasive pest for which we lack individual movement data due in part to the difficulty posed by individual identification. We developed a computer-aided method to identify individual adult spotted lanternfly using wing spot patterns from photos processed in the software I3S and demonstrated the method's accuracy with lab and field validations. Based on 176 individuals in the lab, we showed that digitizing the spots of one wing allowed a 100% reliable individual identification. The errors due to user input and the variation in the angle of the image were largely negligible compared to inter-individual variations. We applied this method in the context of a mark-recapture experiment to assess the feasibility of this method in the field. We initially identified a total of 84 unique spotted lanternflies, 31 of which were recaptured after four hours along with 49 new individuals. We established that the analysis of recaptures can possibly be automated based on scores and may not require systematic visual pairwise comparison. The demonstration of the effectiveness of this method on relatively small sample sizes makes it a promising tool for field experimentation as well as lab manipulations. Once validated on larger datasets and in different contexts, it will provide ample opportunity to collect useful data on spotted lanternfly ecology that can greatly inform management.

KEYWORDS

biological invasion, dispersal, individual recognition, movement, pest, photographic mark-recapture, population size

1 Introduction

The spotted lanternfly (*Lycorma delicatula* (White) (Hemiptera: Fulgoridae), SLF) is an invasive insect in the early stages of its invasion that is spreading across the northeastern United States and has the potential to cause billions of dollars in damage to the wine, timber, and ornamental plant industries due to its phloem-feeding diet (1–3). At the beginning of

invasions, population dynamics data is critical for informing management (4) and gaining knowledge on SLF behavior, dispersal capabilities, and demography is one of the central pillars for managing the invasion (1). Much of this information requires tracking the fates of particular individuals, yet we lack an individual identification method to track SLF in a mark-recapture framework. Demonstrating the applicability of photographic mark-recapture on SLF would open a field of research opportunities that would inform the management of this species. In particular, individual movement data would reveal habitat use to inform where control actions should be enacted and the rate of movement can inform how often actions should be enacted as new individuals move into a location (5, 6).

Non-invasive methods are advised for individual identification in wildlife research, not only for ethical reasons, but also because adverse effects associated with handling and marking, such as changes in behavior or survival, may affect mark-recapture estimates (7). Photographic mark-recapture is a cheap and harmless technique that circumvents the drawbacks of physically marking individuals, as it only relies on the inter-individual variability in permanent natural marks that act like "fingerprints" to visually identify individuals. Photographic mark-recapture has been successfully applied to fish (8–10), amphibians (11–13), reptiles (14–16), and also arthropods (17–19). The SLF likely satisfies several required conditions for individual photo-identification (20): adult wings are covered in spots that likely differ in number and position among individuals (Figure S1, Supplementary Material 1) and the first pair of wings is rigid and unlikely to be distorted in photos. However, whether the inter-individual variability of the wing patterns is sufficient to distinguish individuals must be tested to determine if the technique is suitable for answering scientific questions.

Manual individual photo-identification is a time-consuming technique, since all pairs of photographs must be compared to recognize individuals, a number that increases exponentially with sample size. Several photo-identification programs (e.g. I3S, Wild.ID, APHIS) have been developed to semi-automate this process by allowing users to digitize particular features on images and calculating an index of dissimilarity between features of candidate (unknown) and reference (known) individuals. The software presents users with reference images ranked by similarity for each candidate image, and lets the user decide whether there is a true match. This process considerably facilitates individual photo-identification but still requires a time-consuming step of careful visual comparison from the user to validate correct matches. The original publication for I3S stated that a score of less than 400 indicated a high probability of a possible match (20). To further reduce the amount of user input required, it would be informative to determine if a threshold can be found for scores indicative of non-recaptures, leaving only a fraction of comparisons with intermediate scores to be manually investigated, facilitating the use of this method on highly locally abundant SLF populations.

To use individual photo identification software for non-invasive mark-recapture studies of SLF, three potential obstacles must be overcome. First, biologically, the patterns on the wings must be variable enough among individuals to allow photo-identification itself, even within localities. Indeed, there is no research on whether spot patterns are genetically coded or environmentally driven, which would cause similar spot patterns within localities. Second, technically, when using the software, dissimilarity scores must not be too sensitive to user error in digitizing the wing spots, nor to the positioning of SLF on trees or lightning conditions in the field. Third, logistically, the method must not be too time intensive and require as little user input as possible, which implies that scores themselves should allow for the identification of recaptures and non-recaptures.

We tested whether individual photo-identification is an appropriate method for mark-recapture in SLF. This process involved validation in the lab to assess whether inter-individual variability in wing spot patterns is sufficiently high for individual identification within and among localities and to test the robustness of the method to digitization errors and photos taken at different angles. We complemented the lab validation with a field validation in the form of a test in natural conditions to demonstrate the applicability of this technique to SLF. Finally, we examined the distribution of the pairwise dissimilarity index between recaptures and non-recaptures to further automate the pattern-matching step and reduce the need for user validation.

2 Material and methods

2.1 Lab validation

2.1.1 Aim

The objective of the lab validation was to test, based on images of labeled SLF, whether there is enough inter-individual variation and minimal digitization error to make individual photo-identification possible in SLF, and automate individual identification using digitization scores. We used the program I3S Classic version 4.0 (9) and partly followed the technique developed by Sacchi and collaborators on lizards (16), and reused for the development of photo-identification on other taxa (11, 17). Specifically, we used the same method of comparing pairwise dissimilarity scores for two digitizations of the same image, digitization of two images of the same individual, and digitization of different individuals, but we modified the statistical analysis of the scores produced (see below).

2.1.2 Images

We used 176 adult SLF individuals that had been collected in the field at eight locations in Pennsylvania (7-44 per location, Figure S2) in 2020 for a companion project. Following capture, individuals were frozen at -20°C and then photographed in the lab with a smartphone. The image resolution was considered sufficient when the contours of the wing(s) considered for recognition as well as the spots were clearly discernible. Two images were taken per individual: the first image was taken from a top-down angle directly above the individual, and the second image was taken at a ~45° angle on the left side of the individual (Figure 1B). Individuals are unlikely to be photographed at an angle higher than 45° in the field as the photographer must stay away from the tree to avoid influencing SLF behavior. Therefore, considering the top-down angle and a 45° angle simulates the range of variation in the image angle, and thus the range of image distortion, that may be encountered in the field.

We maintained the top-down and side-angle images of each individual in separate sets to use in the analyses.

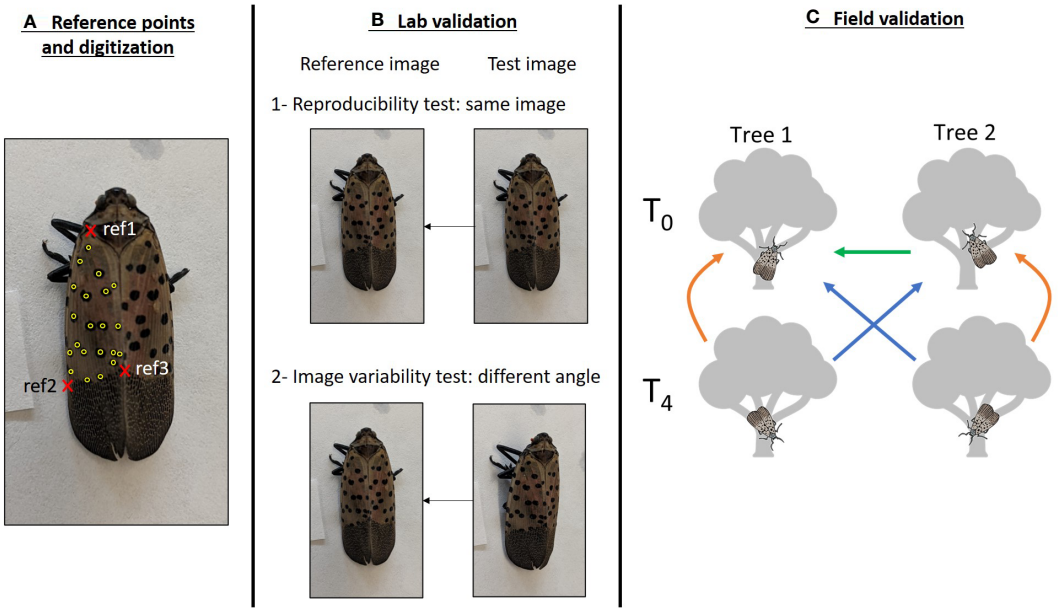

FIGURE 1
Graphical representation of the methodology used to validate the photo-identification of SLF. **(A)** Detail of the digitization of a SLF individual. The three reference points used to orient the individual are in red: wing attachment point (ref1), left (ref2) and right (ref3) margins of the limit between the spotted and dashed gray zones of the wing. Digitized spots are in yellow. Note that spots on the left margin of the wing were not digitized. **(B)** Lab validation. A first set of photos (top-down set) was digitized twice, the first fingerprint was used as a reference, and the second fingerprint was compared to this reference to assess the reproducibility of the digitization (B1). A second set of photos, taken at an angle (side-angle set), was digitized and compared to the top-down reference to assess the impact of the photo angle (B2). **(C)** Field validation. SLF were photographed on two trees during two sessions (T_0 and T_4). Comparisons between images from trees and sessions were performed to assess initial distinctiveness of SLF ($T_{0\ tree1}$ vs. $T_{0\ tree2}$, green arrow), recaptures on the same tree (orange arrows), and recaptures on a different tree (blue arrows). Arrows point from the candidate set of images to the reference.

2.1.3 Image processing

All images were processed in I3S Classic version 4.0 (20). This program allows the digitization of spot patterns of an animal within an area determined by three reference points that are used to align the images. Together the digitized spot patterns and reference points create a fingerprint file. I3S then compares pairs of fingerprints by superimposing reference points and calculating the distance between pairs of spots. It generates an index of dissimilarity that is the sum of the distance between each spot pair divided by the square of the number of spot pairs (20). As a result, pairs of SLF images with low dissimilarity scores have similar spot patterns.

We limited digitization to the left wing of each individual rather than both wings, given that the number of spots on a single wing typically spans 12-30, a range recommended in I3S to optimize both the identification and the amount of time necessary to digitize images (20). The three reference points used to orient the image were chosen to provide the least distortion of the SLF in the area being digitized: the intersection of the first left rib and the right margin, near the wing attachment point (ref1), and the intersections of the margin and the gray zone at the rear of the wing, on the left (ref2) and right side (ref3, Figure 1A). All spots on the left wing were digitized except for the spots on the first left rib that may not be visible depending on the angle of the image as well as on aggregated individuals (Figure 1A). While the effect of the number of digitized spots on the accuracy of the identification is an issue that must be addressed in animals with numerous natural marks that are subsampled during digitization (e.g. 16), we did not test this because we digitized all spots within the defined boundary.

The top-down set of images was digitized and used as the reference database. The top-down images were then digitized a second time by another user and matched against the reference database to measure the reproducibility of digitization by two different users using the "batch compare" feature of I3S. "Batch compare" computes scores for all pairwise combinations between the tested images and the reference database. The side-angle set of images was then digitized and matched against the reference database using "batch compare" to measure the method sensitivity to the angle of the image that may distort distances between points.

2.1.4 Statistical analysis

Scores of reference individuals matched to candidate images were called DmatchX, with X representing the rank of the score among all other pairwise comparisons. For example, the reference individual with second-lowest score (i.e., the second most similar image) is called Dmatch2. First, because we knew the correct matches between all pairs of images, we checked the ranking and score of the correct reference image for each candidate image, using the top-down set of images digitized by another user, and then using the side-angle set of images. Most publications consider the method successful if the correct individual is ranked within the first few matches shown by the program (15, 17, 20, 21). Ideally, for the method to be most effective and automated, the reference image corresponding to the candidate image should be ranked first (Dmatch1), so this was our aim for the lab validation. Once we confirmed that Dmatch1 did in fact correspond to the photo of the same individual, we tested whether

Dmatch1 was higher for the side-angle set of images than for the top-down set of images due to distance distortion using a Wilcoxon signed rank test.

Second, we assessed how well the software was able to discriminate a specific individual by calculating the difference in scores between Dmatch1 and Dmatch2 (i.e., the correct and the "best" incorrect individual) and comparing it to the difference between Dmatch2 and Dmatch3 (i.e., the two "best" incorrect individuals) for each individual using a Wilcoxon signed rank test. This test indicates if the correct match has a distinctively lower score compared to the best other matches, for each individual. It represents a step further from studies that investigated this question by comparing the first match with the average population score (16, 17, 21), in that the extent of the dissimilarity of the focal individual with the most similar other individual is key in ensuring that the correct individual will always be ranked first. Then, we compared the range of Dmatch1 to that of Dmatch2 to determine whether they do not overlap among individuals, which would allow to set a generic threshold score that indicates recaptures of the same individuals.

Third, we tested whether spot patterns were more similar within localities than among localities, suggesting either heritability or environmentally-driven spot patterns, and making it potentially harder to photo-identify individuals within localities, by comparing Dmatch2 obtained within the locality to Dmatch2 obtained from all localities using Wilcoxon signed rank tests. All statistical analyses were done in R version 4.0.5 (22).

2.2 Field validation

2.2.1 Aim

The objective of the field validation was to assess whether this method of photo-identification is robust to the variability in lighting and body positioning of SLF introduced by field conditions and to confirm that the method can be used for photographic mark-recapture of individuals over time.

2.2.2 Images

We photographed SLF found on two red maples (*Acer rubrum*) separated by 12 meters in an urban park in Philadelphia, PA (Figure S2) on August 8, 2022 at 3:30 PM (T_0). SLF visible on tree trunks and lower branches were successively photographed with a smartphone until all adults were photographed, or within a maximum of 5 minutes where abundances were high to standardize sampling effort. During photographing, the photographer avoided getting close enough to the tree to alter the behavior of SLF individuals and zoomed in on the SLF to take photos. Four hours later, the photographer took a second set of photographs on each tree, following the same methods (T_4, Figure 1C). The four sets of images we used in our analyses were tree 1 at T_0, tree 2 at T_0, tree 1 at T_4 and tree 2 at T_4. Since capture of the same SLF individual in multiple photos within a single photo session was likely, the ability to detect duplicate individuals in a single photo session is an additional piece of information to gauge the accuracy of the software.

2.2.3 Image processing

Because SLF often aggregate on trees, many of the photos were of multiple individuals. Therefore, to facilitate digitization, all images were first cropped to be just of single individuals. All images were then digitized by multiple users using the same three reference points as in the lab validation. We looked for individuals photographed in duplicate during a single session on a tree by visually comparing all pairs of images within a set.

The "batch compare" feature was used to compare sets of images and generate scores. The test of the detection of duplicates within a single session on the same tree was done by matching a set of digitized images against itself. After this step, we kept a single image per individual to create a set of unique individuals with a single image for each session per tree to avoid adding an effect of the number of images per individual. We then assessed the ability to detect recaptures by matching T_4 images (candidate) to T_0 images of the same tree and the other tree (reference, Figure 1C), as well as matching fingerprints from Tree 2 at T_0 (candidate) against Tree 1 at T_0 (reference). In the I3S software, identification of recaptures involves a judgment call based on a visual comparison of all pairs of images, and it is how we determined recaptures, independently of their scores.

2.2.4 Statistical analysis

We first assessed whether visually identified recaptures were classified as Dmatch1 for the corresponding individual. Ideally, if this method could be used in a semi-automated fashion based on scores to identify recaptures, recaptured individuals should have lower Dmatch1 scores and greater differences between Dmatch1 and Dmatch2 than individuals that were not recaptured. To test this, we tested whether Dmatch1 was lower for recaptured than for non-recaptured individuals with a Mann-Whitney test. In addition, we determined whether there was a significant difference between Dmatch1 and Dmatch2 for recaptured individuals compared to non-recaptured individuals with a Mann-Whitney test.

We used the same reasoning to assess whether the program could identify duplicated images within a single session: as Dmatch1 is the same image (score = 0), we checked whether Dmatch2 was the duplicated image, and tested whether Dmatch2 was lower in duplicated images compared to non-duplicated images with a Mann-Whitney test. We determined whether there was a significantly higher difference between Dmatch2 and Dmatch3 for duplicated images than for non-duplicated images with a Mann-Whitney test.

3 Results

3.1 Lab validation

For the first analysis where we compared the two top-down images digitized by different users, 100% of Dmatch1 for the test images were the correct reference individual among the 176 individuals forming the database. These Dmatch1 scores were higher than 0 (average ± SD: $Dmatch1_{top\text{-}down}$ = 176 ± 57), and represented the user error. When we compared side-angle images to

the top-down images, 100% of the test images were again matched to the correct reference individual using the program, even if Dmatch1 scores were slightly higher for the side-angle photo set comparison ($Dmatch1_{side\text{-}angle}$ = 379 ± 93, V = 0, p < 0.001, Figure 2A).

There was an average difference of 783 ± 155 between Dmatch1 and Dmatch2 when comparing the two top-down fingerprints, and 629 ± 161 when comparing the side-angle and top-down fingerprints (Figure 2B). These differences were much larger than the differences between Dmatch2 and Dmatch3, which were on average 85 ± 75 for the two top-down fingerprints (V = 15576, p < 0.001) and 89 ± 72 for the side-angle and top-down fingerprints (V = 15575, p < 0.001, Figure 2B).

No overlap was found between Dmatch1 and Dmatch2 among all individuals. Dmatch1 scores were always lower than 428 and Dmatch2 scores were larger than 566 with the two top-down images. Dmatch1 were always lower than 705 and Dmatch2 were larger than 719 with the side-angle and top-down images (Figure 2B).

When comparing the two top-down images, Dmatch2 scores were lower across localities ($Dmatch2_{across}$ = 960 ± 167) than within localities ($Dmatch2_{within}$ = 1176 ± 208, V = 0, p < 0.001, Figure 2C). It was also the case when comparing top-down to side-angle images across versus within localities ($Dmatch2_{across}$ = 1008 ± 147, $Dmatch2_{within}$ = 1247 ± 238, V = 0, p < 0.001, Figure 2C).

3.2 Field validation

For Tree 1, 63 and 53 photos of 58 and 45 individuals were taken at T_0 and T_4, respectively. For Tree 2, 27 and 44 photos of 26 and 35 individuals were taken at T_0 and T_4, respectively (Table 1). Based on the visual comparison to identify recaptures: (1) no individual was captured on both trees at T_0, (2) 34% and 42% of the individuals were recaptured on the same tree at T_4, and (3) no individual was found on a different tree at T_4 from the tree it was on at T_0 (Table 1). Recaptured individuals that were visually identified were always ranked as Dmatch1 by the program, meaning that the user would not have to look beyond Dmatch1 to identify recaptures. In the case of recaptures, Dmatch2 was on average 867 ± 262 points higher than Dmatch1 (Figure 3B). Dmatch1 and Dmatch2 scores were much closer in the case of non-recaptures (W = 39, p < 0.001) with an average difference of 176 ± 153.

Overall, Dmatch1 showed a clear bimodal distribution, where the first mode consisted of all recaptured individuals, and the second mode was all non-recaptured individuals (Figure 3A). In other words, Dmatch1 scores were lower for recaptured individuals than for non-recaptured individuals ($Dmatch1_{recaptures}$ = 473 ± 150, $Dmatch1_{non\text{-}recaptures}$ = 1342 ± 300, W = 4742, p < 0.001). There was however a "gray zone" where recapture and non-recapture Dmatch1 scores

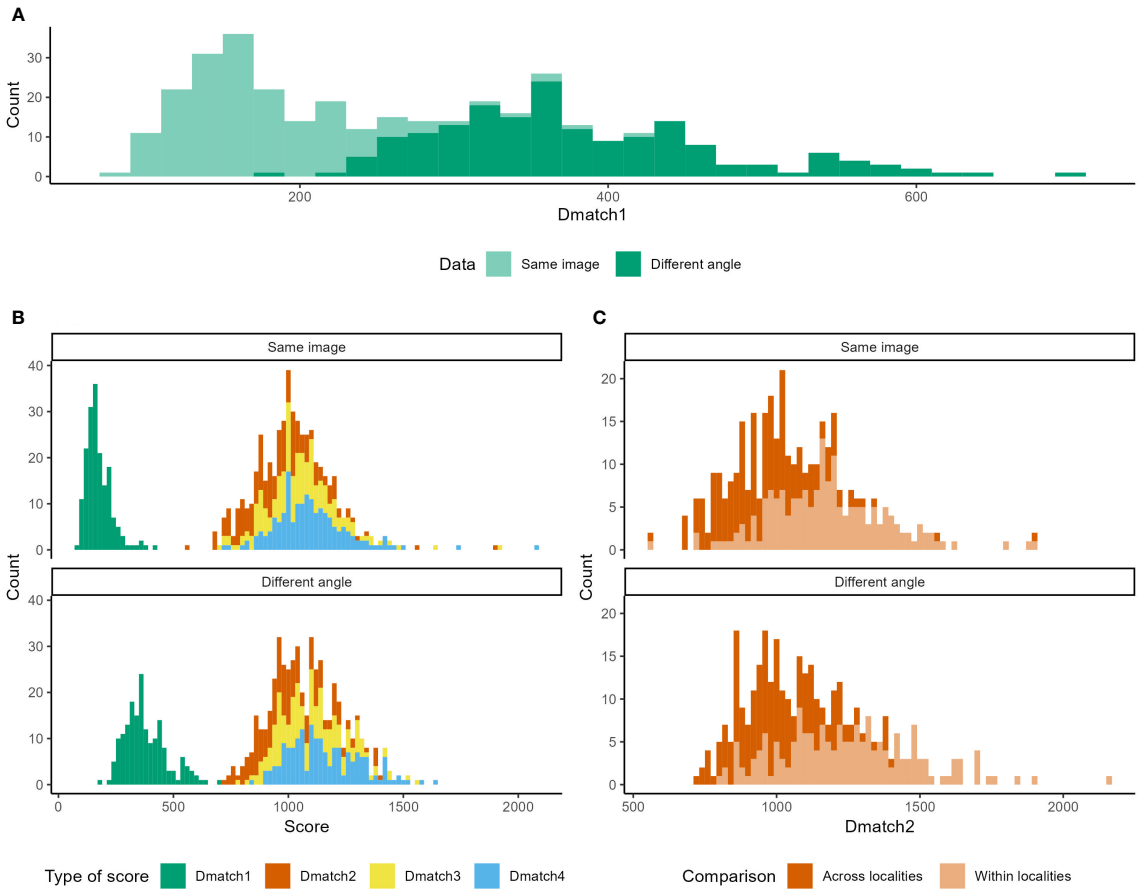

FIGURE 2
Lab validation of the photo-identification of the spotted lanternfly. **(A)** Distribution of scores of the correct match (Dmatch1) when using the same image or an image taken from a different angle. **(B)** Distribution of scores of the correct (Dmatch1), second-, third- or fourth-best match obtained (Dmatch2, Dmatch3 and Dmatch4, respectively). **(C)** Distribution of scores of the second-best match (Dmatch2) when images are compared to individuals from their locality of origin (within locality) or from all localities combined (across localities).

TABLE 1 Summary of the recapture field study.

Reference \ Tested	Tree 1	Tree 2	
	T_4 (45 ind.)	T_0	T_4 (35 ind.)
Tree 1 T_0 (58 ind.)	20 (34%) – 828/933	0 (no movement)	0 (no movement)
Tree 2 T_0 (26 ind.)	0 (no movement)		11 (42%) – 724/807

Number of recaptures between trees and sessions (percentage of initial captures) - Maximal score of recaptures/Minimal score of non-recaptures.

overlapped, since Dmatch1 of recaptures were lower than 828 and Dmatch1 of non-recaptures were larger than 807 (Figure 3A).

For the test of duplicates within a single session, Dmatch2 was always the duplicated individual when it had been identified manually, meaning that duplicated individuals were ranked better than non-duplicated individuals for each individual. In the case of duplicates, Dmatch2 was on average 471 ± 211, significantly lower than the Dmatch2 of non-duplicated individuals (1292 ± 288, W = 6367, $p < 0.001$, Figure S3A). In other words, Dmatch2 showed a bimodal distribution, the first mode consisted of duplicated individuals, and the second mode was all non-duplicated individuals. Dmatch2 and Dmatch3 were much closer in the case of non-duplicated individuals with an average difference of 159 ± 139, while in the case of duplicated individuals this difference was 818 ± 296 (W = 159, $p < 0.001$, Figure S3B). There was a gray zone where duplicated and non-duplicated Dmatch2 overlapped, since Dmatch2 of duplicates were lower than 969 and Dmatch2 of non-duplicates were larger than 741 (Figure S3A).

A sensitivity analysis was conducted to assess the impact of sample size on the evolution of the scores of the best-ranked different individuals (Supplementary Material 2). It was done by resampling the pool of all individuals (lab and field validation, N = 309) and matching it against itself. The procedure was repeated 10 times, and with different sample sizes (n = 10 to 309). Results show that the lowest score for an incorrect match does not decrease linearly but seems to stabilize around 1000 as sample sizes increase (Supplementary Material 2). In all cases, no more than 30% of these incorrect matches had scores < 900 and would have to be visually verified.

4 Discussion

Research on the spread of SLF would benefit from having an individual-based identification method to allow for the tracking of individuals in the field and determine demographic parameters of wild populations. Our results show that the wing spot patterns were unique among the tested individuals and that their semi-automated comparison was a reliable method for individual identification. This study thus constitutes a promising proof-of-concept for photographic mark-recapture in the spotted lanternfly.

The errors due to user input and the variation in the angle of the image were largely negligible compared to inter-individual variation. The dissimilarity score for different individuals was high, and lower across localities than within localities (likely because of higher sample sizes), suggesting that inter-individual variability is high enough within localities for the software, potentially making this methodology applicable at both small and large spatial scales. Future work is needed to confirm these patterns across a wider array of localities.

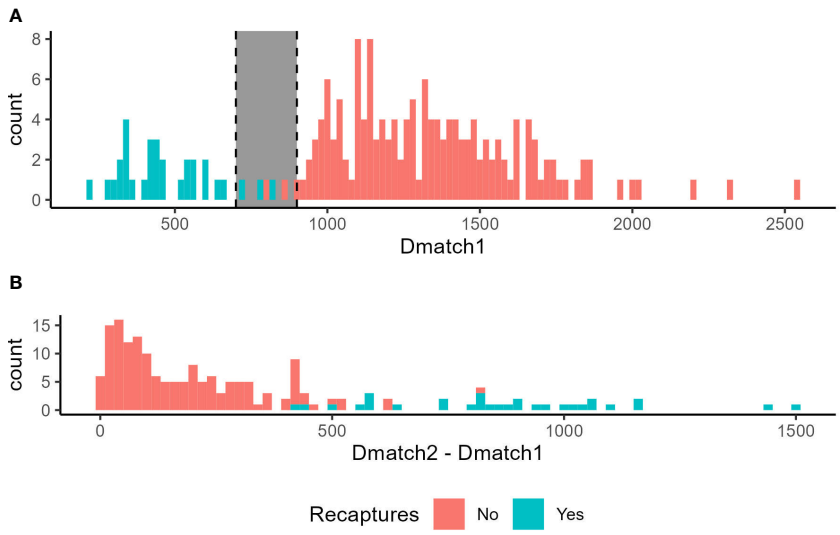

FIGURE 3
Distribution of scores in the field validation depending on whether the individual is a recapture (blue) or not (red). Note that non-recaptures should have no matching images, so Dmatch1 represents a match with a different individual in that case. **(A)** Scores of the best match (Dmatch1) obtained by individuals. The gray zone corresponds to intermediate scores where Dmatch1 potentially overlaps between recaptures and non-recaptures. **(B)** Difference between the best match (Dmatch1) and the second-best match (Dmatch2).

In the case of recaptures in the field, the best match was always the correct individual from our reference database of 84 individuals. This high performance of the method for identifying recaptures is not unique to SLF (8) but seems to exceed what has been found in other species, where correct individuals could be found in the first few best matches of reference databases of 132-358 individuals (10, 16, 17). Moreover, in our dataset, scores of the best match were much lower for recaptures than for non-recaptures, as well as for duplicated images compared to non-duplicated images, suggesting that further automatization of the image analysis is possible to reduce the need for user visual validation. Indeed, the bimodal distribution of the best-match score across individuals suggests an almost clear-cut transition between scores of recaptures and scores of non-recaptures, and between scores of duplicated and non-duplicated images. The original publication for the I3S software stated that a score of less than 400 indicated a high probability of a possible match (20). Based on our analysis, we would expand this to a higher score, and any individual with a best-match score lower than 700 could be considered as a recapture, while an image with a best-match score larger than 900 is likely an unknown individual (non-recapture). Best-match scores in between these values were in a gray zone where recaptures and non-recaptures overlapped. Comparable thresholds were found for duplicated and non-duplicated images (700-1000). Only images with best-match scores in this gray zone would need to be manually checked, potentially drastically reducing the user input time in the analysis to a fraction of the individuals, 3% of the individuals in our field study, compared to the routine use of the program that involves visually checking at least the first match of 100% of the individuals tested.

The limits of this gray zone would have to be calculated and reported on larger sample sizes, in different environmental contexts and times of the day to determine their empirical stability. This method could be less effective for high sample sizes because the more individuals are compared, the more likely it is to find similar individuals. However, our sensitivity analysis suggested that interindividual scores stabilize as sample sizes increase, which supports the conclusions established here on smaller sample sizes (Supplementary Material 2). Overall, absolute threshold values for the gray zone are not necessary, but this concept makes processing the data much easier, and researchers applying this method in the future should look for thresholds, which could be study-specific and determined using appropriate pilot studies.

By using the photo identification of individuals, new opportunities abound for research on the SLF, including the study of ecological or biological questions that cannot be answered without individual identification, like the estimation of movements over long periods of time. For example, knowledge on SLF flight or movement capabilities in the field so far have been limited to observations of groups of individuals (23–25). Studying individual movements can provide better estimates on SLF habitat use and dispersal capabilities. Although the field validation component of our study was not intended to produce biological information, we discovered moderately high recapture rates, though no individuals moved between trees despite trees being only 12 m from each other. This may suggest that SLF had limited movements during that day. Expanding this study to compare different host trees and periods of time would bring significant insight into key elements of their ecology which could be used to parameterize first principles mathematical models.

We also demonstrated that the analysis time can be greatly reduced by analyzing scores, leaving only a fraction of photos with intermediate scores for visual validation. We suggest that future projects conduct a pilot study on a subset of individuals to confirm or adapt the thresholds proposed in this article to fit their study case. User input is still needed for digitizing the sets of images, but as current artificial intelligence programs already offer the possibility for automated species identification (26), we believe that this obstacle will soon be overcome, and allow to fully automate digitization, making the photographic mark-recapture process almost immediate. Finally, similar approaches could be adapted and applied to other species that have visual patterns with high interindividual variability.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

NB conceptualized the idea, collected the data and digitized the images, performed the analysis and wrote the original draft of the paper. JB helped with digitization of the images and critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1112551/full#supplementary-material

SUPPLEMENTARY DATA SHEET 1
Supplementary figures S1, S2 and S3

SUPPLEMENTARY DATA SHEET 2
Sensitivity analysis

SUPPLEMENTARY DATA SHEET 3
Lab validation data

SUPPLEMENTARY DATA SHEET 4
Lab validation data (within populations only)

SUPPLEMENTARY DATA SHEET 5
Field validation data

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Survival and development of *Lycorma delicatula* (Hemiptera: Fulgoridae) on common secondary host plants differ by life stage under controlled conditions

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Host range assessment for emerging invasive insects is a vital step toward fully defining the issues the insect may pose. Spotted lanternfly (SLF) is an invasive species that is rapidly expanding its presence in the United States. The primary hosts facilitating this spread are tree of heaven, a plant from SLF's native range, and the economically important winegrape. Black walnut is also implicated as an important and common host plant. This study investigated the survival and development of SLF on diets that included a variety of crop host plants in the presence or absence of tree of heaven. The following plant species, 'Honeycrisp' apple, 'Reliance' peach, silver maple, and tree of heaven were paired with winegrape or black walnut throughout the study. SLF had strong development and high survival on a diet of winegrape alone, and winegrape or black walnut paired with tree of heaven. Survival parameters were reduced with all other plant pairings. In particular, SLF in the winegrape and peach diet treatment did not develop past the third nymphal instar. A second experiment evaluated the survival of early and late instar nymphs and adult SLF life stages on three specialty crops – 'Cascade' hops, muscadine grapes, and kiwifruit over a two-week period. Nymphs survived longer than adults, with survival of first and second instar nymphs on hops not differing from the control tree of heaven treatment. The adult stage survived best on kiwi and muscadine grape. Our results show tree of heaven and winegrape were the only single plant diets evaluated that are sufficient for complete SLF development, while other host plants may require additional host or hosts of sufficient nutritional quality for SLF survival.

KEYWORDS

spotted lanternfly, *Vitis vinfera* L., *Juglans nigra*, greenhouse, specialty crop

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; EPG, electrical penetration graph; HR, hazard ratio; RH, relative humidity; SLF, spotted lanternfly (*Lycorma delicatula*).

Introduction

Once a novel invasive species becomes established in a new area, factors affecting spread into the surrounding landscape become especially salient. For polyphagous insects, available host plants can be abundant in many ecosystems, while host preference and usage patterns within these ecosystems can appear variable and abstruse. Spotted lanternfly (SLF), *Lycorma delicatula* (White) (Heteroptera: Fulgoridae), is a polyphagous phloem-feeding species established in the USA starting in Berks County, PA (1) with confirmed populations now in 14 states (2). Spotted lanternfly damage – which involves effects from direct feeding, such as loss of vigor, stem dieback, and indirect damage from honeydew excretion causing decreased photosynthetic ability from sooty mold growth (3) – is of great concern for specialty crop growers. At highest risk for economic damage are winegrapes (*Vitis vinifera* L. (Vitales: Vitaceae)), used in the production of wine, raisins, and grapeseed oil (4). Reports from China, Korea, and Pennsylvania reveal SLF damage to additional fruit, vegetable, and tree nut crops (5–7). As risk to susceptible crops from invasive species can be regionally specific due to local biotic and abiotic conditions, it is important to understand host use patterns in each invaded region.

While SLF can fully develop and reproduce on tree of heaven, *Ailanthus altissima* (Mill.) Swingle (Sapindales: Simaroubaceae) (8), SLF fitness feeding on other plant species is more complex. Without tree of heaven, SLF can develop to adulthood on select diets comprised of a single host plant species, though overall fitness is greater when multiple plant species are available (9–11) including tree of heaven (11, 12). Molecular gut content analyses show SLF feed on a variety of species throughout their development (13–15). Together, these results suggest SLF visit multiple hosts to optimize their development and gather necessary nutrients that may be absent from their preferred host, tree of heaven, or that they require multiple plant species to attain adequate nutrition for survival and development (9). In the field, SLF are observed on dozens of plant species throughout their development (16–18). Spotted lanternfly are thought to have their broadest host range during the 1st instar stage, with this range becoming increasingly narrower as it molts into later life stages. Spotted lanternfly nymphs and adults are found on vine and tree species common throughout Eastern US forests (7, 17, 19).

Plant species with vine growth habits usually contain a mixture of woody and herbaceous tissue. As such, all SLF life stages can exploit the various plant parts to access phloem. All SLF stages have been observed feeding on winegrape and poison ivy (*Toxicodendron radicans* (L.) Kuntze (Sapindales: Anacardiaceae)) while other vine species are observed as a feeding source for 1st and 2nd instars only despite yearlong availability (7). Additional vine species grown as specialty crops, such as cucumber, muscadine grape, hop, and kiwifruit, are reported as SLF hosts, though SLF's utilization of these species in the United States is unclear (7, 11, 20, 21). However, SLF are considered pests of kiwifruit (*Actinidia chinensis* Planch. (Ericales: Actinidiaceae), *Act. deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson (Ericales: Actinidiaceae)) in China and Korea (22–25).

Risk of SLF inflicting economic damage in US orchard crops is of concern (26), though their pest potential for most crops including orchards remains understudied (27). Spotted lanternfly are a reported pest of apple in China (Xiao 1992, Zhang 1993), however Lee et al. (28) reported SLF were not able to enter the phloem phase of neither apple nor peach plant tissue *via* EPG and showed low survival of nymphs and adults on these hosts. Nevertheless, high populations of SLF adults have been observed in and around US orchards (29, 30). Further research to clarify their host status is warranted, especially in the context of mixed host diets.

The goal of this study was to investigate the potential for SLF to utilize and develop on single and mixed diets of cultivated specialty crop and wild host plants. We quantified SLF survival on cultivated woody vine hosts over a two-week period for early and late nymphal instars and adults. We also used winegrape and black walnut as the primary hosts to assess survivorship and development as they are commonly encountered species in SLF's current geographic range. Results from this study will add to our understanding on SLF host use and nutritional requirements of each life stage.

Materials and methods

Two-week survival on specialty crops

Three crop plants were evaluated as single diets for SLF: 'Cascade' hops, *Humulus lupulus* L. (Rosales: Cannabaceae) (Great Lakes Hops); muscadine winegrape, *Vitis rotundifolia* Michx. var. Carlos (Vitales: Vitaceae) (Willis Orchard, Catersville, GA); and kiwifruit, *Actinidia* sp. (Ericales: Actinidiaceae) (grown at Appalachian Fruit Research Station (AFRS)). For kiwifruit, *Act. deliciosa* 'Hayward' was grafted onto seedlings of *Act. chinensis* 'Tango' (PP32,617) and pollinated by *Act. chinensis* 'Hombre'. Tree of heaven was used as a control. Tree of heaven plants were grown from field-collected samaras, which had been stratified in a refrigerator at 5 – 7°C for two months. Prior to planting, wings were removed and the remaining seeds from the samaras were soaked in water for 18 h. Seeds were then planted in a tray and placed in an environmental chamber (25°C, 16:8 L:D) to germinate. Once seedlings leafed out, they were transplanted to 0.6 L pots and moved to the greenhouse for maintenance. Healthy trees were then transplanted into 2.7 or 6.5 L pots. All plants for experimental use were maintained in a greenhouse at the AFRS, USDA-ARS, in Kearneysville, WV at a height of ~50 cm (8, 11). At the start of each trial, plants were transported to a quarantine greenhouse at Fort Detrick, MD and placed in a cage (W32.5 x D32.5 x H77.0 cm, 680 μm aperture mesh, BugDorm-4S3074 Insect Rearing Cage, MegaView Science Co., Taiwan). Each cage housed a single host plant in a 6.5 L pot with a water saucer underneath. An 18 L mesh bag covered the saucer and pot and was secured around the base of the plant with a zip-tie to prevent SLF from falling into the water pool. Fifty early instar nymphs (1st and 2nd instars), twenty-five late instar nymphs (3rd and 4th instars), or ten pre-reproductive or reproductively mature adult SLF were introduced into each cage using individuals collected directly from Winchester, VA (APHIS permits P562P-18-03369, P526P-21-04099). Early instar trials were conducted in June and July 2020 (10 – 32°C, average temperature: 20.3°C, 41 – 95% RH, average RH: 59.4%) and May 2021 (17 – 30°C, average temperature: 21.3°C, 22 – 92% RH, average RH: 55.2%); late instar trials were conducted July and August 2020 (10 – 32°C, average temperature: 18.7°C, 40 – 78% RH, average RH: 57.2%) and

July 2021 (17 – 33°C, average temperature: 22.2°C, 44 – 98% RH, average RH: 67.5%); pre-reproductive adult trials were conducted September 2020 (6 – 34°C, average temperature: 16.3°C, 35 – 90% RH, average RH: 62.0%), August 2021 (17 – 33°C, average temperature: 21.8°C, 45 – 100% RH, average RH: 73.1%) and September 2021 (16 – 32°C, average temperature: 20.4°C, 47 – 100% RH, average RH: 75.2%); reproductively mature adult trials were conducted beginning in mid-October 2021 (16 – 35°C, average temperature: 19.4°C, 26 – 98% RH, average RH: 61.8%).

All trials were conducted with natural daylengths. Insects were observed for 14 days, during this time the number of dead SLF was recorded and removed every 2 – 4 days. After day 14, the number of surviving SLF was confirmed. Six cages of early instars (total N = 300), six cages of late instars (N = 150), five cages of pre-reproductive adults (N = 50), and three cages of reproductively mature adults (N = 30) were evaluated for each host. Differences in survival distribution within each life stage were assessed using Kaplan-Meier with log-rank (Mantel-Cox) tests for pairwise comparisons ($\alpha = 0.05$) using the Bonferroni adjustment for multiple comparisons (31) and Cox proportional hazard ratios (HR) to assess instantaneous risk of death. All tests were conducted in R Statistical Software (v2.4.2; 32) using the base, *survival* (33) and *survminer* (34) packages.

Survival and development on winegrape and black walnut supported diets

The following plants were maintained at 30-50 cm in height in 2.7 L pots for use in single and mixed plant species diet trials: cultivated winegrape, *Vitis vinifera* L. var. Riesling (Amberg Winegrapes LLC, Clifton Springs, NY); black walnut, *Juglans nigra* L. (Fagales: Juglandaceae) (Cold Stream Farm, Free Soil, MI); apple, *Malus domestica* Borkhausen (Rosales: Rosaceae) var. Premium Honeycrisp (Adams County Nursery, Aspers, PA); peach, *Prunus persica* (L.) Batsch (Rosales: Rosaceae), var. Reliance (Dave Wilson Nursery, Hickman, CA); and silver maple, *Acer saccharinum* L. (Sapindales: Sapindaceae) (Cold Stream Farm, Free Soil, MI). Tree of heaven was grown as previously described (8, 11) and maintained in 2.7 L pots at 30 cm height.

Spotted lanternfly egg masses were collected by removal from trees in the field (Winchester, VA) in the winter (Jan/Feb). Egg masses were held in ventilated storage at ambient conditions for 4-8 weeks, brought to the quarantine facility and held in a growth chamber at 10° C until brought into the greenhouse for hatching. Thirty neonate SLF 1st instar nymphs (<48 h old) were introduced into a cage (W32.5 x D32.5 x H77.0 cm, 680 µm aperture mesh, BugDorm-4S3074 Insect Rearing Cage, MegaView Science Co., Taiwan) containing two potted plants. Experimental diets evaluated were: 1) winegrape/winegrape; 2) winegrape/apple; 3) winegrape/peach; 4) winegrape/silver maple; 5) winegrape/tree of heaven; 6) winegrape/black walnut; 7) black walnut/black walnut; 8) black walnut/apple; 9) black walnut/peach; 10) black walnut/silver maple; and 11) black walnut/tree of heaven. Each treatment was replicated three times. All cages were started as neonates emerged, between 1st and 29th April 2021, and held in the greenhouse under natural daylength. Plants were replaced as necessary based on a subjective evaluation of plant health, including the amount of honeydew, presence of yellow and dropped leaves or visible microbial growth. We replaced plant on average every three weeks for 1st – 3rd instars and every 2 weeks when 4th instars and adults were present. Survivorship and development were recorded three times per week until all individuals in a cage died. Development was assessed by visual counts of live individuals and collection of dead insects and nymphal molts. A combination of molts and body size was used to determine the life stage of each insect as they progressed through nymphal instar stages.

When found dead, adult females were collected into 95% ethanol and stored at -20°C. For dissections, legs and wings were removed from specimens, and specimens were imaged and dissected using an Amscope SM-3T stereo microscope and camera. The lateral and ventral aspects of all specimens' abdomens were imaged to capture the yellow area showing in these regions, which increases as SLF females reproductively mature (30). Imaged specimens were stored in 95% ethanol at room temperature until dissection. Because specimens were desiccated and showed some degradation from exposure prior to initial collection, they were then soaked in a mixture of 200 µl glycerol with 1000 µl 1× Dulbecco's phosphate-buffered saline solution at room temperature for 24 h prior to dissection. Ovary development was rated using a modified scale based on Nixon et al. (11), such that females were rated as: (1) previtellogenic-I (0-1 immature oocytes/ovarioles detectable; ovaries undeveloped, bright white in color); (2) previtellogenic-II (>1 immature oocytes/ovarioles detectable; bright white in color); (3) vitellogenic-III (ovaries more developed, multiple oocytes on 'string'; beige to yellowish in color); (4) vitellogenic-IV (ovaries contain many eggs; eggs not fully yellowed and not full size, without hardened/thicken surface); (5) postvitellogenic (eggs filled with yellow yolk; surface hardened. Specimens were also examined for any evidence of having been mated (i.e., for whole or pieces of a spermatophore). Bursa copulatrix development was scored as follows: (I) undeveloped, thin exterior wall; (II) somewhat developed, exterior wall somewhat thickened; (III) features of (II) plus a honeycomb structure visible on wall; (IV) features of (III) plus crystals apparent inside. Bursa copulatrix sclerotization was scored as follows: (I) No sclerotization; (II) minor sclerotization, tan or light brown; (III) highly sclerotized, dark brown; (IV) highly sclerotized with black marks present. Survivorship was analyzed using a Kaplan–Meier analysis with pairwise comparisons ($\alpha = 0.05$) using the Bonferroni adjustment for multiple comparisons. Development times of each life stage were compared using ANOVA with Tukey's HSD (honestly significant difference) for mean separation. Analysis was conducted using the base, *survival* (33) and *survminer* (34) R packages (32).

Results

Two-week survival on specialty crops

Overall, nymphal SLF had a lower risk of death when feeding on any single host plant compared with adult SLF, with reproductively mature adults having the greatest risk of death (Figure 1; Early nymph: HR = 1, late nymph: HR (CI) = 0.95 (0.86-1.05) p = 0.331; pre-reproductive adults: HR (CI) = 1.18 (1.01-1.37), p = 0.036; reproductively mature adults HR (CI) = 2.36 (1.9-2.91), p < 0.001). By host plant, tree of heaven as a feeding host held the lowest chance for death for all SLF life stages, followed by, in order, hops, muscadine

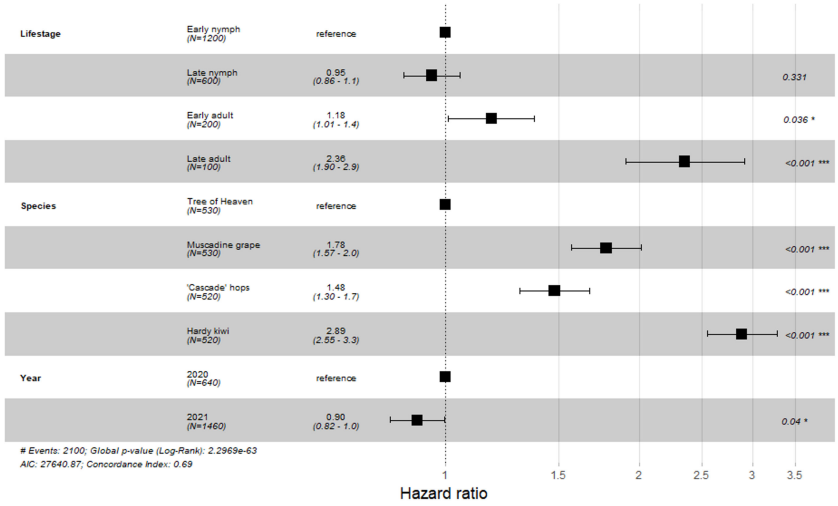

FIGURE 1
Hazard ratio values for 2-week survival study Each variable within a factor are compared to a reference variable (assigned a value of 1.0). Hazard ratios below 1 indicate a decreased risk of death, while values greater than 1 suggest an increased risk of death as compared to the selected reference. Horizontal bars indicate the 95% confidence interval. Numbers on the right side of the figure are the p-values for each sub-variable, with asterisks indicating the degree of significance: * < 0.05, ** < 0.01, *** < 0.001.

grape, and kiwi (Figure 1; tree of heaven: HR = 1; 'Cascade' hops: HR (CI) = 1.48 (1.3-1.7), p < 0.001; muscadine grape: 1.78 (1.57-2.0, p < 0.001; kiwi: HR (CI) = 2.89 (2.55-3.3), p < 0.001).

Survival probability of early nymphal (1st and 2nd) instars over the two-week period was highest for tree of heaven and hops (>65%), followed by muscadine grape (<40%) and kiwi (<10%) (Figure 2A; χ^2 = 343, df=3, p < 0.001). Survivorship for later stage nymphs was, again, highest when feeding on tree of heaven, with survivorship on muscadines significantly higher than on either kiwi or hops (Figure 2B; χ^2 = 86.3, df=3, p < 0.001). Later stage nymphs had greater than 75% survivorship on all hosts until day 10, followed by a sharp decline in survival in the last four days. Pre-reproductive adults only survived well on tree of heaven (>90% at 14 d), with steady decline in survival probability when feeding on the other three host

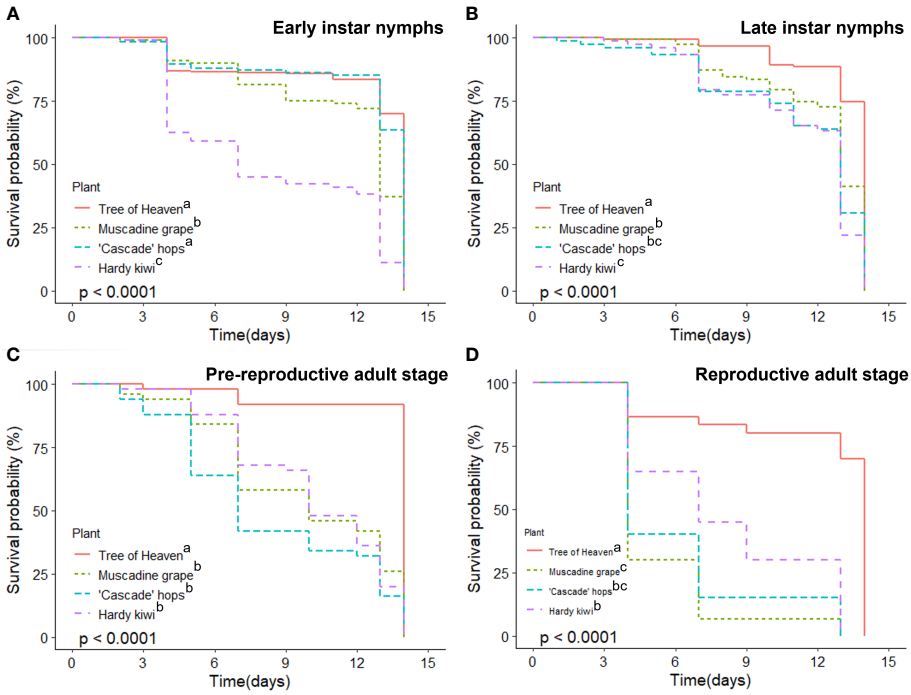

FIGURE 2
Survival of *L. delicatula* at different development stages for 2 weeks on 4 host species. **(A)** Early nymphs (1st and 2nd instars); **(B)** late stage nymphs (3rd and 4th instars); **(C)** pre-reproductive adults (early September); and **(D)** reproductively mature adults (mid-October). Within each panel legend, plants sharing the same letter after their name are not significantly different from one another at α=0.05.

species, ending with less than 25% survivorship on day 14 (Figure 2C; χ^2 = 66.5, df=3, p < 0.001). Reproductively mature adult SLF experienced substantial early die-off beginning on Day 4; adults feeding on muscadine grape and hops had <40% survival probability after 4 days. While tree of heaven sustained SLF survivorship well, SLF feeding on the other host plants had significantly lower probability of survival (Figure 2D; χ^2 = 59.8, df=3, p < 0.001).

Survival and development on winegrape and black walnut supported diets

All host combinations supported SLF development through to adulthood except winegrape/peach diet where no SLF completed development to the 4th instar stage. Among the other treatments, the four diets of winegrape/winegrape, winegrape/tree of heaven, winegrape/walnut, and walnut/tree of heaven had the highest overall survival probability, which includes time spent both in the nymphal and adult stage (Figure 3; χ^2 = 279, df=10, p < 0.001). These diet treatments had the highest percentage of SLF nymphs surviving to adulthood and lived significantly longer as adults (Table 1; $F_{9, 180}$ = 6.74, p < 0.001, ANOVA). Adult SLF fed diets of winegrape only and winegrape/tree of heaven survived over 6 weeks (46.5 ± 5.5 d and 45.2 ± 5.9 d, respectively). While total nymphal development time was a significant factor, there were no pairwise differences among the treatments Table 1; ($F_{9, 180}$ = 2.54, p = 0.009, ANOVA Table 1). Host diet treatments with the highest SLF survival also had lower total average development times, 88.4 d average versus 92.7 d global average. Host diets with the lowest overall survival, percentage survival to adult, and survival as adults were black walnut-based diets: walnut/peach, walnut/apple, walnut/maple, and walnut/walnut. The proportional hazard analysis was in accordance with the log-rank test in terms of ranking the treatment combinations, so is not shown.

Within each life stage, there was no relationship between instar period and total development time (Table 2). In general, SLF that spent a shorter time in the first instar stage spent a longer amount of time in the second instar stage. The same was true for those SLF with longer development periods as first instars had shorter second instar periods. The length of development in the third instar stage was not different among the diet treatments, while the final pre-imago stage was the most variable period, ranging from 22.9-34.9 d, average 26.33 ± 0.35.

A total of 76 adult female SLF were dissected to assess reproductive development, 18 of which were too degraded to score for some, but not all of the parameters. Of these, only one female (from the winegrape/tree of heaven diet treatment) was mated. The most reproductively developed females occurred in the diets most favorable for nymphal development: winegrape/tree of heaven (Previtellogenic-I: 5 females; Previtellogenic-II: 9; n = 18), walnut/tree of heaven (Previtellogenic-I: 8 females; Previtellogenic-II: 3, Vitellogenic-III: 4; n = 21), winegrape/walnut (Previtellogenic-I: 8 females; Previtellogenic-II: 11; Vitellogenic-III: 1; n = 20) (Table 3). No females had fully mature ovaries or oocytes present. Twelve females (18.5%) received a score of (III) for bursa copulatrix development, while seven scored (II), and the remainder scored (I) (Table 3). Five males emerged from the walnut only treatment, but no females were available for dissection.

Discussion

These results confirm and expand the literature on the relationship between SLF fitness and feeding on common specialty crop and wild tree species of the eastern United States. Evaluating SLF survival on three vine specialty crops over two weeks revealed kiwi as an adequate host crop for late instar and early season, pre-reproductive adult survival, while hop plants were as good as tree

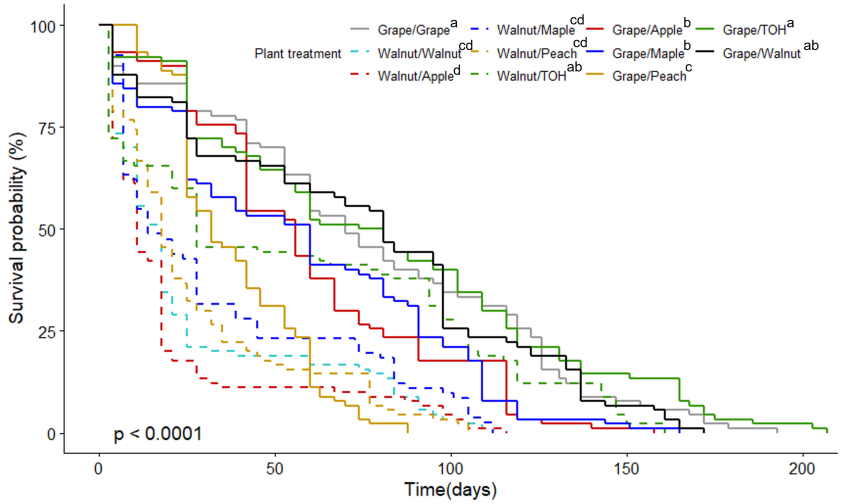

FIGURE 3
Survival curve of SLF on all treatment combinations using grape and walnut-paired diets. χ^2 = 279, df = 10, p < 0.0001. Grape diets are depicted with a solid line and walnut diets are dashed. Color of the lines corresponds to the second plant host in the treatment combination. The grape/walnut treatment is a solid black line. Treatments followed by the same letter are not significantly different from one another at α=0.05. TOH = tree of heaven.

TABLE 1 Development and survival parameters for SLF on single or mixed diet treatments. .

Treatment	Mean nymphal development time (d ± SEM)	Survival to adult (%)	Adult survival (d ± SEM)1
Grape/Grape	90.7 ± 1.6	24	45.2 ± 5.9^{ab}
Grape/Apple	96.1 ± 2.2	14	22.9 ± 3.6^{bc}
Grape/Maple	92.6 ± 2.9	21	20.6 ± 2.4^{c}
Grape/Peach	n/a	0	n/a
Grape/Tree of heaven	88.3 ± 1.0	38	46.5 ± 5.5^{a}
Grape/Walnut	87.7 ± 1.9	41	32.1 ± 3.3^{abc}
Walnut/Walnut	95.4 ± 4.3	5.6	8.6 ± 3.1^{c}
Walnut/Apple	93.8 ± 1.6	6.7	10.0 ± 3.1^{c}
Walnut/Maple	101.7 ± 2.0	3.7	4.7 ± 2.33^{c}
Walnut/Peach	93.2 ± 4.6	5.6	5.0 ± 1.9^{c}
Walnut/Tree of heaven	87.0 ± 1.3	36.7	31.6 ± 4.2^{abc}

Single diets included two plants of the same species. Nymphal development time calculated as time required to go from first hatch until adult emergence reported in days (d) ± standard error of the mean (SEM). All SLF in the grape/peach treatment died before completing nymphal development. Development time was significant, but no pairwise differences were observed ($F_{9,180} = 2.535$, $p = 0.009$; ANOVA, Tukey HSD). Survival times in the adult survival column followed by the same letter are not significantly different from one another at α=0.05. n/a, not applicable; all SLF in this treatment died before completing all four nymphal stages.

of heaven for early instar nymph survival. In the development study, SLF had the highest survivorship and fastest development rates on diets of winegrape/winegrape, winegrape/walnut, or either of those species paired with tree of heaven. Spotted lanternfly fed a diet of peach, maple and apple-paired treatments had low rates of survival to adulthood, even when paired with preferred host plant, winegrape. Black walnut diets generally did not support significant development of SLF alone or in combination with a second plant species, unless paired with winegrape or tree of heaven, highlighting the intricacy of SLF nutritional needs. Female reproductive development was positively associated with development and survival parameters. Still, the specific nutritional requirements for SLF growth, development, and reproductive maturity remain elusive.

Total nymphal development length was numerically shorter for higher quality pairings but showed no uniform pattern within each instar stage. In other Hemipteran pest species, host quality plays a significant role in the length of nymphal instar periods and survivorship, in that high quality hosts decrease instar period length (e.g., 35–37), including the invasive *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) in a similar single and mixed diet study revealed hosts such as peach provided high survivorship and short developmental times (38). While the total development time of

TABLE 2 SLF development time within each nymphal life stage for single and mixed diet treatments.

Treatment	Mean time in life stage (d ± SEM)			
	1st Instar	2nd Instar	3rd Instar	4th Instar
Grape/Grape	23.4 ± 0.5^{cd}	19.2 ± 0.8^{c}	19.7 ± 0.8	28.8 ± 1.9^{ab}
Grape/Apple	24.3 ± 0.3^{cd}	18.3 ± 0.9^{bc}	23.4 ± 1.5	34.9 ± 2.0^{b}
Grape/Maple	26.0 ± 0.7^{d}	20.9 ± 0.6^{c}	21.0 ± 1.3	24.6 ± 2.0^{a}
Grape/Peach	25.4 ± 0.5^{cd}	13.4 ± 1.5^{a}	24.0 ± 1.7	N/A
Grape/Tree of heaven	25.5 ± 0.0^{cd}	14.9 ± 0.8^{ab}	21.1 ± 0.6	27.3 ± 1.0^{a}
Grape/Walnut	23.8 ± 0.4^{bc}	19.1 ± 0.7^{c}	19.3 ± 0.9	24.2 ± 1.0^{a}
Walnut/Walnut	19.0 ± 1.4^{a}	20.7 ± 1.6^{c}	24.2 ± 2.1	28.4 ± 2.6^{ab}
Walnut/Apple	21.1 ± 1.5^{abc}	18.3 ± 2.4^{abc}	24.8 ± 1.2	26.0 ± 1.5^{ab}
Walnut/Maple	19.8 ± 1.9^{ab}	22.5 ± 2.4^{c}	21.8 ± 1.2	30.7 ± 1.3^{ab}
Walnut/Peach	22.4 ± 1.5^{abcd}	16.9 ± 1.7^{abc}	21.5 ± 4.6	26.2 ± 1.8^{ab}
Walnut/Tree of heaven	23.1 ± 0.7^{bcd}	19.0 ± 0.7^{c}	22.0 ± 0.8	22.9 ± 1.1^{a}

Single diets included two plants of the same species. Times within the same column sharing the same letter are not significantly different from one another (1^{st} instar: $F_{10,522} = 7.383$, $p < 0.001$; 2^{nd} instar: $F_{10,402} = 6.094$, $p < 0.001$; 3^{rd} instar: $F_{10,298} = 2.143$, $p = 0.021$; 4^{th} instar: $F_{9,180} = 4.281$, $p < 0.001$; ANOVA, Tukey HSD). No pairwise differences for third instar treatments were significant. N/A, not applicable; no SLF in this treatment survived to the fourth nymphal instar stage.

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TABLE 3 Reproductive development parameters of adult female SLF.

Treatment	N	No. Females	Mean Lateral Yellow Area (mm^2)	Ovary Development				Bursa Copulatrix Development				Bursa Copulatrix Sclerotization		
				n/a	I	II	III	n/a	I	II	III	n/a	I	II
Grape/Grape	19	7	0.138	1	–	6	–	1	3	2	1	1	4	2
Grape/Apple	13	2	0.378	–	2	–	–	–	2	–	–	–	2	–
Grape/Maple	18	5	0.036	–	4	1	–	–	1	–	–	–	3	–
Grape/Peach	0	0	–	–	–	–	–	–	–	–	–	–	–	–
Grape/Tree of heaven	35	18	0.305	4	5	9	–	6	6	3	3	7	6	5
Grape/Walnut	41	20	0.437	–	8	11	1	2	13	–	5	2	13	5
Walnut/Walnut	5	0	–	–	–	–	–	–	–	–	–	–	–	–
Walnut/Apple	6	3	0.278	1	1	1	–	1	2	–	–	1	2	–
Walnut/Maple	3	1	0.063	–	1	–	–	–	1	–	–	–	1	–
Walnut/Peach	4	2	0.062	–	2	–	–	–	2	–	–	–	2	–
Walnut/Tree of heaven	31	18	0.207	5	8	3	2	6	9	1	2	7	7	4

'N' represents the total number of SLF adults emerging from that treatment, 'No. Females' is the number that were female. Only females were dissected. The lateral yellow area describes an area on the side of the abdomen that becomes larger over time and with reproductive maturity. Definitions for the scoring matrix can be found in the Methods section. ' - ' = no female SLF were available to measure, or none were designated in that specific sub-category. n/a, not applicable, the female SLF was not able to be measured for that parameter.

immature SLF was not significantly different among the diet treatments, the number of individuals that survived to adulthood and their subsequent lifespan emphasizes the role of host quality on SLF fitness. Here, SLF longevity and hardiness were compromised when not given access to either tree of heaven or winegrape. The search for hosts providing adequate nutrition may be a primary reason SLF are observed dispersing within and across the landscape (29, 39, 40).

While nutrition is likely a key factor, the outcomes observed here may also be attributed to specific insect and plant physiological features. The kiwi plant used in this study (*Act. deliciosa* 'Hayward' grafted onto *Act. chinensis* 'Tango') has vine-like growth with pubescent stems and tomentose leaves. First and second SLF instars may not possess a proboscis with the length sufficient to get through the plants' physical defenses (41). Indeed, later instar nymphs and adults survived better on kiwi, potentially in part due to larger mouthparts. The leaf and stem characteristics of the common kiwifruit, *Act. chinensis*, are glabrous so early instars may be able to exploit vines of the more widely grown kiwi species (42).

Plant size may have also affected survival of SLF adults. Spotted lanternfly spend much of their adult stage feeding and tend to be found feeding on the trunks of trees, unlike nymphs who access phloem from smaller diameter tree limbs and herbaceous plant material (39). These observations suggest that larger, woody plants may yield a greater resource-to-energy expense ratio than herbaceous plants, an advantage only later SLF life stages can utilize. As such, the 30 cm tall, younger plant material used in this greenhouse study may not have contained sufficient phloem volume for the prodigious feeding behavior of adults and affected their survival, though we tried to compensate for this possibility with frequent plant replacements. Although previous greenhouse studies have shown that SLF can reproduce on these smaller trees (8), here, only one female was mated and none had fully developed ovaries despite some adults living in excess of 6 weeks. This may be due in part to the conditions under which these SLF were held. In studies designed to develop a rearing protocol for this invasive species, females reproduced more reliably when provided with an oviposition substrate such as a tree of heaven log and held in a growth chamber at 12L:12D and (24°C:13°C) compared with those held in a greenhouse with natural light and temperatures between 21-25°C (similar to conditions in our experiment) or in a growth chamber at 16L:8D and ~24°C (8). Our experimental design did not ensure equal adult sex ratios, so further research to assess the impact of these diets on SLF reproductive development is needed to clarify questions about mating and reproductive maturity.

Nevertheless, we can contextualize the results of this study to others in this field. Like others, we continue to see low developmental success and survivorship of SLF on apple and peach plants, suggesting the large presence of SLF observed in orchards may be less of a concern than initially thought. Still, researchers in China have reported damage to peach trees by SLF (21, 43), and others recently found that feeding by SLF on young, non-bearing peach trees resulted in increased frost injury the following spring (LJN, *personal observation*). However, as SLF does not survive well on peach based on results of this study and in other studies, these impacts may be rare (11, 28).

Winegrape continues to be a key host for all life stages of SLF. The present study used the common winegrape, *V. vinifera* 'Riesling'. A similar study assessing the effect of mixed diets on SLF development used a different species of grape, the scuppernong, *V. rotundifolia* (11). Fruits of this species, also called muscadine, are eaten fresh or made into a type of wine. Spotted lanternfly developing on *V. rotundifolia* only completed development to the third instar before dying out (11), similar to the winegrape/peach diet in the current study. SLF reared on *V. vinifera* however could fully develop to adulthood, with some adults living more than 6 weeks. While comprehensive research on the performance of SLF feeding on different *Vitis* spp. has not taken place, it would be warranted due

to the documented damage and preference observed for various grape species.

Results from this study add to the building literature that SLF can survive and develop without access to what is often considered their primary or preferred host, tree of heaven. Interestingly, while *V. vinifera* continues to be a good host by itself, combining it with certain species, specifically peach, increased immature mortality and halted development at the third instar stage. The vine species tested could sustain SLF for about a week with low mortality, though survival likelihood declines rapidly in subsequent days. While tree of heaven is a major predictor of suitable habitat, SLF can likely be found persisting in areas without tree of heaven, but with access to winegrape and to a smaller extent black walnut. Some vineyards have begun removing tree of heaven from wooded areas close to their vines to reduce SLF habitat, a strategy that might not be effective if SLF can persist to a high degree on the grape host or if they can develop successfully on other yet unknown wild hosts, providing source populations for dispersal into vulnerable crops such as winegrape.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

All authors conceived, designed, and conducted the research. JE, SJ, LN, and JU conducted the experiments. JE analyzed the data, conducted statistical analyses, and wrote the initial manuscript. TL and JU secured funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Proactive classical biological control of *Lycorma delicatula* (Hemiptera: Fulgoridae) in California (U.S.): Host range testing of *Anastatus orientalis* (Hymenoptera: Eupelmidae)

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Lycorma delicatula (Hemiptera: Fulgoridae), the spotted lanternfly, native to China, invaded and established in the northeast U.S. in 2014. Since this time, populations have grown and spread rapidly, and invasion bridgeheads have been detected in mid-western states (i.e., Indiana in 2021). This invasive pest presents a significant threat to Californian agriculture. Therefore, a proactive classical biological control program using *Anastatus orientalis* (Hymenoptera: Eupelmidae), a *L. delicatula* egg parasitoid native to China, was initiated in anticipation of eventual establishment of *L. delicatula* in California. In support of this proactive approach, the potential host range of *A. orientalis* was investigated. Eggs of 34 insect species either native or non-native to the southwestern U.S. were assessed for suitability for parasitism and development of *A. orientalis*. Of the native species tested, 10, 13, and one were Hemiptera, Lepidoptera, and Mantodea, respectively. Of the non-native species, eight Hemiptera and two Lepidoptera were evaluated. Host range tests conducted in a quarantine facility, exposed individually mated *A. orientalis* females (Haplotype C) to non-target and target (i.e., *L. delicatula*) eggs in sequential no-choice and static choice experiments to determine suitability for parasitization and development. Additionally, the sex ratio, fertility, and size of offspring obtained from non-target and target eggs were evaluated. Results of host range testing indicated that *A. orientalis* is likely polyphagous and can successfully parasitize and develop in host species belonging to at least two different orders (i.e., Hemiptera, Lepidoptera) and seven families (Coreidae, Erebidae, Fulgoridae, Lasiocampidae, Pentatomidae, Saturniidae and Sphingidae). Prospects for use of *A. orientalis* as a classical biological control agent of *L. delicatula* in the southwestern U.S. are discussed.

KEYWORDS

Coreidae, Lasiocampidae, Spotted lanternfly, Saturniidae, parasitoid, polyphagy, southwestern U.S

1 Introduction

Classical or introduction biological control is the intentional importation, release, and establishment of natural enemies for suppressing damaging populations of invasive non-native organisms to densities that no longer cause economic or ecological harm. This approach aims to reduce pest population densities by re-associating safe (i.e., host-specific) and efficacious natural enemies with the target pest (1). Host range and host specificity testing are important primary steps in identifying natural enemy species that may have deleterious impacts on pest populations while presenting minimal risk to non-target species (1). Host use evaluation studies are mandatory in the United States of America (U.S.) and provide safety data for review by Federal agencies (i.e., United States Department of Agriculture, Animal and Plant Health Inspection Service [USDA APHIS]), that regulate the importation and release of natural enemies for use in classical biological control programs (2). Host range and host specificity testing evaluations are time consuming, often taking years to complete (3). During this time, newly established invasive pest populations tend to increase in density and spread as management plans are slowly developed and implemented. Proactive biological control attempts to reduce or eliminate this window of opportunity for an invasive pest by evaluating candidate natural enemies for potential use in a classical biological control program in advance of the anticipated incursion and establishment of the target pest in the area of concern (3).

Spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), native to China (4), was detected for the first time in the U.S. in Berks County Pennsylvania, in September 2014 (5). By November 2022, *L. delicatula* infestations were confirmed in an additional 13 states in eastern and mid-western areas of the U.S. (Connecticut, Delaware, Indiana, Maryland, Massachusetts, Michigan, New Jersey, New York, North Carolina, Ohio, Rhode Island, Virginia, and West Virginia) (6). *Lycorma delicatula* is a phloem-feeding fulgorid that has a broad host range (7). Direct feeding damage can cause mortality to highly preferred hosts like *Ailanthus altissima* (Miller) (Sapindales: Simaroubaceae) and grapevines (*Vitis vinifera* L. [Vitales: Vitaceae]). Indirect damage results from the excretion of high quantities of honeydew that promote sooty mold growth (7, 8). *Lycorma delicatula* has been recorded infesting forest and ornamental shade tree species in natural and urban areas, respectively (7, 9), and presents a significant risk to economically important perennial agricultural crops (e.g., grapes and nuts) (10, 11). Long-distance dispersal by *L. delicatula* is almost entirely human-assisted. This occurs primarily through the accidental translocation of egg masses that are often laid indiscriminately on inert substrates (e.g., wooden pallets and railcars) that undergo subsequent transportation into uninfested areas (12, 13). This type of inadvertent relocation in the U.S. likely resulted in the establishment of invasion bridgeheads in Indiana (2021) and Michigan and North Carolina (2022) (6). Ecological niche models indicate that *L. delicatula* has a potential distribution that includes large areas of the west coast of the U.S., and other parts of the world (e.g., Europe) (14). For California, a western U.S. state, with an agricultural economy worth ~$50 billion per year (15), *L. delicatula* is viewed as a significant invasion threat that could cause major problems for producers of specialty crops, like grapes and nuts, which are multi-billion-dollar industries (15).

The egg parasitoid, *Anastatus orientalis* Yang and Choi (Hymenoptera: Eupelmidae), was found parasitizing *L. delicatula* eggs in northern China in 2011during foreign exploration for natural enemies for use in South Korea, where *L. delicatula* is also invasive (16, 17). Following the invasion and spread of *L. delicatula* on the east coast of the U.S. there was renewed interest in the potential use of *A. orientalis* as a classical biological control agent (18, 19). Molecular analyses identified six different haplotypes of *A. orientalis* collected from the native range. Importations of *A. orientalis* into the U.S. were initially comprised of Haplotype C, which was first evaluated as a classical biological control agent against *L. delicatula* (20). The majority of *Anastatus* spp. Motschulsky are primary endoparasitoids attacking eggs of Diptera, Dictyoptera, Coleoptera, Hemiptera, Lepidoptera, Orthoptera, and Mantodea (21–27). Numerous *Anastatus* species have been considered or used as classical biological control agents against various pests around the world. For example, *A. japonicus* Ashmead was released in the eastern U.S. for control of *Lymantria dispar* L. (Lepidoptera: Erebidae) (27, 28) and against *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae) in China (29, 30). *Anastatus* sp. was released in Nepal to control *Rhynchocoris humeralis* (Thunberg) (Hemiptera: Pentatomidae) (31). *Anastatus bifasciatus* (Geoffroy) was evaluated to study levels of biotic resistance of central European natural enemies against invasive *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) populations (32, 33). In the eastern U.S., *A. reduvii*, a native species, has been detected as one of the most common parasitoids emerging from eggs of invasive *H. halys* (34). Some *Anastatus* spp. are commercially-available and used for augmentative biological control of *Amblypelta nitida* Stål and *A. lutescens lutescens* Distant (Hemiptera: Coreidae) in Australia (35, 36).

Given the obvious threat posed to California agriculture by the westward migration of *L. delicatula* in the U.S., a proactive biological control program was initiated. Proactive research efforts focused on the suitability of *A. orientalis* (Haplotype C) as a potential classical biological control agent of *L. delicatula* in advance of its expected establishment in California (3). Consequently, the objective of this study was to investigate the physiological host range of *A. orientalis* on native and non-native non-target species from the southwestern U.S. (i.e., California and Arizona) to determine whether or not this natural enemy would be a suitable candidate to release for classical biological control of *L. delicatula* should it eventually establish in California. The results of these studies are presented here.

2 Materials and methods

2.1 Source and collection of test insects

Anastatus orientalis Haplotype C specimens were obtained from colonies established at USDA APHIS PPQ (Plant Protection and Quarantine) Forest Pest Methods Laboratory in Massachusetts,

U.S. Initial *A. orientalis* populations were shipped to the University of California Riverside Insectary and Quarantine Facility (UCR-IQF) as parasitized *L. delicatula* egg masses under USDA-APHIS permit P526P-22-03022 and P526P-22-04208 and California Department of Food and Agriculture (CDFA) permit 3888. Colonies of *A. orientalis* were established in UCR-IQF in October 2019 and reared continuously on cold stored *L. delicatula* egg masses.

Lycorma delicatula egg masses were field collected in winter (December to March) of 2019 to 2022. Collections were made in Pennsylvania, U.S. (Berks, Dauphin, Huntingdon, Lancaster and Lebanon Counties) predominantly from *A. altissima* (>90%). Entire egg masses attached to underlying bark were removed using chisels and shipped or hand carried to the UCR-IQF under USDA-APHIS permit P526P-19-02058 and CDFA Permit 3458. In quarantine, all field collected egg masses were stored at 5°C and 60-75% R.H. Egg masses were randomly selected and used for experiments reported here.

Selection of non-target species for host range testing was made based on phylogenetic relationships amongst species within the family Fulgoridae and their representation in the southwestern U.S. Five native fulgorid genera in California and Arizona, *Amcyle* spp., *Cyrpoptus* spp., *Poblicia* spp., *Scaralina* spp. (described incorrectly as genus *Alphina* spp Stål, Yanega et al. unpublished), and *Scolopsella* spp (37). were targeted for field collections and use in host range tests. Previous host range studies on *Anastatus* suggested that species may potentially have broad host ranges and are capable of utilizing hosts from different orders (32). Consequently, to determine if *A. orientalis* potentially exhibits oligophagy or polyphagy, additional non-target species belonging to Hemiptera (Cicadellidae, Coreidae, Liviidae, Pentatomidae, Reduviidae, and Rhopalidae), Lepidoptera (Erebidae, Lasiocampidae, Saturniidae, and Sphingidae), and Mantodea (Mantidae) were included in host range testing for *A. orientalis* (Table 1). These families were also selected to compliment simultaneous testing conducted by collaborators at the USDA APHIS PPQ Forest Pest Methods Laboratory of potential native and non-native non-target species found in the eastern U.S. All non-target insect colonies used for host range testing, unless otherwise stated, were maintained on each test species preferred host plant species held in cages (BugDorm-2120 61×61×61 cm, MegaView Science Co. Ltd., Taiwan) at the UCR-IQF at 25°C, 65%RH, L:D 16:8. Colonies were checked daily for egg masses which were harvested and used immediately or held at 10°C until used for experiments.

2.2.1 Hemiptera

2.2.1.1 Fulgoridae collections

Fulgorids collected in the Chiricahua mountains near Portal, Santa Cruz County, in southeastern Arizona included *Scaralina* spp. (comprised of three undescribed species and incorrectly placed as *Alphina* genera, Yanega et al. unpublished) and *Cyrpoptus vanduzeei* Ball (Table 1). Adult *Scaralina* spp. were hand collected as they were attracted to mercury vapor and UV lights. Immediately after capture, adult males and females were caged (i.e., sleeve cages made of mesh with fiber spacing of 160 µm (Figure 1) on trunks of oak trees, *Quercus arizonica* Sarg. (Fagales: Fagaceae), at the American Museum of Natural History's Southwestern Research Station, Portal Arizona. Cages were inspected daily for oviposited egg masses which were collected and maintained at ~10° C until use in experiments with *A. orientalis*. Three species of *Scaralina* were collected and relatively low numbers of egg masses per species were obtained. Therefore, all egg masses (n = 9) used for experiments were pooled and referred to as "*Scaralina* spp.". *Poblicia fuliginosa* (Olivier) and *C. vanduzeei* adults were collected during the day from *Baccharis sarothroides* Gray (Asterales: Asteraceae) from different locations in Arizona (Table 1). Adult *P. fuliginosa* and *C. vanduzeei* were maintained on potted *B. sarothroides* plants held in cages. Live insects used for experiments were moved to UCR-IQF under USDA-APHIS Permit number P526P-19-00766 and CDFA Permit number 3457.

2.2.1.2 Cicadellidae collections

Homalodisca vitripennis Germar (Hemiptera: Cicadellidae), a pest of grapes, were collected with sweep nets in citrus orchards in Riverside, California and maintained on potted basil, *Ocimum basilicum* (Lamiales: Lamiaceae), a host plant that supports adult feeding and oviposition.

2.2.1.3 Coreidae collections

Acanthocephala thomasi Uhler, *Chelinidea vittiger* Uhler, *Leptoglossus zonatus* (Dallas) and *Thasus neocalifornicus* Brailovsky and Barrera were included in host range tests. *Acanthocephala thomasi* specimens were hand collected in Portal Arizona (Table 1) and maintained on potted *B. sarothroides* plants. *Chelinidea vittiger* specimens were collected in Riverside, California and maintained on *Opuntia* sp. (Caryophyllales: Cactaceae). *Leptoglossus zonatus* specimens were obtained from research colonies maintained in the Department of Entomology at UC Riverside. *Thasus neocalifornicus* were collected in Sonoita, Arizona and maintained on *Prosopis velutina* Wooton (Fabales: Fabaceae) trees (Table 1).

2.2.1.4 Liviidae collections

Diaphorina citri Kuwayama (Hemiptera: Liviidae) eggs were obtained from colonies maintained in the UCR-IQF building of the Department of Entomology at UCR. *Diaphorina citri*, collected in southern California in 2011 and certified free of the citrus killing bacterium *Candidatus* Liberibacter asiaticus (CLas), were reared on *Murraya koenigii* (Sapindales: Rutaceae), a non-propagative host for CLas.

2.2.1.5 Pentatomidae collections

Banasa dimidiata (Say) (native), *Bragada hilaris* (Burmeister) (invasive), *Chinavia hilaris* Say (native), *Nezara viridula* L. (invasive) and *H. halys* (invasive) were used in host range tests. *Banasa dimidiata* specimens were collected in Riverside, California and maintained on *Hirschfeldia incana* (Brassicales: Brassicaceae) (Table 1). *Bragada hilaris* and *Chinavia hilaris* were obtained from

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TABLE 1 Non-target species tested, selection criteria for use in evaluations, and collection information.

Order	Family	Genera	Species	Native, non-native or invasive	Egg deposition type[1]	Selection criteria (Ref)	GPS coordinates of collection sites[2]/ Commercially obtained	Collection date
Hemiptera	Cicadellidae	*Homalodisca*	*vitripennis*	Invasive	M	Egg masses laid under leaf epidermis	33° 58' 20.26"N - 117° 19' 3.33"W (CA)	April 2020
	Coreidae	*Acanthocephala*	*thomasi*	Native	I	Recorded host family for *Anastatus* sp (29, 32).	31° 54' 54.38"N - 109° 8' 9.17"W (AZ)	August 2021
		Chelinidea	*vittiger*	Native	G	Recorded host family for *Anastatus* sp (29, 32).	33° 58' 29.32"N - 117° 18' 59.98"W (CA)	July 2020
		Leptoglossus	*zonatus*	Invasive	G	Recorded host family for *Anastatus* sp (29, 32).	Colonies maintained at UC Riverside	–
		Thasus	*neocalifornicus*	Native	G	Recorded host family for *Anastatus* sp (29, 32).	31° 40' 42.93"N - 110° 39' 37.16"W (AZ)	August 2021
	Fulgoridae	*Cyrpoptus*	*vanduzeei*	Native	M	Family-level relatedness to *L. delicatula*	31° 54' 48.28"N - 109° 8' 22.75"W (AZ)	August 2020
		Lycorma	*delicatula*	Invasive	M	Target	Pennsylvania, U.S.	2019, 2020 and 2021
		Poblicia	*fuliginosa*	Native	M	Family-level relatedness to *L. delicatula*	Southeastern AZ	August 2021
		Scaralina	*unidentified* spp.	Native	M	Family-level relatedness to *L. delicatula*	31° 53' 12.77"N - 109° 12' 40.37"W (AZ)	August 2019 to 2021
	Liviidae	*Diaphorina*	*citri*	Invasive	G	Eggs readily available from research colonies.	Colonies maintained at UC Riverside	–
	Pentatomidae	*Banasa*	*dimidiata*	Native	M	Recorded host family for *Anastatus* sp (29).	33° 58' 20.26"N - 117° 19' 3.34"W (CA)	April 2020
		Bragada	*hilaris*	Invasive	I	Recorded host family for *Anastatus* sp (29).	Colonies maintained at UC Riverside	–

(Continued)

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TABLE 1 Continued

Order	Family	Genera	Species	Native, non-native or invasive	Egg deposition type[1]	Selection criteria (Ref)	GPS coordinates of collection sites[2]/ Commercially obtained	Collection date
		Chinavia	*hilaris*	Native	M	Recorded host family for *Anastatus* sp (29).	Colonies maintained at UC Riverside	–
		Nezara	*viridula*	Invasive	M	Recorded host species for *Anastatus* sp (21, 29).	34° 3' 46.03"N - 118° 21' 16.12"W (CA)	April 2020
		Halyomorpha	*halys*	Invasive	M	Recorded host species for *Anastatus* sp (29, 31).	34° 3' 46.03"N - 118° 21' 16.12"W (CA)	April 2020
	Reduviidae	*Zelus*	*renardii*	Non-native	M	Beneficial insect. Readily available	Commercially available	–
	Rhopalidae	*Jadera*	*haematoloma*	Invasive	I	Easily collected from field sites	33° 58' 26.67"N - 117° 19' 1.55"W (CA)	April 2020
Mantodea	Mantidae	*Stagmomantis*	*californica*	Native	M	Recorded host family for *Anastatus* sp (22, 23).	33° 40' 12.00"N - 116° 24' 44.43"W (CA)	August 2020
Lepidoptera	Erebidae	*Apantesis*	*unidentified* sp.	Native	I	Recorded host family for *Anastatus* sp (29).	33° 27' 57.48"N - 117° 2' 29.93"W (CA)	June 2020
		Pseudohemihyalea	*edwardsii*	Native	G	Recorded host family for *Anastatus* sp (29).	31° 53' 12.77"N- 109° 12' 40.37"W (AZ)	August 2019 to 2021
	Lasiocampidae	*Gloveria*	*arizonensis*	Native	G	Recorded host family for *Anastatus* sp (29).	31° 53' 12.77"N- 109° 12' 40.37"W (AZ)	August 2019 to 2021
	Saturniidae	*Actias*	*luna*	Non-native	I	Recorded host family for *Anastatus* sp (29).	Commercially available	–
		Agapema	*anona*	Native	I	Recorded host family for *Anastatus* sp (29).	31° 43' 5.15"N - 110° 52' 56.22"W (AZ)	September 2021

(Continued)

TABLE 1 Continued

Order	Family	Genera	Species	Native, non-native or invasive	Egg deposition type[1]	Selection criteria (Ref)	GPS coordinates of collection sites[2]/ Commercially obtained	Collection date
		Anisota	*oslari*	Native	G	Recorded host family for *Anastatus* sp (29).	31° 53' 12.77"N-109° 12' 40.37"W (AZ)	August 2021
		Antheraea	*oculea*	Native	G	Recorded host genera for *A. orientalis* (38).	31° 53' 12.77"N-109° 12' 40.37"W (AZ)	August 2021
		Automeris	*cecrops pamina*	Native	G	Recorded host family for *Anastatus* sp (29).	31° 53' 12.77"N-109° 12' 40.37"W (AZ)	August 2021
			metzli	Non-native	G	Recorded host family for *Anastatus* sp (29).	Commercially available	–
		Eupackardia	*calleta*	Native	I	Recorded host family for *Anastatus* sp (29).	31° 43' 5.15"N - 110° 52' 56.22"W (AZ)	August 2021
		Hyalophora	*euryalus*	Native	I	Recorded host family for *Anastatus* sp (29).	32° 54' 55.26"N - 116° 53' 50.67"W (CA)	May 2021
		Rothschildia	*cincta*	Native	I	Recorded host family for *Anastatus* sp (29).	31° 43' 5.15"N - 110° 52' 56.22"W (AZ)	August 2021
		Saturnia	*walterorum*	Native	I	Recorded host family for *Anastatus* sp (29).	32° 54' 55.26"N - 116° 53' 50.67"W (CA)	May 2021
	Sphingidae	*Pachysphinx*	*occidentalis*	Native	I	Recorded host family for *Anastatus* sp (29).	31° 53' 12.77"N-109° 12' 40.37"W (AZ)	August 2025

(1) Egg type: M = Egg mass [eggs laid very close together with some protective material covering the eggs]; I = Individual eggs; G = Group of individual eggs laid in patches of irregular number of eggs.
(2) CA, California; AZ, Arizona. California and Arizona are southwestern states in the U.S.
(Ref), Reference.

research colonies maintained in the Department of Entomology at UCR. *Halyomorpha halys* and *N. viridula* were established from adults collected from Hancock Park, in Los Angeles, California. All live insects were transported to UCR-IQF under USDA-APHIS Permit number P526P-22-03011 and CDFA Permit number 3887 (Table 1). *Halyomorpha halys* colonies were maintained on a mixed diet of avocados, carrots, apples, green beans, table grapes and *A. altissima*. *Nezara viridula* colonies were maintained on green bean plants and raw peanuts.

2.2.1.6 Reduviidae collections

Egg masses of *Zelus renardii* Kolenati were purchased from Arbico Organics (Oro Valley, Arizona). Purchased eggs were

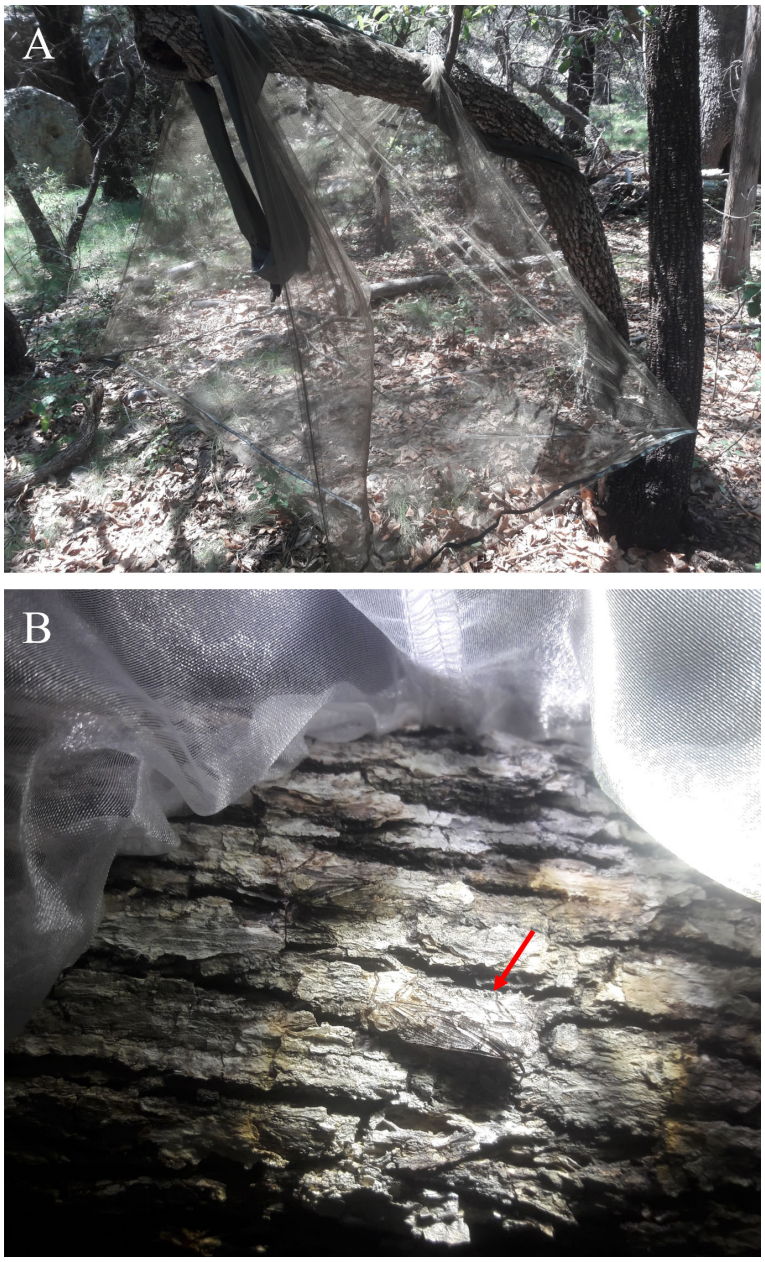

FIGURE 1
(A) Sleeve cages set up on branches of *Quercus arizonica* in Portal, AZ were used to confine adult native fulgorids on putative host plants for mating and oviposition. **(B)** A *Scaralina* sp. specimen captured at night by black lighting is seen resting on the bark of *Q. arizonica* branch enclosed by a sleeve cage (the red arrow indicates position of *Scaralina* sp.).

approximately 2 days of age upon receipt. Egg masses were exposed to *A. orientalis* immediately.

2.2.1.7 Rhopalidae collections

Jadera haematoloma Herrich-Schäffer adults were collected in the Botanic Gardens at the University of California Riverside campus, Riverside, California. Specimens were not feed, adults were kept on ventilated plastic containers, provided with a water-saturated cotton wick, and eggs were collected daily and exposed immediately to *A. orientalis*.

2.2.2 Lepidoptera

All Lepidoptera (Erebidae, Lasiocampidae, Saturniidae, and Sphingidae) species, used in host range testing (except for *Automeris metzli* Sallé which were purchased from an online vendor as pupae) were field collected as adults (Table 1). *Automeris metzli* pupae were held at 10°C for two months to simulate exposure to winter temperatures. After this chilling period, pupae were moved to a temperature cabinet set at 25 ± 2° C and 60% R.H. until adults emerged. Field captured adult moths were kept in bug-dorms (BugDorm-2120 61×61×61 cm, MegaView

TABLE 2 Non-target species tested for host range suitability of *A. orientalis*.

				7 days exposure to *A. orientalis*				24 hours exposure to *A. orientalis*			
Order	Family	Genera	Species	Percent parasitism (± SE)	(n)	Average number of eggs/n (± SE)	Sex ratio (± SE)	Percent parasitism (± SE)	(n)	Average number of eggs/n (± SE)	Sex ratio (± SE)
Hemiptera	Cicadellidae	*Homalodisca*	*vitripennis*	0	5	10 ± 0.32	–	–	–	–	–
	Coreidae	*Acanthocephala*	*thomasi*	100	1	1	1	–	–	–	–
		Chelinidea	*vittiger* *	0	1	37	–	–	–	–	–
		Leptoglossus	*zonatus*	0	11	38.27 ± 2.67	–	–	–	–	–
		Thasus	*neocalifornicus*	0	7	4.71 ± 0.75	–	–	–	–	–
	Fulgoridae	*Cyrpoptus*	*vanduzeei* *	0	2	54.5	–	–	–	–	–
		Lycorma	*delicatula* **	58.24 ± 10.09	13	39.08 ± 3.84	0.77 ± 0.07	28.15 ± 5.04	38	40.66 ± 3.79	0.76 ± 0.04
		Poblicia	*fuliginosa*	12.37 ± 8.42	7	29.86 ± 2.69	0	–	–	–	–
		Scaralina	*spp.*	0	7	26.57 ± 2.57	–	–	–	–	–
	Liviidae	*Diaphorina*	*citri*	0	3	115.67 ± 40.71	–	–	–	–	–
	Pentatomidae	*Banasa*	*dimidiata*	0	1	14	–	–	–	–	–
		Bragada	*hilaris*	0	3	6 ± 0.58	–	–	–	–	–
		Chinavia	*hilaris*	68.79 ± 12.41	11	17.73 ± 2.51	0.22 ± 0.1	7.41	2	30.5	0
		Nezara	*viridula*	18.34 ± 6.87	17[a]	77.35 ± 5.79	0.07 ± 0.05	4.79 ± 1.91	17	59.58 ± 3.78	0.02 ± 0.01
		Halyomorpha	*halys*	48.05 ± 3.5	92[a]	26.85 ± 0.57	0.05 ± 0.02	37.27 ± 13.96	8	23 ± 2.46	0
	Reduviidae	*Zelus*	*renardii*	0	4	24.25 ± 2.02	–	–	–	–	–
	Rhopalidae	*Jadera*	*haematoloma*	0	3	43 ± 3.21	–	–	–	–	–
Mantodea	Mantidae	*Stagmomantis*	*californica*	0	2	150	–	–	–	–	–
Lepidoptera	Erebidae	*Apantesis*	*sp.*	0	2	130 ± 8	–	–	–	–	–
		Pseudohemihyalea	*edwardsii*	16.7 ± 6	6	22.17 ± 3.12	0	–	–	–	–
	Lasiocampidae	*Gloveria*	*arizonensis*	38.74 ± 9.62	13[a]	18.92 ± 1.72	0.11 ± 0.06	23.33 ± 9.55	5	16 ± 1	0.72 ± 0.21
	Saturniidae	*Actias*	*luna*	25.78 ± 6.89	9	19.11 ± 0.79	0.04 ± 0.04	–	–	–	–
		Agapema	*anona*	76.48 ± 14.10	5	42.2 ± 7.43	0	–	–	–	–
		Anisota	*oslari* *	0	16	14.63 ± 1.22	–	–	–	–	–

(Continued)

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TABLE 2 Continued

Order	Family	Genera	Species	7 days exposure to *A. orientalis*				24 hours exposure to *A. orientalis*			
				Percent parasitism (± SE)	(n)	Average number of eggs/n (± SE)	Sex ratio (± SE)	Percent parasitism (± SE)	(n)	Average number of eggs/n (± SE)	Sex ratio (± SE)
		Antheraea	*oculea*	0	1	6	–	–	–	–	–
		Automeris	*cecrops pamina*	19.12 ± 14.41	3	13 ± 3	0	–	–	–	–
			metzli	0	1	10	–	–	–	–	–
		Eupackardia	*calleta*	100	1	12	0	–	–	–	–
		Hyalophora	*euryalus*	100	3	5.5	0.16	–	–	–	–
		Rothschildia	*cincta*	45	2	10	0	–	–	–	–
		Saturnia	*walterorum*	19.60 ± 17.41	5	11.2 ± 1.02	0.5 ± 0.32	42.96 ± 25.7	3	9.67 ± 0.33	0.5 ± 0.2
	Sphingidae	*Pachysphinx*	*occidentalis*	1.56 ± 1.56	4	17.5 ± 2.72	0	–	–	–	–

Male and female parasitoid offspring produced (female sex ratio) on each non-target species, number of repetitions per species (n), average number of eggs used per species per repetition and average parasitism rate of A. orientalis on eggs of each species.
(*) 100% of the A. orientalis females did not parasitize L. delicatula in the sequential exposure.
(**) target host.
[a]non-target host eggs of different ages. See section 1 and Figure 2.

Science Co. Ltd., Taiwan) and maintained outdoors near collection sites or in a temperature chamber (25 ± 2°C; 60% of R.H.) when moved into UCR-IQF for mating and oviposition. Adults were not provided with food or water as test species do not feed in adult stage (except *Apantesis* sp. which was provided 50% honey water solution). Eggs oviposited onto walls of cages or on cardboard oviposition strips were collected daily and either used immediately or maintained at 10°C until used for experiments.

2.2.3 Mantodea

2.2.3.1 Mantidae collections

Adult female *Stagmomantis californica* Rehn & Hebard were collected in Riverside County, California (Table 1) and fed with *H. halys* nymphs and adults. Ootheca, ~48 h of age, were collected and presented to *A. orientalis*.

2.3 No-choice sequential host-testing

Five female *A. orientalis*, less than 24 hours of age, were placed in a test unit with one male and a thin smear of pure honey on the mesh of the unit's lid as a carbohydrate source. Each experimental egg mass-parasitoid test arena was comprised of a clear plastic container 3 cm × 4 cm × 5 cm (180 mL clear RPTE hinged lid deli containers, AD16 GenPak, Charlotte, NC) with a modified lid that had a ventilated mesh window (1.5 cm x 2.5 cm) to facilitate air exchange. One *L. delicatula* egg mass was placed in the test unit and exposed to the five females and the male of *A. orientalis* for seven days. This seven day period is a pre-oviposition period during which host feeding occurs (pers. obs. F. Gomez Marco) at temperatures that simulate the fall (i.e., September when parasitoid oviposition in the field occurs) in Beijing (average daily high 25°C, average daily low 14°C, lights on 6:00 AM, lights off 6:30 PM [i.e., L:D 12.5:11.5], 75% R.H.; referred to as Beijing-fall regimen), the general area where *A. orientalis* was collected for colony establishment in the U.S (16–19). All experiments were conducted under the Beijing-fall regimen. After this one-week pre-oviposition period, females were moved individually and placed singly without males in new separate test units for a total of 274 A. *orientalis* females. One non-target host egg mass [number of eggs in the egg mass and the physical size of the egg mass varied on species being tested (Table 2)] was placed into each test unit containing a single mated female for seven days. After the seven-day exposure period, non-target eggs were removed, and replaced with *L. delicatula* egg masses, and females were left to host feed and oviposit for an additional seven days. Thus, the sequential non-choice tests were performed in this order; target host (pre-oviposition period, seven days) – non-target host (seven days) – target host (seven days). Parasitism of *L. delicatula* egg masses in the exposure trial following exposure to non-target eggs confirmed female competency if no parasitism was observed from non-target exposures. The total time taken to complete each no choice sequential host test cycle for each female was 21 days.

A variation of this experiment that reduced female exposure time to non-target egg masses from five species [*C. hilaris*, *Gloveria*

arizonensis Packard, *H. halys, N. viridula* and *Saturnia walterorum* Hogue & Johnson (Table 1)] was conducted. In this set of experiments, five *A. orientalis* females had a seven day pre-oviposition period with a male and access to a *L. delicatula* egg mass. Following this seven-day exposure period, individual mated females without males were exposed to non-target eggs (Tables 1, 2) for 24 hours then moved to a target *L. delicatula* egg mass for 24 hours to confirm competency.

For both experimental designs, female *A. orientalis*, that did not produce offspring either on the non-target eggs or on the second exposure to the *L. delicatula* egg mass, were classified as "incompetent" and discarded from analyses. This rule was not followed for *Anisota oslari* Rothschild due to the high mortality (100%) of *A. orientalis* females following exposure to eggs of this species, and for *C. vittiger* and *C. vanduzeei*, due the low number of repetitions due to low egg availability (Table 2), which resulted from difficulty in acquiring sufficient test eggs of non-target species for experiments. Each *L. delicatula* egg mass (from pre-oviposition and post-non-target exposure) and non-target species eggs were held under the Beijing-fall regimen for one month, for development of parasitoid larvae. After this four-week period, eggs were transferred to 25°C, 16:8 L:D, and 75% R.H. for emergence of parasitoid offspring following previously published protocols (19). The host species from which *A. orientalis* emerged were recorded. Target eggs that did not produce parasitoids were dissected to detect failed parasitism (i.e., presence of dead parasitoid larvae or pupae were recorded). Percentage of parasitism was calculated as:

$$\%\ Parasitism = \frac{Total\ number\ of\ parasitoids\ (i.e.,\ emerged\ adults\ +\ failed\ larvae\ +\ failed\ pupae)}{Total\ number\ of\ host\ eggs\ exposed} \times 100 \quad (1)$$

Mortality of non-target host species due to exposure to *A. orientalis* was calculated using the Henderson–Tilton formula (Equation 2) (38), which calculates percent mortality based on the initial and final insect counts in the control relative to treatments with parasitoid exposure. Rates of naturally occurring mortality for non-target species host eggs were calculated with control eggs that were held under similar ambient conditions to test eggs but were not exposed to *A. orientalis*. The percentage of mortality by parasitoids was calculated as:

$$\%\ Mortality\ by\ parasitoids = \left(1 - \frac{(Average\ number\ of\ eggs\ in\ controls) \times (Number\ of\ juveniles\ after\ exposure\ to\ A.\ orientalis)}{(Number\ of\ eggs\ exposed) \times (Average\ number\ of\ juveniles\ in\ controls)}\right) \times 100 \quad (2)$$

Using percent mortality caused by parasitoids and percent parasitism, percent mortality of non-target hosts resulting from parasitoid activity, but that did not result in *A. orientalis* offspring (i.e., excess mortality [due to host feeding and/or oviposition attempts]) was calculated with the equation:

$$\%\ Excess\ mortality = \%\ Mortality\ by\ parasitoid - \%\ Parasitism \quad (3)$$

Percent excess mortality caused by parasitoids to non-target hosts was compared with percent mortality in controls not exposed to parasitoids when the number of successfully completed repetitions for each treatment exceeded a minimum of three.

2.4 Choice host-testing

To assess host preference on parasitization by *A. orientalis* when given a simultaneous choice between eggs of non-target species and *L. delicatula*, choice host-tests were performed with two non-target species, *G. arizonensis* and *H. halys*. Pairs of egg masses of target and non-target species were presented simultaneously to female parasitoids ~48 hours of age in exposure arenas which were constructed using two stacked transparent U-shaped acrylic risers 15cm×15cm×15cm (SW Plastics F2191, Riverside, CA), that formed a rectangular cage 15cm×15cm×30cm with two open sides. One open face was covered with white semi-opaque no-see-um netting (Skeeta, Bradenton, FL) and the other was fitted with a 30cm-long sleeve sewn from no-see-um netting). Choice tests were run either for 24 hours or seven days. Inside arenas, egg masses were separated by 26 cm and randomly placed on the floor of arenas for each repetition. After exposure time, each group of eggs (target, non-target, and control eggs not exposed to parasitoids) were isolated in ventilated clear plastic test arenas (3 cm × 4 cm × 5 cm, see section 2) and held under the Beijing-fall regimen for four weeks before being moved to 25°C until parasitoids or immature non-target species emerged from eggs, or eggs were classified as dead and dissected for evidence of parasitism when possible.

2.5 *Anastatus orientalis* offspring sex ratio, fertility and size when reared from target and non-target hosts

Anastatus orientalis offspring that emerged from non-target host species and target host (i.e., *L. delicatula*) eggs were evaluated for offspring sex ratio, fertility of males and females, and adult size. Parasitoid sex ratio was calculated as the number of female parasitoids divided by the total number of female and male parasitoids combined that emerged from each experimental egg mass. Three different offspring fertility evaluations were performed on five non-target host species: *Actias luna* L., *Agapema anona* Ottolengui, *G. arizonensis*, *H. halys* and *P. fuliginosa*. First, males and females emerging from the same non-target host species (< 48 hours of age) were set up in test arenas (see section 2 for details). Second, males that emerged from non-target host species were coupled with unmated *A. orientalis* females that emerged from *L. delicatula* eggs. Finally, females that emerged from non-target host species were coupled with *A. orientalis* males that emerged from *L. delicatula* egg masses. All mating couples were exposed to *L. delicatula* egg masses for seven days and a thin smear of pure honey on the mesh of the ventilated lid of the test arena as a carbohydrate source. After seven days, male-female pairs were removed, and each egg mass was held under the Beijing-fall

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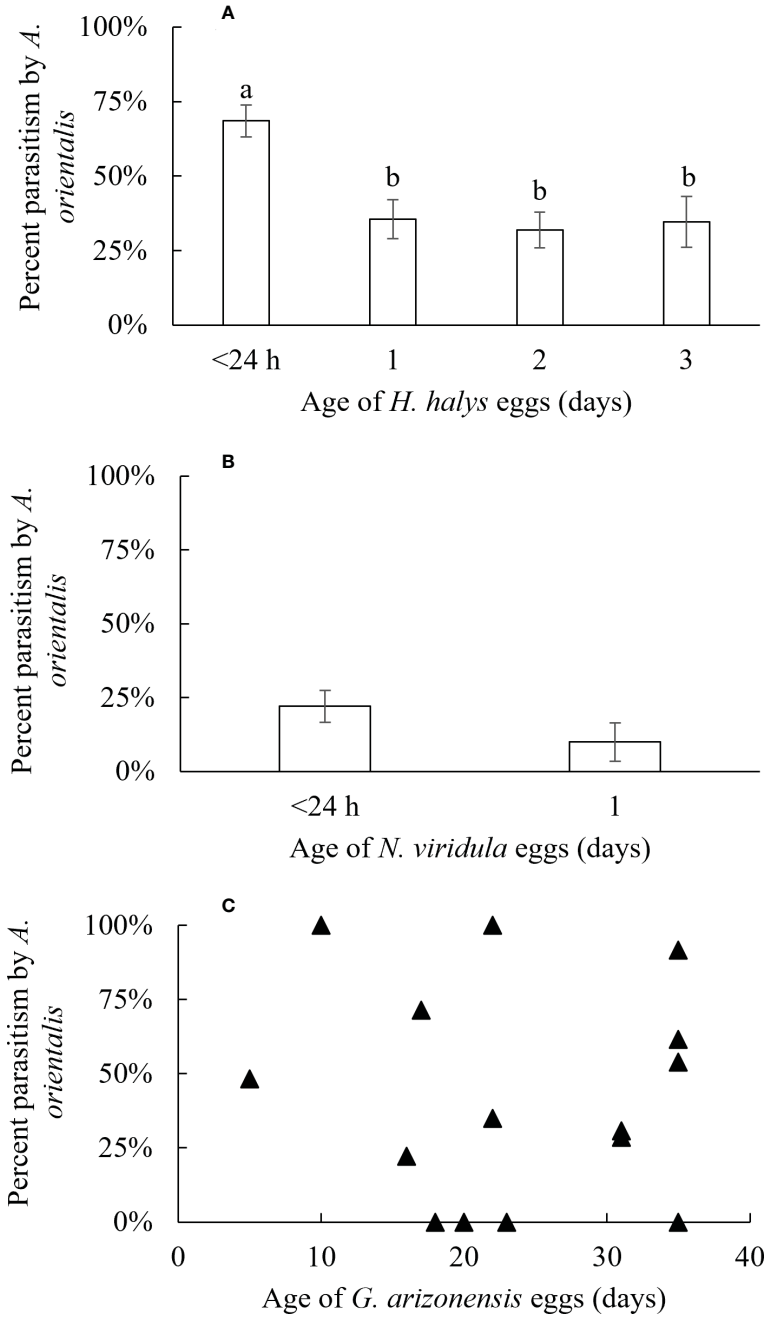

FIGURE 2
Percentage of parasitism (± SE) by *A orientalis* on non-target host eggs of **(A)** *H. halys* (<24 hours; n = 27, 1 day; n = 23; 2 days; n = 27, 3 days; n = 17), **(B)** *N. viridula* (<24 hours; n = 11, 1 day; n = 6) and **(C)** *G. arizonensis*. Different letters indicate significant differences for percent parasitism of eggs of different ages.

regimen for four weeks then moved to 25°C for the emergence of parasitoids and non-target hosts.

To evaluate the effect of host species on the size of *A. orientalis* male and female parasitoids that successfully emerged from non-target and target host eggs, measurements of right hind tibia lengths were used as a proxy for parasitoid size and subsequent fitness (i.e., parasitoids with larger hind tibia are assumed to be bigger and more fit than parasitoids with smaller tibia) (39, 40). Excised right hind tibiae were placed onto glass slides and covered with a second glass slide. Hind tibia length was measured from its point of attachment on the femur to the attachment point with the tarsi using a Leica S8AP0 microscope. Slide mounted hind tibiae were photographed at a magnification of 25 × with an attached Leica DMC2900 camera and length was measured using the Leica Application Suite version 4.6.2. A total of ~10 randomly selected *A. orientalis* males and females from each non-target host were measured and compared to 10 randomly selected males and females reared from *L. delicatula*.

To evaluate the effects of non-target host egg age on parasitism rates/host acceptance of *A. orientalis*, the age of eggs from all non-target host species exposed to *A. orientalis* was recorded. Data from

eggs exposed to *A. orientalis* for seven days in the non-choice experiment (see section 2) were used. Three species, *G. arizonensis*, *N. viridula*, and *H. halys* resulted in sufficient repetitions and/or age variability to be analyzed. *Gloveria arizonensis* Packard eggs age ranged from 5 to 35 days old. *Nezara viridula* egg age used in this study were ≤24 hours of age. Finally, *H. halys* egg age ranged from <24 hours to three days of age. Percent parasitism was compared between egg age for each of these three species.

2.6 Statistical analysis

All statistical analyses were conducted in R version 4.1.3 (41) using the development environment RStudio (42). Percent excess mortality that resulted from non-reproductive behavior of *A. orientalis* was compared with natural-occurring mortality rates in the paired controls using a generalized linear model (GLM) with a quasi-binomial distribution to account for high variance in data sets. The relation between the hind tibia size of the male parasitoids offspring and the sex ratio of parasitoid offspring emerging from the same host was analyzed using linear regression. Differences in mean hind tibiae lengths between male and female parasitoids emerging from different non-target species eggs and target eggs were analyzed using ANOVA followed by a Tukey posthoc test at the 0.05 level of significance using the package 'multcomp'. All means are presented ± standard error (SE).

7 Results

7.1 No-choice sequential host-testing experiments

In addition to the target host *L. delicatula*, eggs from a total of 34 non-target host species, however, eggs of three *Scaralina* spp. were pooled as *Scaralina* sp. giving a functional total of 32 species that were exposed to *A. orientalis* females in no-choice sequential host testing experiments. From the total of 244 female *A. orientalis* that completed the entire sequential exposure series (non-target and target), 23 (9.4%) females failed to parasitize the non-target host and the target host. They were considered incompetent and were excluded from data analyses. There were three exceptions for non-target hosts; *C. vittiger* and *C. vanduzeei*, due to the low number of repetitions because of the low numbers of non-target eggs available for testing, and *A. oslari* due to the high mortality of parasitoids (n =16; 100% of females tested died) after exposure to non-target eggs (Table 2). Additionally, 41 (16.8%) parasitoids were able to parasitize non-target host eggs and then failed to parasitize *L. delicatula* eggs. The results from these trials were included in data analyses.

Anastatus orientalis parasitized five species in the order Hemiptera: *A. thomasi* (Coreidae), *C. hilaris*, *H. halys* and *N. viridula* (all Pentatomidae) and *P. fuliginosa* (Fulgoridae) (Table 2). *Anastatus orientalis* parasitized 10 species in the order Lepidoptera: *A. luna*, *A. anona*, *Automeris cecrops pamina* Neumoegen, *Eupackardia calleta* Westwood, *Hyalophora euryalus* Boisduval, *Rothschildia cincta* Tepper, *S. walterorum* (Saturniidae), *G. arizonensis* (Lasiocampidae), *Pseudohemihyalea edwardsii* Packard (Erebidae) and *Pachysphinx occidentalis* Edwards (Sphingidae) (Table 2). The maximum percent parasitism of eggs for non-target hosts in Hemiptera and Lepidoptera were obtained on the native pentatomid, *C. hilaris*, and the native saturniid, *A. anona*, at 68.79% ± 12.41 and 76.48% ± 14.10, respectively.

Percent non-target host mortality of eggs exposed to *A. orientalis* was corrected with their paired controls (Equation 2) and excess mortality for non-target hosts following exposure to parasitoids was calculated (Equation 3). Excess mortality was compared across nine non-target hosts species (four suitable for parasitism and five unsuitable for parasitism, Figure 3) with their paired controls. Percent excess mortality resulting from death other than parasitism that resulted in the emergence of an adult parasitoid (i.e., mortality from host feeding and/or failed parasitism) for non-target host species were not affected by exposure to *A. orientalis* and no significant differences were found for nine species (*A. oslari*; $F_{1,18} = 0.184$, $P = 0.67$, *C. hilaris*; $F_{1,19} = 0.11$, $P = 0.75$, *D. citri*; $F_{1,4} = 0.82$, $P = 0.41$, *H. halys*; $F_{1,116} = 0.097$, $P = 0.75$, *J. haematoloma*; $F_{1,18} = 3.5$, $P = 0.13$, *L.* zonatus; F1,13 = 0.69, P = 0.42, *N. viridula*; $F_{1,30} = 1.98$, $P = 0.16$, *S. walterorum*; $F_{1,6} = 0.71$, $P = 0.43$, *T. neocalifornicus*; $F_{1,8} = 4.89$, $P = 0.058$) (Figure 3).

Of the five non-target host species exposed to *A. orientalis* females for 24 hours, three pentatomids, *C. hilaris*, *H. halys* and *N. viridula*, and two Lepidoptera species, *G. arizonensis* (Lasiocampidae) and *S. walterorum* (Saturniidae), were all parasitized by *A. orientalis*. The maximum average percent parasitism was observed for *S. walterorum* (42.96 ± 25.7) and the minimum average percent parasitism was recorded for *N. viridula* (4.79 ± 1.91) (Table 2).

The effect of non-target host egg age on parasitism by *A. orientalis* was evaluated for three species, *G. arizonensis*, *H. halys* and *N. viridula*. Rates of parasitism decreased as non-target host egg age increased for *H. halys* ($F_{1,92} = 1.54$, $P < 0.001$), and no effect of egg age on parasitism was observed for *N. viridula* ($F_{1,17} = 1.54$, $P = 0.23$). Similarly, increasing age of *G. arizonensis* eggs did not affect parasitism rates ($F_{1,13} = 0.07$, $P = 0.79$) (Figure 2).

7.2 Choice host-testing experiments

The two non-target species used in choice experiments, *G. arizonensis* and *H. halys*, were parasitized in both exposure periods, 24 hours and 7 days, when exposed to *A. orientalis* in the presence of *L. delicatula* egg masses (Figure 4). For all the *G. arizonensis* vs *L. delicatula* choice trials (n = 12), four parasitoids failed to parasitize one of the two hosts species exposed; *G. arizonensis* and *L. delicatula* eggs were not parasitized three times and one time, respectively. For *H. halys* vs *L. delicatula* experiments (n = 19), three *A. orientalis* females did not parasitize either the

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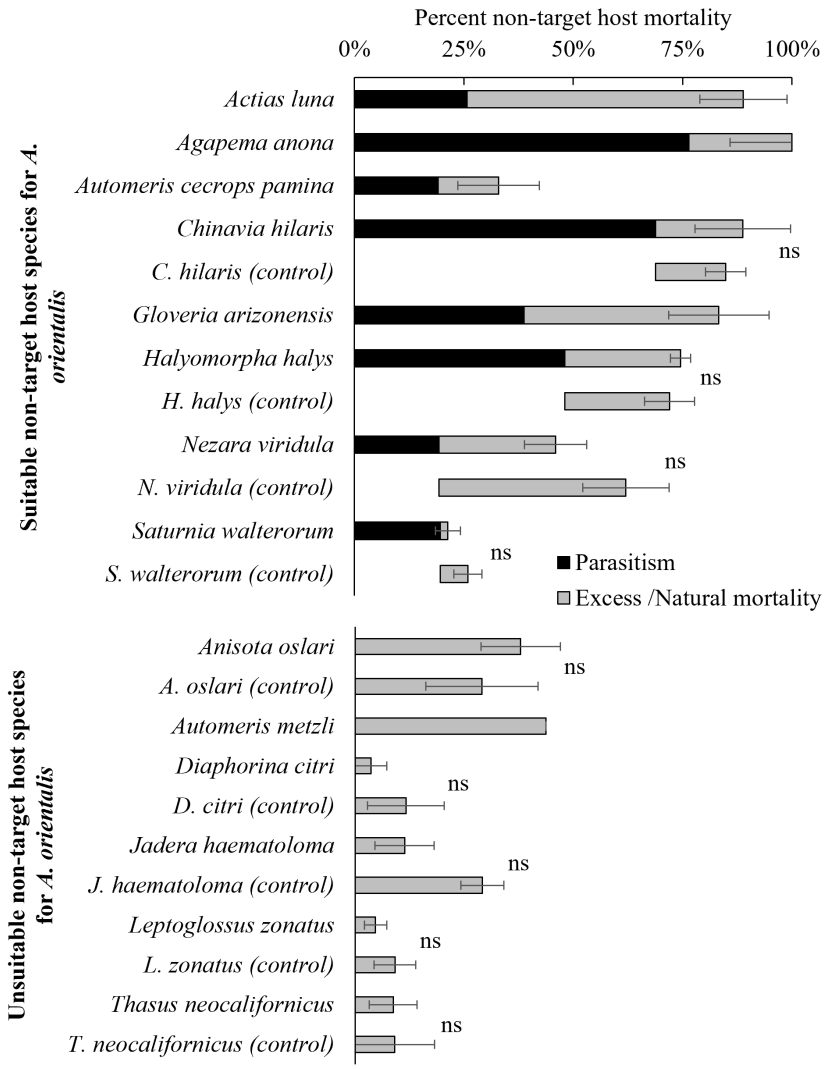

FIGURE 3
Percent non-target host mortality after exposure to *A. orientalis* from which parasitoids emerged (suitable hosts) and failed to emerge (unsuitable hosts). Species with no excess/natural mortality following parasitoid exposure were excluded from figure (Correction of mortality for exposed non-target host was null [Equation 2]). Total percent mortality of non-target hosts is the sum of the percentage of parasitism (in black) and the excess mortality (in grey) (± SE). (ns) Indicates no significant differences between the percent excess mortality after exposure to *A. orientalis* and the percentage of eggs alive without exposure to *A. orientalis* (control or naturally-occurring mortality).

target or the non-target host. Eight parasitoids failed to parasitize one of the two host species exposed; *H. halys* eggs were not parasitized in three trials and *L. delicatula* eggs were not parasitized in five trials.

7.3 *Anastatus orientalis* offspring from non-target hosts; sex ratio, male and female fertility, and size

7.3.1 Sex ratio

A total of 15 non-target host species were suitable hosts for *A. orientalis* and a total of 1,870 parasitoids, 1,780 males and 90 females, emerged from susceptible non-target species following seven-day exposure time. The highest female sex ratio was obtained from *S. walterorum* eggs (0.5 ± 0.32) (there was one exception, *A. thomasi*, which only one egg was exposed and parasitized and resulted in a female parasitoid). Seven non-target species did not produce females after being parasitized by *A. orientalis* (i.e., parasitism of *A. anona*, *A. cecrops pamina*, *E. calleta*, *P. edwardsii*, *P. fuliginosa*, *P. occidentalis* and *R. cincta* eggs produced only male offspring) (Table 2).

The five non-target species exposed to *A. orientalis* for 24 hours resulted in a total of 137 parasitoids, 110 males, 24 females and three larvae. The highest sex ratio between these five species was obtained from *G. arizonensis* eggs (0.72 ± 0.21).

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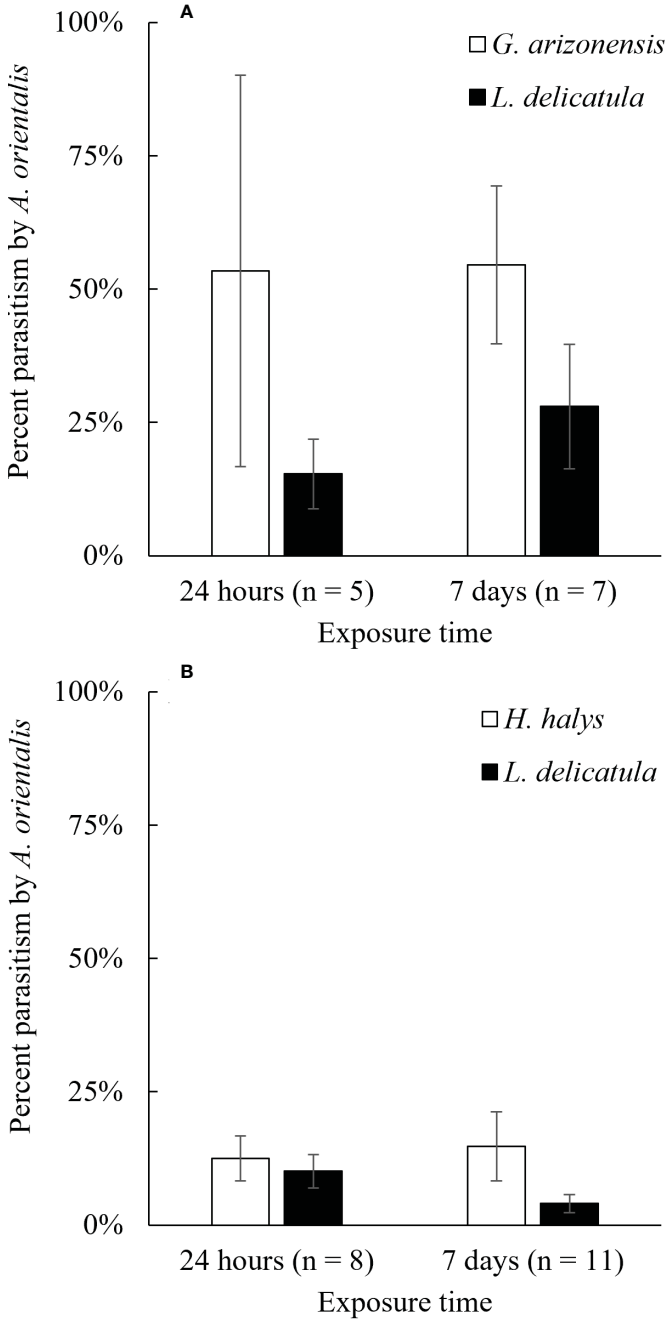

FIGURE 4
Average percent parasitism of non-target host eggs **(A)** *G. arizonensis* and **(B)** *H. halys*, when presented simultaneously with the target host, *L. delicatula* for two different exposure times, 24 hours and 7 days (n = number of repetitions).

Only *C. hilaris* and *H. halys* did not produce females (i.e., male offspring only produced) after being parasitized by *A. orientalis* (Table 2).

7.3.2 Fertility

Male parasitoids were observed mating with females reared from the same non-target host species and female offspring were subsequently produced confirming mating was successful (*A. orientalis* is arrhenotokous and female offspring are produced from fertilized eggs). Consequently, male-female pairs of *A. orientalis* that emerged from the same non-target host species produced male and female offspring. (Table 3). Males that emerged from non-target host species successfully inseminated females emerging from *L. delicatula* egg masses as female offspring were produced from these male-female pairings (Table 3).

TABLE 3 Parasitism rates and offspring sex ratio (i.e., proportion of female offspring) produced by i) *A. orientalis* couples that emerged from five different non-target host species or ii) males that emerged from non-target host species mated with unmated females that emerged from *L. delicatula* eggs or iii) females that emerged from non-target host species mated with males that emerged from *L. delicatula* eggs (n = number of repetitions).

	Host source of *A. orientalis* mating pairs and resulting parasitism rates and offspring sex ratio											
	Male-female pair from same non-target host species				Male from non-target host, Female from *L. delicatula*				Female from non-target host, male from *L. delicatula*			
Non-target host species	Percentage of parasitism	(n)	sex ratio	(n)	Percentage of parasitism	(n)	sex ratio	(n)	Pecrentage of parasitism	(n)	sex ratio	(n)
Actias luna	10.39 ± 0.81	3	0.33 ± 0.19	3	58.19 ± 8.02	3	0.53 ± 0.26	3	–		–	
Agapema anona	–		–		11.64 ± 8.02	3	0.57	2	–		–	
Gloveria arizonensis	20.89 ± 13.27	7	0.88 ± 0.07	3	0	3	–		13.04*	1	–	
Halyomorpha halys	30.96 ± 7.93	13	0.36 ± 0.1	11	–		–		–		–	
Poblicia fuliginosa	–		–		0	1	–		–		–	

*Parasitoids failed to emerge and parasitism was calculated by counting numbers of parasitoid larvae (alive) in dissected eggs.

7.3.3 Size

A wide range of offspring sizes as well as a pronounced sexual dimorphism with larger females and smaller males was observed for *A. orientalis* when reared from non-target host species (Table 4). Average male hind tibia lengths ranged from 0.309 ± 0.005 mm (host: *P. edwardsii*) to 0.587 ± 0.022 mm (host: *G. arizonensis*). Average hind tibia lengths for females ranged from 0.613 ± 0.007 mm (host: *C. hilaris*) to 1.04 mm (host: *A. thomasi*) (Table 4). The largest *A. orientalis* males emerged from the target host, *L. delicatula* ($F_{6,62}$ = 100.9, $P < 0.001$). The largest female emerged from the non-target host, *A. thomasi*. All other non-target hosts from which females emerged were smaller when compared with females that emerged from *L. delicatula* eggs ($F_{3,26}$ = 285.4, $P < 0.001$) (Table 4). The average size of male offspring was significantly correlated with the sex ratio of the parasitoids emerging from the same host species ($F_{1,6}$ = 7.668, P = 0.032; R^2 = 0.561) (Figure 5). Non-target hosts that produced *A. orientalis* males were smaller and the sex ratio of emerged parasitoids was lower (i.e., male biased) and larger parasitoids typically emerged from host eggs that had female –biased sex ratios (Figure 5).

8 Discussion

This is the first study to assess the physiological host range and specificity of *A. orientalis* (Haplotype C) with respect to potential non-target species that occur in the southwestern U.S. In the quarantine laboratory, no-choice tests assessing the developmental suitability of non-target host species for *A. orientalis* demonstrated that 15 out of 34 non-target host species were suitable hosts for *A. orientalis*. This work indicated *H. halys* eggs could successfully support development of *A. orientalis* (offspring sex ratio was strongly male biased), a finding contrary to previous reports (43). *Antheraea* sp. (Saturniidae) eggs (i.e., *A.*

TABLE 4 Average hind tibia length (mm) of *A. orientalis* females and males that emerged from eggs of non-target species.

Host species	Average of hind tibia length (mm)			
	Males	n	Females	n
Acanthocephala thomasi	–	–	1.04	1
Chinavia hilaris	0.485 ± 0.005 cd	9	0.613 ± 0.007 c	4
Gloveria arizonensis	0.587 ± 0.022 b	10	0.881 ± 0.018 b	6
Halyomorpha halys	0.492 ± 0.013 c	10	0.629 ± 0.008 c	10
Lycorma delicatula	0.689 ± 0.018 a	10	0.959 ± 0.007 a	10
Nezara viridula	0.367 ± 0.004 e	10	–	–
Pachysphinx occidentalis	0.501	1	–	–
Poblicia fuliginosa	0.433 ± 0.011 d	10	–	–
Pseudohemihyalea edwardsii	0.309 ± 0.005 f	10	–	–

Different letters indicate significant differences between hind tibia size of parasitoids that emerged from different host species.

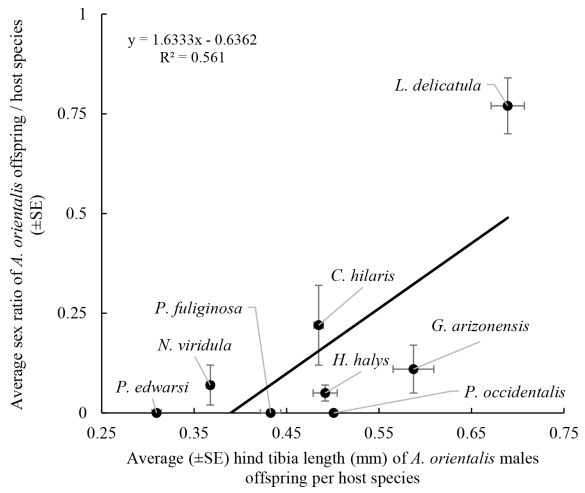

FIGURE 5
Relation between hind tibia length of *A. orientalis* offspring males emerging from each host (average values from Table 4) and sex ratio obtained from the same host (average values from Table 2).

pernyi) have been reported to be suitable reproductive hosts for *A. orientalis* (43). However, parasitization of *Antheraea oculea* Neumoegen eggs by *A. orientalis* was not recorded in this study. Five new hemipteran hosts in three families (Coreidae [*A. thomasi* {native}], Fulgoridae [*P. fuliginosa* {native}], and Pentatomidae [*C. hilaris* {native}, *H. halys* {invasive} *N. viridula* {invasive}]) and ten lepidopteran hosts in four families (Erebidae [*P. edwardsii* {native}], Lasiocampidae [*G. arizonensis* {native}], Saturniidae [*A. luna* {non-native}, *A. anona* {native}, *A. cecrops pamina* {native}, *E. calleta* {native}, *H. euryalus* {native}, *R. cincta* {native}, *S. walterorum* {native}] and Sphingidae [*P. occidentalis* {native}]) were identified as suitable hosts and are added to an increasing list of identified species that *A. orientalis* can successfully use as reproductive hosts in the laboratory (Table 2).

In terms of percent parasitism, eggs of two non-target species, *C. hilaris* (Pentatomidae [native]) and *A. anona* (Saturniidae [native]), were similar to or better hosts, than the target, *L. delicatula*. In the first case, *C. hilaris*, percent parasitism (68.79% ± 12.41; sex ratio 0.22 ± 0.1) was similar to the target, *L. delicatula* (58.24 ± 10.09; sex ratio 0.77 ± 0.07). This result may need to be interpreted cautiously, because *C. hilaris* eggs masses had on average a smaller number of eggs (17.73 ± 2.51) when compared to the average number of eggs per egg mass (39.08 ± 3.84) from the target, *L. delicatula*. In the second case, *A. anona*, percent parasitism was higher (76.48 ± 14.10; sex ratio 0) than the target *L. delicatula* (58.24 ± 10.09), and both egg masses were approximately equal in size (i.e., 42.2 ± 7.43 eggs and 39.08 ± 3.84 eggs for *A. anona* and *L. delicatula*, respectively). Collectively, data reported here and findings from companion studies (i.e., Broadley et al. [USDA], Submitted) suggest that *A. orientalis*, is at a minimum oligophagous, but more likely to be a polyphagous species.

Similar results from host specificity tests from other *Anastatus* spp. have been reported further supporting findings that *Anastatus* spp. potentially have broad host ranges. Stahl et al. (32, 33) studied the physiological host range of *A. bifasciatus* (this species is native to Europe) for use as a biological control agent against *H. halys*, an invasive pest in Europe. In this study, *A. bifasciatus* successfully parasitized eggs of eight pentatomid species (including *N. viridula* [tested in this study]) and 14 lepidopteran species belonging to seven different families (Endromidae, Erebidae, Lasiocampidae, Notodontidae, Papilionidae, Saturniidae, and Sphingidae). Results reported here indicate that *A. orientalis* can also parasitize hosts eggs from Erebidae, Lasiocampidae, Saturniidae and Sphingidae. Host range tests that expose *A. orientalis* females to eggs of Notodontidae and Papilionidae, two lepidopteran families with species representation in the southwest U.S., may be warranted to further understanding of potential non-target host use (the family Endromidae is not present in North America). For *Anastatus* spp., results reported here and those of Stahl et al. (32, 33), indicate strongly that selecting non-target species which are restricted to close taxonomic relatedness to the target pest (e.g., families in the Fulgoroidea [Hemiptera: Auchenorrhyncha]) may be inadequate as *Anastatus* spp. may tend to be generalists capable of parasitizing species across different orders.

No additional significant levels of excess mortality (i.e., mortality from causes other than parasitism) to non-target species exposed to *A. orientalis* was observed (Figure 3). Failure to detect excess mortality of non-target eggs exposed to *A. orientalis* when compared to levels of naturally-occurring mortality in control eggs not exposed to *A. orientalis* may have at least two explanations: i) parasitized non-target eggs develop successfully when parasitoid larvae and/or envenomation failed to kill the host egg (host eggs are incapable of encapsulating parasitoid eggs and larvae), or ii) *A. orientalis* females only host fed on eggs that were parasitized and it is possible that *A. orientalis* is a concurrent parasitoid (i.e., host feeds on parasitized eggs). Thus, egg mortality from host feeding alone was not observed and egg mortality was attributed to solely to parasitism. Additional studies confirming the lack of excess mortality due to unsuccessful parasitization or host feeding following exposure of non-target host species to *A. orientalis* are needed. Excess mortality of non-target host eggs following exposure to *A. orientalis* should be considered an important deleterious non-target impact if it occurs (44).

For three non-target host species tested, *A. oslari* (Saturniidae [native]), *C. vanduzeei* (Fulgoridae [native]) and *C. vittiger* (Coreidae [native]), all parasitoid females exposed to egg masses of these species failed to parasitize *L. delicatula* egg masses in sequential exposure tests. For *C. vanduzeei* and *C. vittiger*, too few non-target eggs were available for experiments and replication was low for each test species and therefore were not excluded from the results. Interestingly, for female *A. orientalis* exposed to *A. oslari* eggs excess egg mortality increased slightly but not significantly, and all females (n=16) died following exposure to *A. oslari* eggs prior to sequential exposure to *L. delicatula* egg masses. These results, a slight but non-significant increase in excess egg mortality and premature mortality of females, suggest that *A. orientalis* females may have host fed on *A. oslari* eggs and egg contents were possibly toxic to parasitoids. This could be explained by chemical protection of eggs by secondary plant compounds, like tannins, which are used as chemical defenses by host plants (i.e., *Quercus* spp.) of *A. oslari* (45). Sequestration of protective

compounds, like tannins, in *A. oslari* eggs could reduce survivorship rates of third trophic level organisms like *A. orientalis* (46).

To evaluate a more realistic exposure time of *A. orientalis* to non-target species, 24 hour exposure tests (as opposed to a 7 day exposure period) were performed with five non-target species (*C. hilaris*, *G. arizonensis*, *H. halys*, *N. viridula* and *S. walterorum*). *Anastatus orientalis* was able to parasitize the five non-target species in this shorter exposure period following the seven-day pre-oviposition exposure period on *L. delicatula* eggs. These findings suggest that prior host exposure, especially to the target, *L. delicatula*, does not deter use of subsequent non-target host eggs. In addition, when non-target and target host eggs were exposed to *A. orientalis* females at the same time (i.e., choice experiments), that non-target species were attacked under both exposure time scenarios (i.e., 24 hours and 7 days). These results further suggest that *A. orientalis* is probably a generalist parasitoid capable of using any suitable non-target host species upon encounter. In many instances, female parasitoids engaged in parasitism within minutes of introduction into test arenas and contact with non-target eggs (pers. obs. F. Gomez Marco).

The age of non-target host eggs can affect the acceptance behavior of *A. orientalis* females and rates of successful parasitism. For example, percent parasitism by *A. orientalis* was higher on young eggs (<24 hours of age) vs. older eggs (>24 hours to 3 days) of *H. halys*. However, for two other non-target species, *G. arizonensis* and *N. viridula*, tested in egg age acceptance studies, no significant effect of egg age on parasitism was found. Therefore, age of non-target eggs and the non-target species may be an important covariables to consider when host tests are being designed and executed. Additionally, defensive chemical compounds (see above) may also affect the acceptance behavior of parasitoids (and survivorship rates) and this may also warrant consideration in design, analysis, and interpretation of host range tests (46).

The sex ratio of *A. orientalis* offspring was strongly dependent on host egg size. All non-target host eggs which were parasitized by *A. orientalis* with an average egg size visibly (not measured in this study) smaller than *L. delicatula* produced a male biased sex ratio (< 0.5) and smaller adult males and females. However, the sex ratio from hosts which produced smaller *A. orientalis* males decreased, indicating that there was a strong correlation between offspring size and offspring sex ratio. Only one non-target host with eggs larger than *L. delicatula* eggs (pers. obs. F. Gomez Marco) was used in our tests (*A. thomasi*) and this resulted in the largest female parasitoid (hind tibia size of 1.04 mm) obtained from host-range studies. These findings tentatively support conditional sex allocation theory, where, with decreasing host quality (i.e., host egg size in this study), parasitoid offspring sex ratio becomes more male biased and males are generally smaller, both of which correlate with decreased fitness (47). In support of results presented here, two previous studies, Hou et al. (48) and Stahl et al. (32), reported more male-biased sex ratios when *Anastatus* spp. were reared on host eggs that were smaller than the target host. Interestingly, differences in *A. orientalis* offspring sex ratio from two non-target host species, *C. hilaris* and *H. halys*, existed when egg masses were exposed for 24 hours (*C. hilaris* and *H. halys*: 0, no females) or seven days (*C. hilaris*: 0.22 ± 0.1, *H. halys*: 0.05 ± 0.02). This finding might indicate a preference of *A. orientalis* females to first oviposit (within at least the first 24 hours) non-fertilized eggs (i.e., produce male offspring) and then oviposit fertilized eggs (i.e., produce female offspring) when exposure times are longer and there is more time to repeatedly assess host quality. Sex ratio of offspring has important demographic implications as it affects rates of population grown. Male-biased progeny production on non-target hosts of marginal quality may limit or negate adverse population-level impacts on non-target species (32).

Anastatus orientalis offspring reared from non-target host species successfully parasitized *L. delicatula* egg masses. Percent parasitism and sex ratios resulting from mated couples that emerged from the same non-target host species were similar to values recorded for *A. orientalis* reared continuously on *L. delicatula*. Interestingly, males that emerged from *G. arizonensis* and *P. fuliginosa* when paired with unmated *A. orientalis* females that emerged from *L. delicatula* failed to produce any offspring. Due to the low number of repetitions for these experiments this finding should be viewed with caution. However, no obvious biological explanation (i.e., size of the males or inability to copulate with females) was observed to explain these results as unmated *A. orientalis* females should be able to oviposit unfertilized eggs that produce male offspring without mating with males that emerged from *G. arizonensis* and *P. fuliginosa*.

In laboratory studies in a quarantine facility, sequential no-choice and choice exposure studies that exposed female *A. orientalis* to non-target eggs of 34 native and non-native species in three orders and 12 families and target eggs (*L. delicatula*) for either 24 hours or 7 days, indicate that this egg parasitoid potentially has a wide host range as it successfully parasitized 15 species in 6 families in two orders (Hemiptera and Lepidoptera). Results presented here are for *A. orientalis* (Haplotype C). Other haplotypes of *A. orientalis* have been identified and are being assessed to determine if differences (i.e., greater specificity) in host preferences exist (20). A well-founded criticism of host-range tests is that parasitoids are constrained in small ventilated containers spaces with easily accessible hosts for long periods of time (i.e., 24 hours to 7 days) and are unable to engage in behaviors (e.g., rapid abandonment of patches with sub-optimal hosts) that could reduce or eliminate non-target use (49–52). Consequently, in the absence of comprehensive field data on host use and non-target species - target species - parasitoid phenology in the native range (i.e., China) from where *A. orientalis* was sourced, it is difficult to determine if high levels of non-target host use observed in host range tests reported here occurs in the field. Until detailed field data from the native range are available and based on results of host range tests reported here that suggest *A. orientalis* [and possibly most species of *Anastatus* (23–27, 32, 33)] has a very broad host range, use of this natural enemy in classical biological control of *L. delicatula* in the western U.S. should be assessed critically and with extreme caution.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

FG-M conceived and designed the experiments, performed collections and experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. DY performed collections, reviewed drafts of the paper, and approved the final draft. MR performed collections, reviewed drafts of the paper, and approved the final draft. MH obtained the funding, conceived and designed the experiments, performed collections authored or reviewed drafts of the paper, and approved the final draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Life history traits of spotted lanternfly (Hemiptera: Fulgoridae) when feeding on grapevines and tree of heaven

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The invasive planthopper, spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), feeds on a broad range of plants including species of economic importance such as grape. Although SLF feeds on wild and cultivated grape, the effect of grapevines on the insect's life history traits is unknown. This study examined the effect of cultivated Concord grapevines (*Vitis labrusca*) and the insect's preferred host tree of heaven (TOH), *Ailanthus altissima*, on SLF development, survival, reproduction, and body mass. Newly emerged nymphs were allowed to feed on either TOH, Concord grapevines or a mixed diet of Concord grapevines plus TOH through adulthood until death. Development, mortality, and oviposition of paired adults were tracked daily to calculate the SLF rate of development, survival, and reproduction among treatments. When feeding exclusively on Concord grapevines, SLF was able to develop and reproduce but had higher mortality, slower development, and produced fewer eggs. SLF fed on the mixed diet of grapevines plus TOH exhibited faster nymphal development, laid more eggs, and had higher body mass compared with those fed only on grape or TOH. SLF had greater survival when fed on either the mixed diet or on TOH alone. We conclude that Concord grapevines are a poor-quality host for SLF, but when combined with TOH, SLF fitness increases above that of feeding on TOH alone. This study supports the elimination of TOH as a part of SLF vineyard management practices.

KEYWORDS

spotted lanternfly, grape, development, mortality, reproduction, fitness, tree of heaven, concord

1 Introduction

Lycorma delicatula (White) (Hemiptera: Fulgoridae), commonly known as the spotted lanternfly (SLF), is an invasive planthopper introduced into the United States. SLF is native to southeast Asia and was first detected in Berks County, Pennsylvania (PA) in 2014 (1). Despite efforts to control and contain its populations, SLF has spread to numerous states in the Northeast, Mid-Atlantic, and Midwest regions of the U.S. The insect is highly

polyphagous and can reach high numbers making it difficult to control. In Asia, 73 plant species within 32 families have been reported as hosts for SLF nymphs and adults (2). Worldwide, SLF has been reported in association with over 100 plant taxa, many of which are crops, representing a threat to U.S. agriculture, especially grapevines (3).

SLF's preferred host is *Ailanthus altisimma*, commonly known as tree of heaven (TOH), which is a deciduous invasive tree native to China and first introduced to the U.S. as an ornamental species in Philadelphia, PA in 1784 (4). Other common hosts include *Acer* spp. (maple), *Juglans nigra* (black walnut), *Salix* spp. (willow), and *Vitis* spp. (grapevines) (5, 6). Despite its broad host range, SLF seems to be particularly detrimental to TOH and grapevines. High numbers of SLF individuals have been observed in vineyards in early fall in the SLF quarantine zone of Pennsylvania (7). SLF harms plants directly by feeding on phloem sap and indirectly by excreting honeydew leading to the growth of sooty mold (5). SLF causes loss of plant vigor and inhibition of photosynthesis in its host plants (6, 8). Extensive SLF feeding suppresses photosynthesis, sap flow and carbohydrate storage in grapevine roots compromising vine health (9). Economic losses in vineyards are associated with reductions in yield, increased use of insecticides for SLF control, and vine decline (7, 9).

SLF is univoltine; adults lay eggs in the fall from September to the first hard freeze. The eggs overwinter and hatch in the spring; the resulting nymphs undergo four nymphal stages before reaching adulthood in July and August. After mating and undergoing reproductive maturation for several weeks, female SLF lay egg masses on a variety of surfaces, including tree trunks, plant stems, posts, rocks, vehicles, and outdoor equipment (2, 10). Eggs can easily be moved by humans to other geographical regions aiding dispersal to distant sites (10). It is unknown how many egg masses can be laid by one mated female in its lifetime and the length of their preoviposition period. However, it has been reported that each SLF female can lay at least two egg masses before the first frost, and each egg mass contains between 20-50 eggs (2, 10). The duration of the nymphal stages is likely to vary with local environmental conditions due to the strong influence of temperature on insect development (11, 12). The optimal growing temperatures for SLF are 15-30°C and the growing degree days (GDD) required for development into their second, third, fourth instars, and adults have been calculated as 166.6, 208.7, 410.5, and 620, respectively (11). The base threshold temperature is the minimum temperature needed for an insect to develop; the base temperature for SLF has been calculated to be 10.4°C for egg development (12) and about 13°C, 12.43°C, 8.48°C, and 6.29°C for first through fourth instars, respectively (11).

Besides temperature, host plant diet also affects SLF development and life cycle duration (13). For several years after introduction to the U.S, it was assumed that SLF could not survive and reproduce without TOH. However, recent studies showed that SLF can complete its life cycle without TOH and reproduce on other hosts, including grapevines (8, 13, 14). SLF nymphs successfully develop into adults when fed on single diets of TOH and black walnut (13). Similarly, mixed diets of TOH and either apple or black walnut support SLF development to adulthood and reduce time of development (13). In wild conditions SLF nymphs and adults are often found on TOH, multiflora rose (*Rosa multiflora*), and grape (*Vitis aestivalis*) suggesting that these may be preferred hosts for different life stages (15). However, under controlled conditions, grapevines (*Vitis rotundifolia* Var Carlos) seem to only support SLF development to the fourth instar. Despite the economic importance of the grape and wine industry in the U.S., assessed at several billion dollars, the effect of commercial grape cultivars on SLF life history traits has not been investigated.

The goal of this study was to determine the effects of host plant diet on SLF life history traits using TOH and grapevines (*Vitis labrusca*) as single hosts and in combination as a mixed diet. We measured development rate in days and GDD, mortality rate, and reproductive success as the number of eggs laid, hatch rate, and adult dry mass. We hypothesized that SLF fed on mixed diets of grapevines plus TOH would have a shorter development time, lower mortality rate, higher reproductive success, and greater dry mass than when fed on either host alone. The findings of this study contribute to our current knowledge of SLF biology and may help with the design of SLF management strategies in vineyards.

2 Methods

2.1 Research site

This study was carried out under field conditions in Alburtis, PA within the Pennsylvania SLF quarantine zone from May to November of 2021. The field site was located at coordinates 40° 26' 43.368" N, 75° 37' 34.752" W in an area of approximately 1,200 m^2 of land surrounded by trees and shrubs. Most trees near the field site were *Juglans nigra* (black walnut) and *Carya illinoinensis* (pecan). Adjacent to the site was a pond, a corn field, and cattle. The ground was covered by grass, over which black weed barrier (FLARMOR Pro Garden, 20 x 40 m) was placed to prevent grass overgrowth.

2.2 Plant material

Seeds of *Ailanthus altissima* were collected in the fall and winter of 2017-2020 from wild trees in State College, PA. The seeds were sown in a germination tray (25.4 x 50.8 cm with drain holes, Tru Leaf Market, Salt Lake City, UT) with growth media mix [Sunshine Mix 4 (peat moss, perlite, starter nutrient charge, dolomitic limestone, and long-lasting wetting agent), Sungro Horticulture, Agawam, MA]. The first set of 1,000 seeds sown in February 2021 were placed in a tray without seed alteration. The next set of 1,000 seeds sown in April had the seed coat manually removed by gently peeling the outer skin. Seeds sown with the seed coat removed had a higher percentage germination than seeds with the seed coat intact: 17.6% and 5.3%, respectively. Seeds with the seed coat intact germinated after 4 weeks, while seeds without the seed coat germinated within 2 weeks. Seedlings of about 10 cm in height were transplanted into 11.43 cm pots (Greenhouse Megastore, Sacramento, CA). TOH plants of about 20 cm tall were transplanted again into 9.46-liter pots (Greenhouse Megastore, Sacramento, CA) at Berks County, PA in June 2021. The growth

media in the 9.46-liter pots consisted of a mixture of Sunshine Mix 4 and topsoil (Scotts Premium, Home Depot, State College, PA) at a 2:1 ratio. Plants were fertilized with 37 g of Osmocote plus (N:15, P:9, K:12), plus micronutrients six weeks after germination. Each plant was further supplemented once with a 500 ml solution of 10% chelated iron and 8% nitrogen (Sequestrene Iron 330 Fe, ProSolutions LLC, Maryville TN) 10 weeks after germination. The solution was prepared by diluting 4 g of the fertilizer in 4L of water. Bare root canes of Concord grapevines (*Vitis labrusca*) of ~2.5 cm stem diameter were purchased from Amberg Grapevines, LLC (Clifton Springs, NY) and planted in April of 2021. The vines were planted in 9.46-liter pots containing growing media (Sunshine Mix 4) and topsoil (Scotts Premium) in a 2:1 ratio. The vines were fertilized as described for TOH above. Grapevines of ~ 30 cm tall were used for the experiments in late May; subsequently, the vines were pruned regularly to a height of ~35 cm and fruit clusters were removed as they developed. TOH and grapevine plants were grown from February to May under greenhouse conditions (14:10 h of light: dark) at the Pennsylvania State University, University Park, PA. In early June, the plants were transported to Alburtis, PA for the experiments.

2.3 Insects

SLF egg masses were wild collected from Blue Marsh, PA (40° 23' 60" N, 76° 4' 11.99" W) in March 2021. The egg masses were either scraped off trees by cutting underneath the bark with a sharp knife or the masses were collected from smaller branches that were cut into pieces. The egg masses were then stored in plastic storage bins (79 x 51 x 38 cm) in a cooling chamber kept at 4°C for 60 days. After removal from the cooling chambers, the egg masses were placed in mesh cages [(90 x 60 x 60 cm), Jinhua Quiangsheng Outdoor Products, Zhejiang China] with TOH plants in ambient conditions at the research site for 3 weeks until nymphs emerged. Freshly emerged SLF nymphs were collected daily and immediately placed into their designated treatment cages.

2.4 Survivorship and development of spotted lanternfly in grape and tree of heaven

Newly emerged SLF nymphs were transferred to mesh cages (90 x 60 x 60 cm) containing one of the following plant treatments: TOH, Concord grape, or Concord plus TOH. Each cage was infested with five first-instar SLF nymphs that hatched the same day. The survival and development of SLF individuals from each cage was recorded every day until death. Throughout the season, plants were monitored for disease and replaced as needed to sustain the SLF individuals. Once the nymphs emerged as adults, individuals coming from the same plant treatment were paired into male and female couples and isolated in a cage containing the same combination of plants in which the nymphs developed. Grapevine and TOH plants used for adult feeding were ~45 cm tall and 5-months old from the time they were transplanted. Dead SLF males were replaced with new ones from the same plant treatment. Dead female adults were not replaced after oviposition. Adult survival was monitored until adults died naturally when temperatures reached 0 °C.

2.5 SLF oviposition

We recorded the number of SLF couples that laid egg masses, the number of egg masses laid by each SLF couple until first frost, the number of eggs within each egg mass, and the number of nymphs that hatched from those eggs. Within each cage of adult pairs, a polywood (7 x 60 cm) substrate was added for oviposition. SLF females laid their egg masses on either the Polywood, the side of the mesh cages, or on the plant itself. Egg masses laid on plants were collected by cutting the plant piece where they were laid, while egg masses laid on the cages were carefully scraped out and placed into 50 ml plastic tubes covered with mesh lids to allow air flow. Eggs laid on the polywood were left on that substrate and placed in plastic bins (79 x 51 x 38 cm). The egg masses were stored in a cooling chamber for 6 months at 4 °C.

2.6 SLF egg mass hatch

The collected egg masses were removed from the cooling chamber and acclimated to the ambient temperature in mesh cages (90 x 60 x 60 cm) in a greenhouse in April 2022. The number of eggs per egg mass was counted under a stereoscope (SZ30, Olympus, Tokyo, Japan) after gently brushing over the protective wax layer with a wet paper towel to reveal the eggs underneath. The number of hatched SLF nymphs were counted and divided by the number of eggs laid to calculate the percent of egg hatch. Hatch rate was recorded to document a successfully completed life cycle of the adult pairs.

2.7 SLF adult weight gain

Weight gain was determined for each SLF individual from the survivorship experiment that successfully developed into an adult. The adults were collected as they died, placed individually in properly labelled 5 ml tubes (Thermo Scientific) and stored at 4° C. Subsequently, each SLF adult was placed in a paper bag (7.6 x 5.1 x 15.2 cm) and dried in an oven at 60°C until their weight remained constant. The weight of each specimen was determined using an analytical scale accurate to 0.1 mg (Ohaus Adventurer™ Analytical Balance model AX124/E). The weight of each adult was standardized by the number of GDD it accumulated before dying; the standardized dry weight values were used for the statistical analyses.

$$Standardized\ \ Dry\ \ Mass = \frac{Adult\ \ SLF\ \ Mass\ \ (mg)}{Adult's\ \ Total\ \ GDD}$$

2.8 Weather data

Temperature (°C), humidity (%), and rainfall (mm and mm/h) were recorded daily at the research site using a Davis 6152 wireless Vantage Pro2 Weather Station (Scientific Sales Inc. Lawrenceville, NJ. USA). Measurements with the weather station began June 30, 2021. Temperature data prior to June 30 was collected using Weather Underground weather history (TWC Product and Technology LLC 2014, 2022).

2.9 Experimental design and data analysis

To determine differences in development and survival of SLF nymphs on different plant diets, each experimental unit was comprised of five nymphs enclosed in a mesh cage with its respective plant treatments. For adults, the experimental unit comprised one couple (male and female) enclosed in a mesh cage with the same plant treatment in which they developed as nymphs. The experimental units (cages) were set up in a completely randomized design at the research site

2.9.1 Development

SLF development was analyzed by calculating the number of days and the number of GDD required for each nymph to molt into the next developmental stage (instar or adult) using the following formula described by Herms (16).

$$GDD = \left(\frac{Max\ \ temperature + Base\ \ temperature}{2}\right) - Base\ \ temperature$$

GDD calculations that resulted in a negative value were replaced with 0. Calculating the GDD using the average of the maximum temperature and base temperature (Modified Average Method) has been reported to be more accurate than the average of the maximum and minimum temperatures (Average Method) because it accounts for periods of time when the temperature is above the base threshold even if the average temperature is below it (16). Development still occurs when the average temperature is below the base threshold if the maximum temperature surpasses the base temperature (16). The base temperatures used for calculating GDD for first through fourth instar nymphs were 13.00°C, 12.43°C, 8.48°C, and 6.29°C, respectively (11). GDD were summed for each individual per instar to obtain the accumulated GDD. We averaged the number of days and the number of GDD it took the nymphs within each experimental unit to develop into their next stage; this value was used as an independent replication for statistical analyses. Differences among treatment means for the GDD per instar and the number of days spent in each instar were analyzed with one-way Analysis of Variance (ANOVA). Significant differences between treatment means were elucidated with the Tukey test at alpha = 0.05. GDD data from first, fourth, and total instars were transformed using inverse squared. For the second instar we used the inverse transformation, and for the third instar we used an inverse square-root transformation to meet the assumptions of normality and equal variances before pursuing the ANOVA. Data for development time in days were transformed using the inverse for the first and second instar, log base 10 transformation for the third, and inverse square root transformation for the fourth instar to meet the assumptions of normality and equal variances.

2.9.2 Survival

We calculated the percentage of nymphs that survived per instar and the percentage of adults that survived from emergence to first frost for each experimental unit. Each data point from an experimental unit was used as an independent replicate. Differences in survival rates per treatment and SLF biological stage were analyzed using a generalized linear model (GLM) that best fitted the error distributions of proportion data. We fitted a binomial model with a logic link function and tested the significance of the model terms using an analysis of deviance. Overdispersion was tested using the deviance and Pearson Goodness of Fit tests (17); in the presence of overdispersion, a quasibinomial model was fitted (17). Multiple comparisons between treatment pairs were assessed using the glht-tukey method implemented in the multcomp R package (18). In addition, we constructed Kaplan-Meier survival curves for the nymphal stage (first to fourth instar) to better visualize the survival probability of SLF feeding on different plants. When all the nymphs within a cage died, that experimental unit was registered as dead or 1 in the data base, while experimental units with nymphs alive were evaluated as "censored" or zero in the data base. To calculate the time to death, we averaged the days alive of each nymph per instar within each experimental unit and used that value for the K-Meier model. Statistical differences among treatments were determined using the log-rank test (19).

2.9.3 Life table analysis

The number of days SLF spent in each instar was used to construct a life table. Life table analysis displays the proportion of experimental units alive in each treatment at the beginning of each life stage or instar. The probability of surviving the period was calculated by the average proportion of experimental units alive by the end of each life stage divided by the number of experimental units alive at the start of the life stage. Percent probability of death was calculated using the average percent mortality for experimental units within each life stage. Cumulative number of days of survival beyond each life stage (*Age * Tx*) was the average cumulative survival days of each experimental unit.

2.9.4 Oviposition

To assess the effect of each plant treatment on SLF oviposition, we calculated the percentage of couples that laid egg masses out of the total number of initial pairs, the average number of eggs laid per egg mass, and the percentage of nymphs that hatched from those eggs. When a single female laid more than one egg mass, data were averaged for that female. Data for couples that came from the same experimental units in their nymphal stage were averaged and the resulting number used as an independent datum for the statistical analyses. The number of experimental units for adult SLF couples was 51 for TOH treatment, 8 for Concord, and 30 for Concord plus TOH treatment. From these, the total number of independent

replications per treatment was 45 for the TOH treatment, 4 for Concord, and 26 for Concord plus TOH. Differences in the average number of eggs laid per egg mass and the total number of eggs per treatment were assessed with one-way ANOVA followed by the Tukey test. Differences in the percentage of nymphs that hatched between treatments were analyzed using Chi-square. The number of GDD and days from female emergence to the first egg mass laid (preoviposition period) were calculated as explained above for SLF development. Differences among treatment means were analyzed with one-way ANOVA followed by a Tukey test at alpha = 0.05.

2.9.5 Weight gain

The dry weight of SLF adults was standardized by dividing the individual's weight by the total GDD accumulated by each adult using the base temperature of 10.4 °C (12). The standardized data were then analyzed using a one-way ANOVA followed by a Tukey test at alpha = 0.05.

3 Results

3.1 Spotted lanternfly development

SLF nymphs feeding on Concord grapevines developed slower than nymphs feeding on Concord plus TOH or TOH alone (Table 1). The average number of GDD required for nymphal development across treatments increased gradually from first to fourth instar [mean ± SEM:123.42 ± 1.83 (n=120), 136.1 ± 2.3 (n=116), 214.4 ± 5.3 (n=105), 296.7 ± 7.3 (n=81), respectively] for all treatments. There were no significant differences in the number of GDD and development time in days between treatments for the first instar (Table 1, rows 2-4). Nymphs feeding on the single-host Concord diet began to display significantly slower development (required more days and GDD to molt) by the second instar compared to the single-host TOH or the mixed-host diet of Concord plus TOH (Table 1, rows 5-7). SLF feeding solely on Concord vines required on average between 3.7 to 6.1 more days to develop into the third instar, and between 3.5 to 13.1 more days to develop into their fourth instar than those fed on mixed-host diets or TOH alone. Second instars fed on Concord alone required two more days to develop than those fed on mixed-host diets (Table 1). The total number of GDDs required to develop from first instar to adult eclosion were between 146 to 206 greater when fed on Concord compared with other diets, but the development time in days did not differ statistically among treatments (Table 1). Overall, SLF individuals fed on Concord grapevines required the greatest number of GDDs to develop through the nymphal stages (894.2 ± 34.3), whereas individuals feeding on the mixed-host diet of grape plus TOH required the fewest GDDs (688.2 ± 14.9).

TABLE 1 Average growing degree days and number of days required for each spotted lanternfly (SLF) instar to develop when fed on Concord grape, tree of heaven (TOH), or the combination of Concord and TOH.

SLF Instar	Plant Treatment	N	Average GDD ± SEM	Average development time (days ± SEM)	df	GDD		Development time (days)	
						F-value	P-value	F-value	P-value
First	Concord + TOH	26	121 ± 3.4a	16.7 ± 0.4a	2, 117	2.48	0.089	1.22	0.299
	TOH	47	120.9 ± 3.3a	16.5 ± 0.4a					
	Concord	47	127.3 ± 2.6a	17.0 ± 0.3a					
Second	Concord + TOH	25	126 ± 3.2a	13.9 ± 0.4a	2, 113	6.73	0.002	5.01	0.008
	TOH	46	132.5 ± 3.9a	14.9 ± 0.4ab					
	Concord	45	145.3 ± 3.6b	15.9 ± 0.4b					
Third	Concord + TOH	23	178.2 ± 5.4a	17.6 ± 0.6a	2, 102	18.29	<0.0001	15.83	<0.0001
	TOH	45	205.4 ± 7b	20.0 ± 0.7a					
	Concord	37	248 ± 9.4c	23.7 ± 0.7b					
Fourth	Concord + TOH	23	265.3 ± 10.1a	22.5 ± 0.9a	2, 78	12.33	<0.0001	24.9	<0.0001
	TOH	45	290.6 ± 7.5b	26.0 ± 2.4b					
	Concord	13	373.6 ± 22.4c	35.6 ± 0.8c					
Total	Concord + TOH	23	688.2 ± 14.9a	70.5 ± 1.6a	2, 78	19.48	<0.0001	0.09	0.912
	TOH	45	748.9 ± 14.5b	77.4 ± 1.6a					
	Concord	13	894.2 ± 34.3c	90.8 ± 3.8a					

Different letters indicate significant differences among treatment means obtained with the Tukey test at alpha=0.05 following ANOVA. N= number of experimental units, GDD= number of growing degree days, SEM= Standard error of the mean, df= degrees of freedom (treatment, error), F-values and P-values were obtained with one way ANOVA.

3.2 Survival

SLF survival varied at different stages of development and by host-plant diet. The average survival of nymphs across treatments was 64.88% for first instars, 90.87% for second instars, 89.48% for third instars, and 82.2% for fourth instars (Table 2). SLF survival was also affected by host plant diet; third and fourth instars had significantly lower survival when fed on Concord grapevines alone compared to those fed on either TOH or the mixed-host diet of grape plus TOH (Table 2). The average survival rate of SLF nymphs from first instar to adult emergence was lowest on Concord (6.3%) compared with TOH (37.7%) and the mixed host diet [(50.6%), (Table 2, rows 14-16). The average survival of adults to the first frost in November 2021 was 58.36% across treatments. Adults fed on Concord had the lowest survival rate compared with those fed on either TOH or the mixed host diet treatment (Table 2, rows 17-19). Adult SLF individuals feeding on Concord alone also had the shortest life span before the first frost of the season (17 ± 5.1, n=6) compared with those feeding on TOH alone (47.4 ± 2.5 days, n=40) and the mixed host diet (42.5 ± 6 days, n=16), ANOVA $F_{2,59} = 7.26$, $P<0.05$)]. Overall, the lowest survival rates across treatments were for adults and first instar nymphs. The highest survival rates of SLF nymphs and adults were for individuals fed on the mixed host diet and the TOH treatments (Tale 2, column 6).

The Kaplan-Meier Survival Curve (Figure 1; Table 3) shows the SLF survival probability throughout the four nymphal instars. SLF fed on TOH and the mixed diet of grape plus TOH had a cumulative survival rate above 80% throughout all four nymphal stages while SLF fed on Concord reached 50% survival probability before day 60, which occurred in the fourth instar (Figure 1). The log rank test showed significant differences in survival probability between SLF fed on the mixed diet and Concord ($\chi^2 = 17.1$, $P<0.05$), and between nymphs fed on Concord and those fed on TOH ($\chi^2 = 52.4$, $P<0.0001$). There were no significant differences in survival probability between the mixed diet treatment and TOH alone ($\chi^2 = 3.1$, $P >0.05$).

A life table summarizes the cumulative probability of survival at the beginning of each instar (Lx) and the probability of surviving the instar [(Npx), (Table 3)]. SLF nymphs feeding on the mixed diet consistently had the highest probability of survival in their first to third instar, whereas fourth instar nymphs had a higher probability of survival when fed on TOH and the mixed diet treatment (Table 3, column 4). Nymphs fed on TOH alone also survived the greatest number of days after each consecutive instar (Table 3, column 7).

TABLE 2 Survival of SLF nymphs and adults when fed on Concord grape, TOH, or the combination of grape and TOH. The "Initial No. of nymphs" describes the total number of individual nymphs in each treatment at the beginning of the experiment.

Instar	Plant Treatment	Initial No. of nymphs	Initial No. of Experimental Units	Experimental Units Alive (%)	SLF Survival (%)	df	F/Z Values	P- Value
First	Concord + TOH	132	26	100	68.59a	2, 124	0.853	0.428
	TOH	255	51	100	60.4a			
	Concord	251	50	100	65.67a			
Second	Concord + TOH	90	26	100	95.52a	2, 117	2.3159	0.103
	TOH	154	47	92.157	89.79a			
	Concord	165	47	94	87.31a			
Third	Concord + TOH	84	25	96.2	95.2a	2, 114	13.174	7.12e-06
	TOH	130	46	90.2	94.4a			
	Concord	135	46	92	78.84b			
Fourth	Concord + TOH	78	23	88.5	91.3a	2, 101	-7.1366	9.5e-13
	TOH	117	44	86.3	93.2a			
	Concord	87	37	74	62.26b			
Average Nymph Survival Rate	Concord + TOH	132	26	88.46	50.6a	2, 124	26.121	3.41e-10
	TOH	255	51	86.27	37.7a			
	Concord	251	50	24	6.3b			
Adults before first frost	Concord + TOH	66	23	88.5	72.7a	2, 76	-3.5488	3.87e-04
	TOH	104	44	86.3	58.7a			
	Concord	16	12	24	43.7b			

The "Experimental Units Alive (%)" describes the percentage of experimental units remaining in each instar with at least one SLF individual alive. "SLF survival (%)" represents the percent of individuals that survive per life stage out of those that molted into that stage. Mortality rates of each nymphal instar and adults were analyzed using a generalized linear model (GLM). Different letters indicate significant differences among treatments using the *post hoc* glht-Tukey test implemented in the multcomp R package (17). df =degrees of freedom (treatment, error), F/Z: F-values obtained from fitting quasi-binomial models and Z values were obtained from fitting binomial models.

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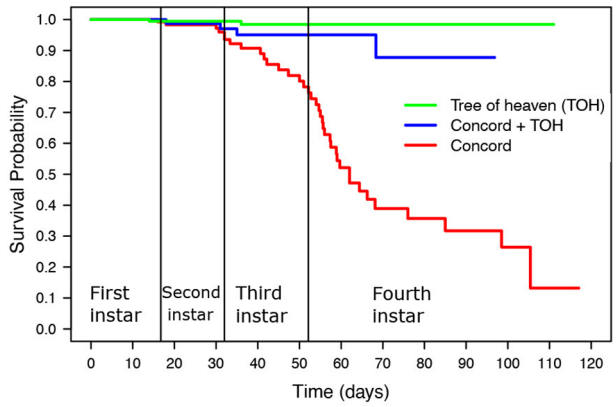

FIGURE 1
Kaplan Meier survival curves of SLF nymphal instars fed on tree of heaven (TOH), Concord, and Concord plus TOH.

3.3 Spotted lanternfly reproduction

The pre-oviposition period (time from adult emergence to first egg mass laid) in SLF ranged from 30-50 days, which corresponded to 250-500 GDDs using a base temperature of 10.4°C (12) (Table 4, column 3). There were no significant differences in the number of days or GDD during the pre-oviposition period among treatments ($F_{3,52} = 1.15$, P = 0.338). The number of SLF females that laid at least one egg mass was greatest in the single diet of TOH. However, paired SLF females fed on Concord plus TOH laid the greatest number of egg masses [(column 4), ($\chi^{2\ =}$ 21.221, df = 12, P = 0.04724). Similarly, SLF females fed on Concord plus TOH laid significantly more eggs than those fed on TOH alone ($F_{3,53} = 5.16$, P = 0.003; Table 4). Females fed on the mixed diet laid on average 2.58 egg masses and 94.89 eggs per female, whereas those fed on TOH laid on average 1.72 egg masses and 48 eggs per female. SLF females fed on Concord only laid one egg mass containing 45 eggs. The average number of eggs per egg mass ranged from 20 to 45 (Table 4). The number of first instar nymphs that hatched from these egg masses was <10.5% for all treatments with no significant differences in percent hatch among treatments ($\chi^{2\ =}$ 29.87, df = 45, P = 0.9597, Table 4).

3.4 Adult weight gain

Adult dry mass was influenced by host diet and gender (Figure 2). Females on average weighed 43 mg more than male

TABLE 3 Life table comparison of SLF in each instar fed on either Concord, TOH, or Concord plus TOH plants.

Treatment	Instar	Proportion of Individual Surviving (Lx)	Probability of Surviving the Instar (Npx)	lx*px	Percent Probability of Death 100 qx	Cumulative Number of Days Lived beyond Age*Tx
Concord + TOH	First	1.00	0.686	0.69	31.41	96.65
	Second	0.69	0.955	0.66	4.48	82.71
	Third	0.66	0.952	0.62	4.80	65.07
	Fourth	0.62	0.913	0.57	8.70	42.56
TOH	First	1.00	0.604	0.60	39.60	108.31
	Second	0.60	0.898	0.54	10.21	93.54
	Third	0.54	0.944	0.51	5.60	73.41
	Fourth	0.51	0.932	0.48	6.80	47.40
Concord	First	1.00	0.657	0.66	34.33	92.36
	Second	0.66	0.873	0.57	12.69	76.38
	Third	0.57	0.788	0.45	21.16	52.65
	Fourth	0.45	0.623	0.28	37.74	17.00

The total proportion of experimental units alive at the beginning of each instar (Lx), Npx describes the survival probability in each instar, lx*px depicts the proportion of experimental units alive to the total initial experimental units, the "Percent Probability of Death 100 qx" is the percent mortality per instar, and the cumulative number of days SLF is alive after each instar is denoted by Age*Tx.

TABLE 4 Reproduction parameters of SLF individuals grown on Concord grape, TOH and Concord plus TOH.

Treatment	SLF Couples that Oviposited (%)	Pre-Oviposition		Total Egg Masses	Avg. Number of Eggs per Egg Mass ± SEM	Avg. Percent of egg Hatch
		Avg. GDD ± SEM	Avg. Days ± SEM			
Concord +TOH	73 (n=26)	384.5 ± 14.2	44.2 ± 1.51	49	34.9 ± 2.27	10.2
TOH	45 (n=49)	370.3 ± 16.5	43.5 ± 1.53	38	25.8 ± 2.12	5.7
Concord	9 (n=11)	168.2 ± 0	34 ± 0	1	45.0 ± 0	0

Average number of growing degree days (Avg. GDD) and average number of days (Avg. Days) from female emergence to oviposition; SEM= standard error of the mean.

adults. Females fed on Concord plus TOH had the highest dry mass ($\bar{x}$ = 114.2 ± 4.9 mg, n= 17), followed by those fed on TOH alone ($\bar{x}$ =85.3 ± 6.7 mg, n=35). Females fed on the single Concord diet had the lowest dry mass with an average of 44.6 ± 11.3 mg ($F_{2,54}$ = 8.9, P< 0.001; n=5). Weight gained by male adults showed a similar trend to those of females; males fed on Concord plus TOH had significantly greater dry mass ($\bar{x}$ =59.4 ± 2.6 mg; n=18) than males fed on TOH ($\bar{x}$ =43.2 ± 2.3 mg; n=34) or Concord alone [($\bar{x}$ =27.4 ± 11 mg; n=4), ($F_{2,53}$ = 13.71, P< 0.001)].

3.5 Weather data

The maximum temperature recorded at the Alburtis PA field site was 35.5°C while the minimum temperature recorded was -4.4°C, on August 13 and November 2, respectively. The maximum temperatures above 33°C occurred in the months of July and August 2021. The minimum temperatures below 0°C occurred in the first week of November 2021 (Supplementary Figure 1). Daily rainfall recorded at the field site measured 5 peak rainfall days with over 20 mm of rain. Days with more than 20 mm of rainfall were August 18 and 22-23, September 1 and 23, and November 3, 2021 (Supplementary Figure 2). Hurricane Ida was a category 4 Atlantic hurricane that affected Pennsylvania August 30-September 5 as a tropical storm. The storm hit the field site on September 1 with total rainfall of 56.9 mm for the day. Maximum percent humidity consistently ranged from 90-100% while the minimum percent humidity varied between 34 and 96%. Peaks in minimum percent humidity occurred simultaneously with rainfall (Supplementary Figure 3).

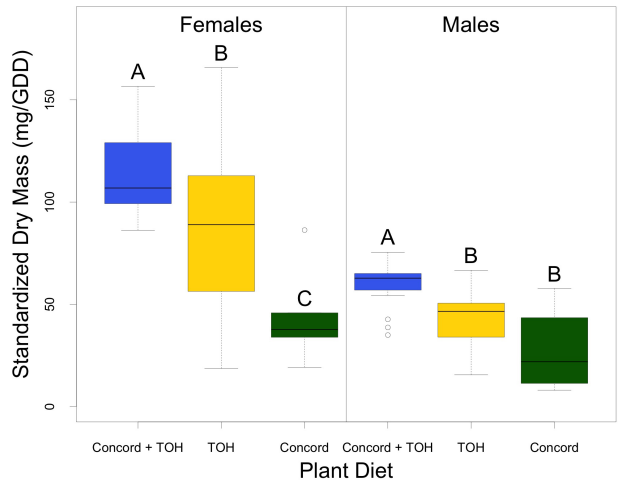

FIGURE 2
Weight gained by SLF adults fed on different diet treatments. Dry mass was standardized by dividing the raw dry mass by the growing degree days. Horizontal bars represent the medians, the box represents the interquartile range, the whiskers represent the range of the data scores, and dots outside of the plots are outliers. Differences among treatment means were analyzed with one way ANOVA. Differences between treatment pairs were analyzed with the Tukey test (alpha = 0.05) following ANOVA. Different letters indicate significant differences among treatment means. Data did not require transformations to meet the assumptions of normality and equal variances before doing the ANOVA.

4 Discussion

The results of this study show that host plant diet has a strong influence on SLF fitness and biology. SLF individuals were able to develop and reproduce when feeding exclusively on Concord grapevines; however, there was high percentage mortality of nymphs from the first instar to adult emergence (93.66%), development was slower for these nymphs, and adults laid fewer eggs than those feeding on a mixed diet or on TOH alone. SLF individuals feeding on a mixed diet of grape plus TOH had faster development to adulthood and laid more egg masses than those fed on single diets of either grape or TOH. At the nymph stage, survival was highest when feeding on TOH and the mixed diet.

Development rates also varied among diet treatments. SLF developed faster when fed on mixed diets of grape and TOH; there was no influence of diet on the development of first instars, but as the nymphs reached their second, third and fourth instar, there were significant differences in development between SLF feeding on different host plants. Nymphs fed on a mixed diet of grape and TOH developed faster than those fed on Concord alone. No significant differences in development were observed for first instar nymphs, possibly due to low nutritional requirements of that stage to enhance survival. Second instar nymphs developed the slowest when fed on grape as a single diet. In general, nymphs required the lowest GDD when fed on mixed diets and the highest when fed on Concord grape alone (Table 1). These differences in rates of development may relate to the nutritional quality of a mixed diet versus a single host diet (20). Studies have shown that TOH is a high-quality host plant for SLF (8, 14), which may be due in part to their shared native range and history of host plant preference or co-evolution (5). The GDD required for second-fourth instar nymphs to develop were lower than those reported in a previous study (11)

regardless of the calculation method used, i.e., the Average Method (not shown), and the Modified Average Method (16). Thus, the lower GDD ranges found in this study were likely due to different experimental conditions, i.e., field vs laboratory, microclimate inside experimental cages, differences in humidity, or stress from other abiotic factors. The microclimate within the cages could have been slightly different from the temperature recorded by the weather station due to the mesh enclosure and placement of cages on a black weed barrier, which may have increased the microclimate temperature in the cages, and the mesh obstructs some of the airflow, raising temperatures. This and previous studies agree that SLF can develop without access to TOH, but their development time is slower, their mortality is higher, and their oviposition is reduced (6, 13, 14).

Host plant diet also affected SLF survival. The average survival rate and the survival probability of SLF nymphs was lowest when feeding exclusively on Concord grape, and highest when feeding on the mixed diet and on TOH. These results suggest that Concord grape alone is a poor diet for SLF compared with the other treatments. Our results agree with a previous study in which mixed diets of TOH plus either apple, black walnut, grapevine (*Vitis rotundifolia*, var. Carlos), or peach improved SLF survival compared with single host diets (13). There was a significant decrease in the survival probability for SLF fed exclusively on Concord grapevines by the third and fourth instars, while survival on TOH and mixed-host diets remained above 80% through the four nymphal stages (Figure 1). Survival probability was similar for SLF feeding on the mixed diet and on TOH alone (Figure 1). Various studies have demonstrated that mixed diets improve growth rates in polyphagous herbivores compared with less diverse diets (21) and SLF is a highly polyphagous insect, with a reported host range of at least 100 different plant taxa (3). Two hypotheses have been proposed to explain this phenomenon; (i) the nutrient balance hypothesis proposed by Pulliam (22) argues that a mixed diet allows herbivores to switch between diets with contrasting nutrient content; and (ii) the dilution of toxin hypothesis proposed by Freeland and Janzen (23), which argues that mixed diets allow for dilution of plant secondary metabolites by feeding on plant material with different allelochemical content (22, 23). Studies with various herbivore species strongly support the nutrient balance hypothesis (21, 24), whereas the effect of toxic plant allelochemicals seems to depend on the food nutrient composition (25, 26). SLF feeds on plant phloem for which nutrient compositions are known to vary among plant species and with abiotic factors, developmental stage, and time of the season (27). Further, SLF dispersal capabilities may allow the insect to regulate its nutrient intake by feeding on multiple hosts.

SLF survival also varied for different developmental stages. The lowest average survival rates across treatments were found in adults and first instar nymphs compared with second through fourth instar nymphs (Table 2). This is likely due to disparate nutritional requirements of different life stages and possibly variation in tolerance to secondary compounds found in their diet. SLF is known to vary in its host preference at different stages of development (2, 5). Although highly polyphagous, adults are known to narrow their host plant preferences compared to nymphs (15). Early instar nymphs have been observed to feed on young plant growth and on herbaceous plants, whereas adults seem to prefer woody host plants and tissues (3). When feeding on grapevines, early instar nymphs feed exclusively on shoots and the veins on the undersides of leaves. Third and fourth instars can feed on shoots and cordons, whereas adults feed on shoots, cordons, large branches, and tree trunks (7). Variation of feeding sites within a single plant species may be associated with morphological variations in SLF mouthparts at different stages of development, and with differences in plant sap flow rate through the growing season (7, 28). Besides, the effects of host plant diet and insect developmental stage, we did not find an effect of local environmental conditions on SLF mortality, except for the first frost that killed the adults on November 2-4 of 2021. Surprisingly, Hurricane Ida on September 1 had no effect on SLF mortality. The cages had fallen over from the strong winds, but there were no spikes in mortality on the days following.

The mixed diet also improved SLF reproduction compared with single host diets of either grape or TOH. Fertilized females fed on the mixed diet laid the greatest number of egg masses and total eggs followed by those fed on TOH. Our results show that access to a mixed host diet containing TOH doubles the number of eggs oviposited by females when compared to a single diet of TOH. From the SLF females that fed on just Concord grapevines, one of them oviposited a single egg mass, but none of these eggs hatched. Poor quality diet is linked to poor reproductive rate and low-quality eggs, which can also force early reproduction to ensure a next generation (29). The average percent of eggs that hatched was very low for all treatments, which may have been due to our experimental conditions. Low percent egg hatch could have been affected by premature placement of the egg masses into cooling chambers, the storage period and temperature, or the acclimation to greenhouse conditions. It has been reported that prolonged egg storage beyond one month at 5 °C decreases SLF egg hatch rate (30). In a field study conducted in Berks (Pennsylvania) in 2017, egg hatch ranged from 51.5 to 84.2%, but egg hatch seems to be highly dependent on winter temperatures (2). The time from female emergence to oviposition ranged from 4-6 weeks, which is similar to previous field observations (2), indicating that the insect has a relatively short time to lay eggs before the first frost in the northeast U.S. Although male and female SLF couples were put together in cages soon after emerging, we have no record on when mating occurred. The insects showed a visible increase in the size of their abdomens (not measured) before they started laying eggs. This suggests that egg production and maturation seem to require a large accumulation of body reserves through food consumption. The pre-oviposition time did not differ among females reared on different host diets; however, more research should be conducted to explore the effects of diet on duration of the SLF preoviposition period and oviposition rates.

Diet type had a strong effect on the body weight of SLF adults. Females reared on the mixed diet gained more weight than those fed on single host diets, and males gained more body weight when fed on the mixed diet compared with those fed on TOH or Concord alone (Figure 2). Body weight is an indicator of insect health (31) and is associated with the nutritional quality of their host plants (32,

33). Also, the high variance within treatments can be explained by the presence or absence of eggs within the female's abdomen. Since the couples were actively laying egg masses at the time of death and sample collection, there may have been females that were unable to lay all their eggs or to mate. The ability to successfully mate can contribute significantly to the dry mass of both males and females due to the transfer of a large spermatophore from the male (5).

In summary, the results of this study show that SLF development, reproduction, and body mass benefit from a mixed diet with TOH compared to feeding solely on grapevines or TOH. SLF survival was highest when fed on either the mixed diet or on TOH. When feeding exclusively on Concord grapevines, SLF was able to develop and reproduce but its fitness was greatly reduced. Our results suggest that SLF management in vineyards could benefit from limiting access to TOH to reduce insect fitness, but more research is needed to compare variations of mixed diets on the insect's life cycle.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

FA designed the study. EL conducted the experiments. EL and FA analysed the data and wrote the manuscript. KH contributed to the identification of the research site, logistics in experimental set up and provided valuable input to the manuscript. All authors read, contributed to revisions, and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1091332/full#supplementary-material

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Persistence and distribution of dinotefuran in tree of heaven

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Spotted lanternfly (SLF) (*Lycorma delicatula* (White)), an invasive planthopper discovered in Pennsylvania, U.S.A. in 2014, feeds for approximately six months by sucking phloem sap from trunks and limbs of tree of heaven, *Ailanthus altissima*, along with several native trees and woody vines. Basal trunk sprays of dinotefuran, a systemic neonicotinoid insecticide, are commonly used to reduce SLF densities and spread. Information on dinotefuran persistence and within-tree distribution can help identify optimal timing of annual basal trunk sprays, facilitating efficient use of available resources. We applied dinotefuran to 20 uninfested *A. altissima* trees in early April then periodically sampled foliage to monitor insecticide residues. Foliar dinotefuran residues averaged (± SE) 7.8 ± 1.1 and 6.3 ± 1.2 in July and August, respectively, then dropped significantly to 2.6 ± 0.5 ppm in September. In a second study, 20 *A. altissima* trees were similarly treated with dinotefuran basal trunk sprays in early June. Trees were felled to collect foliage and phloem from branches and the trunk in either mid-July or September. Foliar residues averaged 12.7 ± 1.3 and 14.6 ± 2.2 ppm in July and September, respectively. For trees felled in July, residues were detected in phloem collected from below the spray line on trunks of seven trees and above the spray line on three trees, averaging 8.6 ± 4.4 and 7.4 ± 2.9 ppm, respectively. In trees felled in September, phloem from below spray lines of seven trees averaged 3.7 ± 1.3 ppm but dinotefuran was not detected in phloem from above the spray line on any trees. Dinotefuran was not detected in phloem sampled from any branches in either July or September. Results suggest dinotefuran basal trunk sprays applied between late May and mid June should persist long enough to effectively control SLF late instars and adults.

KEYWORDS

***Ailanthus altissima*, dinotefuran, spotted lanternfly, *Lycorma delicatula*, basal bark spray, insecticide residues**

1 Introduction

Spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), an invasive planthopper native to China and Taiwan, became established in Korea in 2004 (1) and was subsequently detected in the United States in Pennsylvania in 2014. Since then, established populations of SLF have been identified in localized areas of at least 14 states (2).

Predictive models based on climate and host distribution suggest SLF could potentially become established across much of the eastern U.S (3, 4). Although SLF adults typically engage in migratory flights and disperse to nearby areas in late summer or fall (5), long distance spread occurs when people accidentally transport SLF life stages into new areas.

Research in field sites in Pennsylvania has confirmed the univoltine life cycle of SLF. Egg hatch begins in mid April and peaks in May (6, 7). Nymphs feed throughout summer, completing four instars. Adults, which first appear in late July, feed intensively in aggregations during their four month life span (8). Mating can occur from early September through late October and oviposition occurs from mid September to early November (7, 8). Each female lays 1-2 egg masses containing 30 to 50 eggs on tree trunks or branches or on hard, solid items including boulders, bricks, outdoor equipment and vehicles (6–9). Egg masses overwinter until hatching begins the following spring.

Adults and all nymphal stages feed on phloem sap, excreting copious amounts of honeydew, which leads to growth of black sooty mold (*Capnodium* spp. [Dothideales: Capnodiaceae]) on host trees, vegetation and outdoor items below infested trees (6, 8). Black sooty mold reduces photosynthetic area of foliage, potentially affecting plant vigor as well as appearance, and contaminating agricultural crops (6, 8, 10). Wasps and ants are often attracted to the sweet honeydew, causing further annoyance to residents in affected areas. Given the relatively long duration of SLF adult activity and the high densities SLF populations can reach, this insect can be a major nuisance for residents in affected areas. To date, SLF is not known to have caused tree mortality, although feeding has killed individual shoots or small branches of black walnut (*Juglans nigra* L. [Fagales: Juglandaceae]), maples (*Acer* spp. [Sapindales: Sapindaceae]) and other native trees. Intensive feeding combined with black sooty mold can also reduce yield, quality or simply render fruit from infested trees, grapevines (*Vitis riparia* Michx [Vitales: Vitaceae]) and hops (*Humulus* spp. [Rosales: Cannabinaceae]) unmarketable (8, 11).

Although SLF can feed on several trees and woody vines, tree of heaven (ToH) (*Ailanthus altissima* (Mill.) [Sapindales: Simaroubaceae]) is the most preferred host for SLF feeding and reproduction (6, 8). ToH, native to China, was introduced into the U.S. in 1784 and was widely planted in urban areas through the 19th century (12). It has subsequently spread across much of the U.S. Today, ToH is considered to be an undesirable invasive because of prolific seed production by female trees and high germination rates (13), its ability to colonize disturbed sites and outcompete more desirable vegetation, and the unpleasant odor of crushed leaves or twigs (14). Tree of heaven can also reproduce clonally *via* sprouts from lateral roots (13, 15, 16) and may root graft with other ToH, monopolizing available nutrients in a site (16). Because ToH is highly intolerant to shade (12, 13, 17), it is rarely present in closed canopy forests but often grows along forest edges.

Early efforts to eradicate or contain SLF in Pennsylvania involved treating male ToH with dinotefuran, a systemic neonicotinoid insecticide commonly applied as a basal trunk spray (18). Although dinotefuran can be applied *via* trunk injection, basal trunk sprays are relatively efficient and can be used on trees that are small or otherwise difficult to treat with trunk injection. High rates of SLF mortality were consistently observed following dinotefuran treatment (19, 20). At the same time, female ToH in areas with SLF infestations were removed or killed with herbicide (18). This encouraged SLF to feed on the treated trap trees and also limited further ToH reproduction.

While SLF eradication is no longer a realistic objective, dinotefuran continues to be widely used for control of SLF in Pennsylvania and more recently infested states (21). Because dinotefuran is highly water-soluble, it is translocated relatively rapidly in trees compared to imidacloprid, another systemic neonicotinoid insecticide, but is less persistent (22–26). For example, in ash (*Fraxinus* spp. [Lamiales: Oleaceae]) trees treated in May, foliar imidacloprid residues continued to increase through the growing season while dinotefuran levels were dropping by late summer (25–27). Recent studies have shown other insecticides, including cover sprays of broad spectrum pyrethroid products, can effectively control SLF nymphs or adults (28, 29). However, given concerns about insecticide drift, impacts on nontarget insects and the difficulty of effectively spraying tall trees, dinotefuran remains an essential tool for SLF management.

Identifying the optimal timing for basal trunk sprays of dinotefuran is an essential aspect of SLF containment and management programs, given that feeding extends for at least six months, and label restrictions prohibit multiple applications in a single year. In a previous study, dinotefuran residues in foliage sampled from ToH treated in May persisted into September, whereas in trees treated in April, residues sharply declined between August and September (20). Spring applications of dinotefuran reduce early instar densities, protecting trees and vines from feeding, honeydew and sooty mold growth. Whether insecticide residues remain adequate to control fourth instars and adults in late summer or autumn when feeding and honeydew production are most intense, however, remains a key question for pest managers. Additionally, when SLF nymphs are not controlled, mature adult females commonly engage in short-range dispersal flights (5, 30), sometimes invading vineyards and orchards where late season insecticide sprays just before or during harvest, are especially problematic. Because resources are rarely sufficient for multiple insecticide applications in a single year, understanding translocation and persistence of dinotefuran can help pest managers efficiently control SLF densities while limiting unnecessary applications.

Systemic insecticides such as dinotefuran are transported in xylem tissue (27, 31) and accumulate in leaves, which function as a major sink for water and nutrients during much of the growing season. Insecticide residues in foliage samples are frequently used to quantify insecticide concentrations, monitor insecticide persistence over time or to compare treatment timing, application methods or other factors. All SLF nymphal stages and adults, however, feed on phloem in tree branches and trunks (6, 8, 32). Observations of high and often rapid SLF mortality following dinotefuran application (19, 20, 33, 34) suggest that either dinotefuran moves into the phloem, i.e., *via* transverse rays, or the mouthparts of SLF insects penetrate phloem and encounter insecticide in xylem vessels. Evaluating dinotefuran presence and concentrations in phloem could help to fully understand options for optimizing SLF control.

We conducted two studies in 2019 to assess dinotefuran persistence and within-tree distribution following basal trunk sprays applied to healthy ToH in sites in Michigan, well beyond any known SLF infestation. In the first study, dinotefuran was applied in early April and residues were quantified in samples of ToH foliage collected periodically until late September when leaves were dropping. Based on previous research and experience, we expected dinotefuran residues would remain relatively high for at least two months before declining in mid to late summer. We also evaluated whether tree diameter affected foliar dinotefuran concentrations at each sampling period. We expected to find little or no relationship between residue levels and tree diameter, given that label application rates are based on tree DBH (diameter at breast height) and the thin outer bark of ToH seemed unlikely to prevent rapid movement of dinotefuran into xylem tissue.

In the second study, we quantified residues in ToH foliage and phloem collected on two post-treatment dates following basal trunk sprays of dinotefuran applied in June. Foliage and phloem samples were collected from trees felled in either July or September to compare dinotefuran levels in the two tissues and to assess potential effects of aspect, sampling dates and tree DBH on dinotefuran concentration. Phloem samples were collected from below and above the spray line on trunks of trees felled in July and trees felled in September. We expected phloem residues below the spray line to decrease over time as insecticide was transported to the canopy but whether dinotefuran would be detectable above the spray line, especially in September, was unknown. Given the many reports of rapid mortality of SLF nymphs and adults on trees treated with dinotefuran (18–21), we anticipated that dinotefuran would be present in phloem from branches, although perhaps at lower levels than in foliage. We also were interested in determining whether the relative sun exposure of leaves and branches affected dinotefuran levels or persistence.

2 Materials and methods

2.1 Study sites

Study 1 was conducted with ToH growing in an unmanaged, ~0.1 ha strip of land in Lansing, Ingham County, Michigan. The site was in an industrial area with an overstory composed entirely of ToH, and an herbaceous layer of poison ivy (*Toxicodendron radicans* (L.) Kuntze [Sapindales: Anacardiaceae]), and Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch [Vitales: Vitaceae]). On 2 April 2019, 24 ToH trees with DBH ranging from 10.9 to 34.8 cm DBH and averaging 19.3 ± 1.4 cm were selected and tagged. Twenty trees were assigned to a basal trunk spray of dinotefuran and four were left as untreated controls. Brush was cleared around each tree to facilitate access.

Study 2 was conducted in an ~0.4 ha, even-aged plantation of ToH established in 1976 at MSU's W.K Kellogg Forest in Augusta, Kalamazoo County, MI. A few northern red oak, *Quercus rubra* (L.) [Fagales: Fagaceae] trees grew along the plantation borders while black cherry (*Prunus serotina* (Ehrh.) [Rosales: Rosaceae]) saplings and European buckthorn, (*Rhamnus cathartica* (L.) [Rosales: Rhamnaceae]) grew between and within the rows of ToH. Herbaceous vegetation was dominated by poison ivy, multiflora rose (*Rosa multiflora* (Thunb.) [Rosales: Rosaceae]) and wild raspberry (*Rubus* sp. [Rosales: Rosaceae]) shrubs. On 16 May 2019, brush was cleared at the site (using hand tools) to facilitate access to the trees and to allow a skidsteer to maneuver between and within rows. We tagged and measured DBH of 26 trees across the plantation. Tree DBH ranged from 7.1 to 37.6 cm DBH and averaged 18.8 ± 1.2 cm. Six trees were randomly selected to be left as untreated controls while the remaining 20 were treated with a basal trunk spray of dinotefuran. Even-numbered treated and control trees were felled in mid-summer while odd-numbered treated and control trees were felled in late summer (see below).

Cumulative growing degree days corresponding to each treatment and sampling date were acquired from data recorded by MSU EnviroWeather stations located at the MSU Horticulture Teaching and Research Center, approximately 13 km from the Study 1 site, and from the MSU Kellogg Biological Station, approximately 8 km from the Study 2 site. Cumulative growing degree days were calculated using the Baskerville-Emin method with a base 10°C developmental threshold and a starting date of 1 January. Growing degree day accumulations corresponding to treatment and sampling dates are reported here for potential application in other regions with different weather regimes.

2.2 Dinotefuran application

Trees in Study 1 and Study 2 were treated with dinotefuran on 9 April (25 GDD [growing degree days]) and 6 June (291 GDD) 2019, respectively, using the same insecticide rate and application method. Twelve water soluble packets of Transtect® were added to 3.8 liters (one gallon) of distilled water in the tank of a low-pressure 7.5 liter garden sprayer. Formulated insecticide was applied as a basal trunk spray at a rate of 59 ml (2 oz) per 2.54 cm DBH (1.4 g active ingredient per 2.5 cm DBH) to tree trunks from approximately 1.5 m high down to the base, ensuring the entire trunk was covered and the appropriate amount of insecticide was applied. Spray was applied at low pressure to minimize any drift around tree trunks and care was taken to avoid any spray contact with designated control trees.

2.3 Sampling

To account for the often irregular crown shape of ToH (17), composite foliage samples from Study 1 trees were comprised of shoots from branches on at least three different aspects, whenever available. Leaf-bearing shoots were clipped from treated and control trees on 25 July (770 GDD),107 days post-treatment. Foliage samples from each tree were placed into labeled bags, returned to the MSU Forest Entomology Laboratory in coolers with blue ice, then frozen. In the lab, leaflets were stripped from petioles, and petioles and woody twigs were discarded. Sampling was repeated on 20 Aug (1077 GDD) and 30 Sept (1425 GDD), at 133 days and 174 days post-treatment, respectively.

For Study 2, half of the trees in the plantation were destructively sampled on 16 July 2019 (771 GDD), 40 days post-treatment. The spray line on each tree trunk was marked, then a skidsteer felled ten of the treated trees and three untreated control trees. Trees were cut at approximately the top of the spray line.

Leaves were collected with hand pruners from canopy branches on three to four aspects of the felled trees, depending on crown structure, and bagged separately by aspect for each tree. Phloem samples were collected from the same canopy branches using drawknives to remove long strips of bark and phloem beginning near the trunk and extending distally until the branch was ≤ 4-5 cm in diameter. Heavy overcast conditions, however, limited our ability to confidently assess relative amounts of sun or shade exposure of individual branches. Drawknives were also used to remove 0.5 to 1.0 m long strips of bark and phloem from the upper half of the trunk on the felled trees. Samples from above the spray line were collected 2.5 to 4 m above ground and samples from below the spray line were collected 0.5 to 1.0 m above ground, within the area that had been sprayed. Phloem readily separated from xylem and outer bark in the branch and trunk samples. Phloem samples from different branches and from above and below the spray line on tree trunks were placed into individual bags. All drawknives and hand pruners used for sampling were sterilized with 70% ethanol between each sample to avoid contamination.

Remaining trees in Study 2 in the plantation were felled and sampled on 17 September (1478 GDD), 103 days post-treatment, using the same methods as above. Exposure to sun, which could presumably affect insecticide concentration or persistence, was qualitatively ranked for each branch that was sampled as 1 if it was fully shaded, 2 if it was partially shaded and 3 if it was fully exposed to sunlight.

Foliage and phloem samples collected in July or in September from the Study 2 trees were bagged, transported in a cooler with blue ice to the MSU Forest Entomology Laboratory, then frozen as in Study 1. Leaflets were stripped from shoots and petioles in the laboratory, then re-frozen. Frozen foliage and phloem samples were shipped overnight to collaborators at the USDA APHIS laboratory in Buzzards Bay, MA on 22 October 2019 for insecticide residue analysis.

2.4 Residue analysis

Foliage and phloem samples were removed from bags, air-dried for at least two weeks, then ground in a commercial blender to a fine powder. Blenders were tripled rinsed, scrubbed with soapy water (LIQUINOX® detergent, Alconox Inc., White Plains, NY) and a bottle brush, sprayed with 95% ethanol then rinsed in dionized water to ensure any insecticide residue was removed. Personnel changed nitrile gloves between samples to further minimize any risk of cross contamination.

Analysis of insecticide residues in ToH leaves collected from trees in Study 1 and Study 2, and in ToH phloem from Study 2 trees was determined using commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits (FujiFilm/Horiba; Kyoto, Japan and Wako Chemical, USA Corporation, Richmond, VA). A 0.5 g sample of processed plant material was weighed into a 50 mL plastic centrifuge tube and extracted in 10 mL of pure methanol for 3 hrs on a table-top shaker. Sample tubes were spun down in a high-speed centrifuge for 10 min and the supernatant diluted a minimum of 20x to avoid matrix effects from the kit due to the methanol. Sample aliquots were added to a 96-well plate, developed and the absorbance value calculated according to the manufacturer's instructions using provided standards of 1.5 ppb to 30 ppb. The effective lower limit of kit detection following sample preparation and dilution is 0.6 ppm.

2.5 Statistical analysis

Normality of dinotefuran residues in foliage from Study 1 trees was assessed with a Shapiro-Wilk test and residual plots (PROC MIXED, PROC UNIVARIATE, SAS 9.4) and a square root transformation was applied to normalize residue data (Pr <W = 0.1456).

A one-way ANOVA with repeated measures (PROC MIXED, SAS 9.4) was used to compare differences in foliar dinotefuran residues among the three sample dates with an *a priori* significance level of $\alpha = 0.05$. The Kenward-Roger correction was used for calculating denominator degrees of freedom because it is more conservative than the MIXED default and is generally recommended for repeated measures analysis to minimize the risk of an increased Type 1 error rate generated by improperly fitted covariance structure. The Tukey-Kramer multiple comparison test was applied when the ANOVA results were significant to identify significant differences among sampling dates. Additionally, linear relationships between foliar dinotefuran residues and tree diameter were assessed with simple linear regression (PROC REG).

Results of a Shapiro-Wilk test and residual plots (PROC UNIVARIATE, SAS 9.4) showed dinotefuran residues in leaf samples collected from the Study 2 trees in the plantation were not normally distributed and data were not normalized by transformation. A two-way nonparametric ANOVA was therefore performed on ranked foliar insecticide residues to assess differences among leaves collected from branches on different aspects of the canopy and between the two sample dates (PROC RANK, PROC MIXED, SAS 9.4). Dinotefuran was not detected in any of the phloem samples collected from branches on either sampling date.

A composite foliar residue value for each tree in Study 2 was calculated by averaging residues in the leaves from the three to four sampled branches in July and again in September. Composite foliar residue values in trees were normal on both sampling dates. An independent t-test was used to assess differences in foliar residues between samples collected from trees felled in July versus September (PROC TTEST, SAS 9.4). Within each month, differences between foliar and trunk phloem residues and between residues in phloem from above and below the spray line were evaluated with paired t-tests. Simple linear regression (PROC REG, PROC UNIVARIATE, SAS 9.4) was applied to assess relationships between foliar residue levels and tree DBH for trees sampled on each date.

3 Results

3.1 Study 1

As expected, foliar dinotefuran residues from Study 1 trees were significantly higher in foliage from treated trees than in untreated controls, which had no dinotefuran, across all months (F = 20.52; df = 1,21.9; $P < 0.001$) and differed among post-treatment sample dates (F = 5.63; df = 2,34.6; $P = 0.0076$) (Figure 1). Residues averaged 7.8 ± 1.1 ppm and ranged from 0.7 to 17.0 ppm in July, 6.3 ± 1.2 ppm and 0 to 20.0 ppm in August, and 2.6 ± 0.5 ppm and 0 to 8.8 ppm in September. Residues were significantly higher in samples collected in July (770 GDD) than in September (1425 GDD) ($P < 0.001$) and in August (1077 GDD) compared to September ($P = 0.0013$), but the drop in average dinotefuran concentration between July and August was not significant ($P = 0.5412$) (Figure 1). Residues in 14 trees were lower in August than in July, while values increased in the six trees between July and August. Between August and September, residues in 18 of the 20 treated trees had declined and overall residues in September were 50% lower than in August and 66% lower than in July. Residues in two trees increased slightly in September from August, but residue values were substantially lower in these trees from the July values. Tree size did not affect foliar residues in any of the sampling periods; simple linear regressions yielded R^2 values of 0.02 ($P = 0.48$), 0.03 ($P = 0.41$) and 0.002 ($P = 0.85$) in July, August and September, respectively.

3.2 Study 2

Dinotefuran residues were detected in foliage from all treated trees sampled in mid-July (771 GDD), 40 days post-treatment, and in mid-September (1478 GDD), 103 days post-treatment. Mean foliar residue levels averaged 12.7 ± 1.32 and 14.6 ± 2.18 ppm in the ten trees felled and sampled in July and the other ten trees sampled in September, respectively. While average foliar residues were approximately 5% higher in September than in July, the difference was not significant (t = -0.55; df = 1,23; $P = 0.5895$). Results from the two-way ANOVA confirmed the similarity in foliar residues between samples collected in July and September (F = 0.40; df = 1,87; $P = 0.53$). Residues also did not differ among foliage samples collected from branches at different aspects (F = 0.02; df = 3,87; $P = 0.89$). Mean foliar residues ranged from 11.9 ± 1.90 ppm (eastern aspect) to 13.5 ± 2.69 ppm (southern aspect) in July and from 12.4 ± 3.78 ppm (southern aspect) to 16.3 ± 5.50 ppm (northern aspect) in September. As in Study 1, tree DBH did not affect mean foliar residues in trees sampled in either July ($R^2 = 0.16$; $P = 0.25$) or September ($R^2 = 0.0002$; $P = 0.97$). Leaves from one tree sampled in September exhibited an unusually high dinotefuran concentration but excluding this outlier had little effect on results ($R^2 = 0.03$; $P = 0.621$). All branches that were sampled to collect foliage and phloem from trees felled in September were either partially (Rank 2) or fully exposed to sun (Rank 3). There was no evidence that sun exposure affected foliar dinotefuran residues.

Dinotefuran residues in phloem samples collected from above and below the spray line on trunks of the felled trees varied substantially and were often too low to be detected. Phloem samples collected in July from below the spray line yielded detectable dinotefuran residues in seven of the ten felled trees, averaging 8.6 ± 4.4 and ranging from 0.8 to 32.2 ppm. Three trees had detectable levels of dinotefuran in phloem from above the spray line, with concentrations ranging from 1.6 to 10.6 ppm and averaging 7.4 ± 2.9 ppm. Phloem from only one tree had detectable dinotefuran residues in samples from both above (9.8 ppm) and below the spray line (0.8 ppm).

In September, none of the phloem samples collected from above the spray line on the trunks of the ten felled trees had detectable dinotefuran residues. Seven of these trees had measurable dinotefuran residues in phloem from below the spray line, ranging from 0.8 to 10.8 and averaging 3.7 ± 1.3 ppm.

Overall, residues in phloem samples collected from tree trunks were significantly lower than residues in foliage collected from the same trees in July (t = 3.60; df = 9; $P = 0.0058$) and September (t = 4.31; df = 9; $P = 0.002$). On average, phloem residues in samples from below the spray line were 53% lower than foliar residues in July and 83% lower than September foliar residues. Phloem residues in samples from above and below the spray line did not significantly differ in July (t = 0.99; df = 9; $P = 0.35$) but were significantly higher below the spray line than above the spray line in September (t = 2.49; df = 9; $P = 0.03$). None of the phloem samples collected from branches had detectable dinotefuran residues, regardless of sample date or aspect.

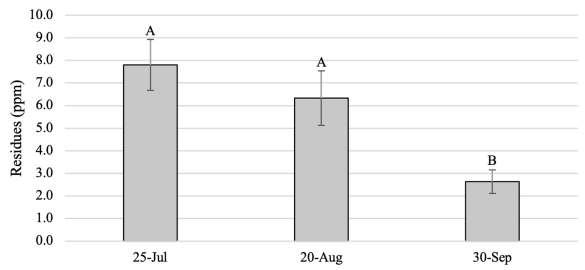

FIGURE 1
Mean (± SE) dinotefuran residues (ppm) in foliage samples from *Ailanthus altissima* trees in Study 1. Samples were collected periodically in 2019 following a 9 April 2019 basal trunk spray. Letters above bars indicate significant differences ($P < 0.05$) (n = 20 trees).

4 Discussion

Basal trunk sprays of dinotefuran remain a key tool for managing SLF infestations to reduce insect density, protect the health of trees and other hosts, and lessen the annoyance or anxiety experienced by residents during outbreaks. Identifying the optimal time to apply dinotefuran, however, remains an essential question for pest managers dealing with established SLF populations along with newly discovered infestations. Regulatory personnel, IPM specialists and resource managers desire a high level of SLF control that is also cost-effective and logistically practical (21). Launching dinotefuran applications in spring could be advantageous when extensive areas require treatment, especially if

personnel or funding are likely to be limited later in the season. Reducing densities of SLF early instars in an area also decreases feeding and honeydew production by later life stages, minimizing potential injury to host plants. Conversely, in other situations, SLF infestations may not be discovered until late summer or autumn when brightly colored 4th instars or the large adults are more easily observed. High densities of SLF can also appear in previously uninfested areas following migratory flights by mature adults in late summer or fall (5).

In our studies, as in most research with systemic insecticides, residues in samples of foliage were quantified to evaluate persistence of dinotefuran. Sampling leaves to assess insecticide concentrations causes minimal injury to trees and facilitates repeated sampling over time. Tree DBH, which ranged from 10.9 and 7.1 cm up to 34.8 and 37.6 in Study 1 and Study 2, respectively, did not affect foliar dinotefuran residues in any sampling period. This is not surprising since the amount of insecticide applied to any tree is based on the DBH of the tree. Previous studies with ash trees, which are ring porous like ToH, have shown that systemic insecticides are carried in xylem vessels in the outer ring of sapwood from the trunk to the canopy, where expanding buds and leaves act as a strong sink for xylem (27, 31, 35). It is notable, however, that the thicker outer bark on large trees relative to the smaller trees in this study did not limit absorption nor affect translocation of dinotefuran applied *via* basal trunk sprays in early April (Study 1) or June (Study 2). Age of the largest trees in Study 1 are unknown, but records show that the mature trees in the plantation used for Study 2 were 48 years old at the time of treatment and sampling. A high proportion of trees in most areas where SLF is established will likely be of similar size and can be efficiently treated with basal trunk sprays instead of more laborious trunk injections. Since ToH trees can reportedly attain diameters of >1.5 m (14), however, further evaluation of insecticide translocation in very large trees may be warranted.

While foliar residues are ideal for monitoring insecticide presence over time or comparing different treatments, SLF feeds by sucking phloem sap from tree branches, trunks, and woody vines. Several studies have reported high and relatively rapid SLF mortality following dinotefuran application (19, 20, 28, 33, 34), indicating that these insects must encounter lethal levels of insecticide as they feed. We anticipated dinotefuran residues would be relatively high in phloem samples collected from below the spray line on tree trunks, e.g., the area where the dinotefuran spray was physically applied. We also expected to detect some level of dinotefuran in phloem samples from above the spray line and in branches, indicative of dinotefuran translocation to the canopy. Movement of dinotefuran from xylem into phloem *via* transverse rays could presumably result in the consistently high SLF mortality observed on treated trees (19, 20). However, in the ten Study 2 trees sampled in July, only 40 days post-treatment, dinotefuran was undetectable below the spray line in three trees and above the spray line in seven of the trees. The lack of detectable dinotefuran residues in phloem from any of the branches sampled on the Study 2 trees was also unexpected, particularly given the insecticide levels in leaves from those same branches. It is possible that dinotefuran in the phloem samples from the branches was present at concentrations below the detection limit of 0.6 ppm of our assay. While LC_{50} values for dinotefuran corresponding to SLF mortality are unknown, it seems unlikely that residues consistently below detection limits would cause the high SLF mortality rates previously observed in multiple infestations (19, 20).

A possible mechanism to account for these seemingly contradictory observations is that while SLF need to access nutrients in phloem, their mouthparts may penetrate phloem and reach xylem tissue in the outer sapwood ring, which could result in the insects encountering a lethal dose of insecticide. Research has suggested that phloem-feeding emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire [Coleoptera: Buprestidae]) larvae may similarly encounter insecticide when early instar galleries score the outer xylem in ash trees (26, 35). Further research into the mechanics of SLF feeding is needed to understand how these insects encounter systemic insecticides, particularly small early instars with short stylets (36).

When young ash (*Fraxinus* spp.) trees were injected with ^{14}C-labelled imidacloprid, another systemic neonicotinoid, residues in subsequent foliage samples varied depending on the position of branches relative to injection sites, and with the height of branch whorls (31). Translocation patterns of ToH and ash, both ring porous trees, are probably similar but in our Study 2 trees, foliar residues were not affected by aspect of the leaf-bearing branches we sampled. Basal trunk sprays, which are applied around the entire circumference of the tree, may facilitate a more even distribution of insecticide throughout the canopy than trunk injection. Additionally, we hypothesized that higher transpiration rates in leaves fully exposed to sun could result in more rapid translocation or higher residues, at least initially, than in shaded branches. However, we found no evidence that exposure to sunlight affected insecticide translocation rates or persistence in foliage. Virtually all foliage-bearing branches on trees in both Study 1 and Study 2 were at least partially exposed to sunlight, while branches below the canopy or those that were shaded by adjacent trees were dead, a pattern consistent with the low shade tolerance exhibited by ToH, and its rarity in closed canopy forests (17, 37).

Although dinotefuran LC_{50} values for SLF have not been determined, we assumed that trees with high dinotefuran concentrations in leaves would be more toxic to SLF nymphs and adults than trees with lower residues. Foliar residues from Study 2 trees, treated in June (291 GDD), averaged 12.8 ± 1.3 and 14.6 ± 2.2 at 40 and 103 days post-treatment, respectively, while residues in Study 1 trees, treated in early April (25 GDD), averaged 7.8 ± 1.1 and 6.3 ± 1.2 ppm in samples collected in July and August, 107 and 133 days post-treatment, respectively. Generally lower foliar residues in Study 1 trees compared with Study 2 trees may reflect the poor Study 1 site conditions, reflected in lower respiration and translocation rates. Study 1 trees were in a narrow, highly disturbed strip of land bordered by parking lots, while Study 2 trees were on a relatively high quality site with minimal disturbance. Variability in foliar residues among Study 1 trees, as evidenced by standard errors, increased between July and August (albeit slightly), and between July and Sept for Study 2 trees, a pattern consistent with differences among trees in insecticide translocation. Increased foliar residues in Study 2 trees between 40 and 103 days post-treatment presumably reflects continued translocation of insecticide from the lower trunk to canopy branches and leaves. Six of 20 Study 1 trees had higher

residues in mid August (133 days post-treatment; 1077 GDD) than in late July (771 GDD), indicating translocation of insecticide was still occurring between 107 and 133 days post-treatment in some trees.

Collectively, these results suggest basal trunk sprays should provide effective control of SLF for at least 100 days and probably for as much as 135 days post-treatment in most trees. However, residues dropped sharply in Study 1 trees during the 41 days between the mid August and late September samples, when residues averaged < 3 ppm (174 days post-treatment). Similarly, the number of Study 1 trees with relatively low foliar residues, e.g., ≤ 5 ppm, increased from seven trees in the July samples, to 12 trees in August and 18 trees in September. In an earlier study, dinotefuran residues in trees treated in mid to late May remained relatively stable in September (20).

Early season treatments to reduce densities of early instars would presumably limit feeding, honeydew production and associated impacts in a given area throughout the summer. However, applications made too early will likely result in trees with relatively low and rapidly declining residues from late August through October, a period when SLF adult feeding, dispersal and migratory flights are likely to peak (38). Delaying dinotefuran basal trunk sprays until late May or mid June should provide effective control of late instars and SLF adults in October, although early instar feeding and local dispersal would still occur. Understanding more about translocation and persistence of dinotefuran and other systemic insecticides including imidacloprid would be valuable for SLF programs and more broadly for insect pests of other trees.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DM and PL conceived the project, acquired funding, and designed the experiments. DM oversaw the experiments, coordinated and participated in field work including insecticide applications, sampling and data collection. PL directed sample analysis of the insecticide residues. JK assisted with field work, processed samples and analyzed data. JK and DM drafted the manuscript with contributions from PL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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An effective trap for spotted lanternfly egg masses

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Spotted lanternfly (SLF) (*Lycorma delicatula* (White)), an invasive planthopper discovered in Pennsylvania, USA in 2014, continues to spread and is now present in 14 states with substantial infestations present in seven states. Population projections using adult SLF trapping or visual counts are not reliable due to the transient, migratory behavior of the adults which make population forecasts difficult. Another approach to population monitoring is utilization of the stationary egg mass stage, but counting small cryptic egg masses throughout the canopy of large trees in dense woodlots is arduous and prone to error. After several field seasons testing various trapping configurations and materials, we have identified an efficient, simple, low-cost trap termed a 'lamp shade trap' that is attached to the lower trunk area of an SLF host tree. SLF females readily enter the trap and lay eggs on the thin, flexible trap surface. A vertical trap orientation was superior, and the most productive woodlots yielded an average of 47 and 54 egg masses per trap, and several traps had over 100 egg masses. There were 1,943 egg masses tallied from 105 traps placed at six locations in two states. Egg mass counts in the area above and below the traps and on nearby control trees yielded very few egg masses in comparison. Selection of trees 15 to 20 cm in diameter for trap placement is most efficient, yielding good egg mass abundance while minimizing the amount of trap material used. The lamp shade trap has potential as an effective tool to identify SLF in new areas, gauge SLF population levels in woodlots and can also be used to collect and monitor egg masses for research purposes.

KEYWORDS

spotted lanternfly, *Lycorma delicatula*, trapping, egg masses, *Ailanthus altissima*

1 Introduction

The spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is an invasive, destructive fulgorid that has gained a strong foothold in the eastern United States since it was first discovered in Pennsylvania in 2014 (1). In a short period of time this sap feeding insect native to Taiwan and China (2) has greatly expanded its range and there are now portions of 14 states with established populations as well as detections of SLF in two additional states (3). This insect is typically a pest of the invasive tree of heaven (TOH), *Ailanthus altissima* (Miller) Swingle (Sapindales: Simaroubaceae) but feeds on a broad

variety of host plants and trees (2, 4, 5). In the state of Pennsylvania, it has and continues to cause devastation to a number of commodities and industries including grape, forest timber and ornamental tree production (6–8).

The potential for this pest insect to expand its population is heightened by the young nymphs (1st to 2nd instars) which are very active and polyphagous and remain widely dispersed as they mature on a large variety of plants (4). The 4th instar (red form) and newly molted adults begin to congregate in large numbers on TOH and other preferred host trees where heavy feeding commences for several weeks before adult courtship and mating activities. During the mating period there are aggressive adult migration and dispersal events (9). Due to their extreme mobility the prediction and estimation of nymph and adult SLF population levels is confounded by an insect which does not actively seek out traps due to the lack of an effective lure (10). SLF aggregation behaviors are not well understood and may be driven by a combination of changing nutritional levels in their host plants as well as for their own needs (11) making it almost impossible to estimate and predict population levels.

Another approach to predicting SLF populations is monitoring of the overwintering egg stage. SLF typically lays eggs from September until early December and egg masses can contain 30 to 50 eggs covered in a yellow-brown waxy covering (12). Egg masses are laid mostly on the bark of trees but can be found on almost any flat surface including vehicles, stones, fence posts, buildings and backyard play equipment (1, 13). Location of egg masses can vary within a tree and are correlated to tree height. For example it was found that egg masses on TOH were concentrated towards the lower 2.5 m of the tree in areas where trees were < 6 m in height (14). Another study (15) found egg masses concentrated above 6 m on TOH when tree height ranged from 5.5 - 23.8 m. SLF egg mass survey activities are common during winter months and have been used to assess populations and gauge infestation levels (15), however the cryptic nature of SLF egg masses and the fact that eggs are most likely higher up in the canopy make visual egg mass surveys unreliable.

Observations by the authors of SLF egg mass locations (e.g. underside of limbs, on fence posts, play equipment, wheel wells of vehicles and on a rusty lid from 55-gallon drum) and that masses were sometimes clustered, indicated to us that perhaps oviposition behavior was not completely random and could be directed. In the fall of 2018, we initiated investigations to see if SLF females had oviposition preferences that would induce them to lay their eggs on a trap or removable surface. In subsequent years we continued to test materials and environments and made incremental progress in identifying what worked and what didn't. We noted certain preferences in materials or eliminated trap designs or approaches that female SLF failed to interact with. In 2022 we settled on a single trap design that combined two key attributes that we were able to identify: a preferred material for egg laying and a protected area/environment that we hoped would induce egg laying behaviors.

Trap appearance is that of a lamp shade and the traps are very efficient at concentrating SLF egg masses. Very few egg masses were noted above and below the traps or on nearby trees. These lamp shade traps provide an environment and a substrate on which SLF females readily oviposit. The traps have potential to be a valuable research tool not only to collect and monitor SLF egg masses but potentially for detecting SLF in new areas, for monitoring SLF in areas of concern and potentially for estimating and predicting SLF population levels in infested areas and woodlots.

2 Materials and methods

2.1 Study sites

Traps were deployed on TOH at locations where adult SLF populations were known to be medium to high. All sites were dominated (>80%) with mature TOH of various diameters with very little understory. Six sites were used from six different counties in southeastern Pennsylvania and in northern Delaware. Site locations are noted in Figure 1. Additional location information and the name designations used in this report were as follows: Blandon (Blandon, PA; Berks Co.; 40.442, -75.880); Route 422 (Royersford, PA; Montgomery Co.; 40.172, -75.504); Easton (Easton, PA; Northampton Co.; 40.678, -75.194); Delaware (New Castle, DE; New Castle Co.; 39.710, -75.568); Keystone (Fairless Hills, PA; Bucks Co.; 40.170, -74.753); Route 30 (Wrightsville, PA; York Co.; 40.029, -76.550).

2.2 Trapping approach 2018-2021

Initial trapping designs in 2018 consisted of 30 x 50 cm burlap or cotton fabric materials and an artificial bark product (PINVNBY, available from Amazon Inc.). Materials were stapled directly to TOH trunks and a few other tree species at diameter breast height (DBH; 1.4 m) and at the base of the trees. Attachment was flush (Figure 2A) or the material was stapled at the top allowing it to hang off the trunk (Figure 2B). We placed a total of 75 objects at three locations where SLF adults were active.

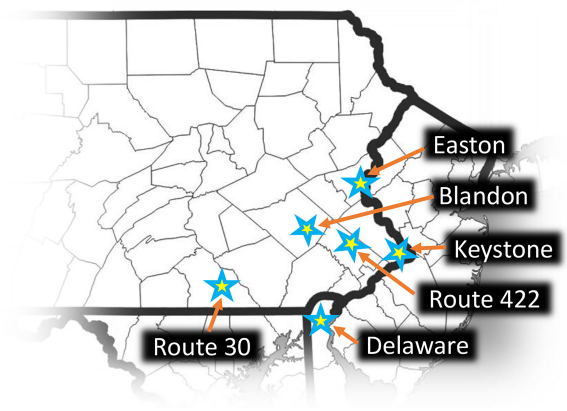

FIGURE 1
Study sites where lamp shade egg mass traps were deployed. Map used by permission from www.amaps.com.

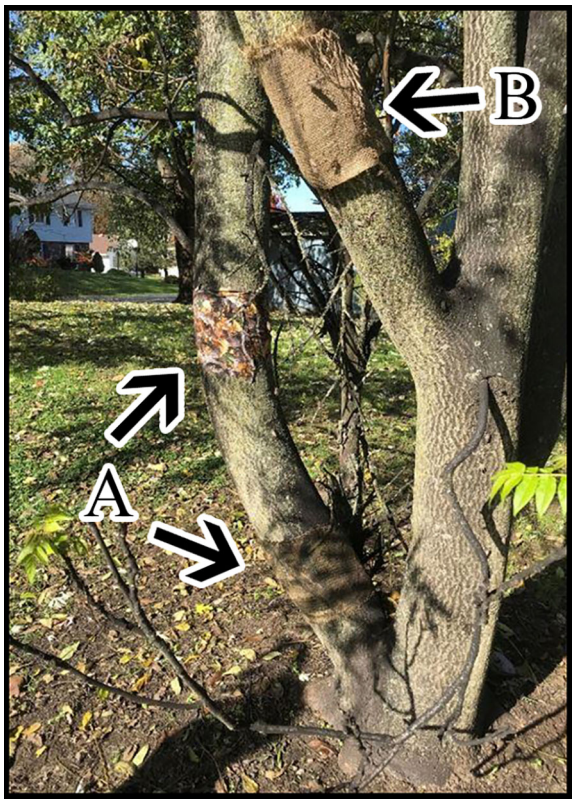

FIGURE 2
Fabric pieces arranged on a TOH as indicated by arrows, **(A)** camo fabric wrapped around the trunk at 1.4 m and burlap fabric wrapped around the base of the tree, **(B)** hanging burlap fabric stapled along the top edge.

Trapping designs in 2019 included ground traps at the base of TOH and a wide variety of designs that were attached to TOH at DBH. The ground traps consisted of bundles of four 0.3 m long by 0.1 m diameter cardboard or aluminum tubes (dryer ducting, General Electric, Boston, MA), held together by zip ties with a piece of 6 mm thick tarp staked over to keep it secure to the ground to protect from the elements (Figures 3A, B). Traps attached to TOH included a 30 x 30 cm sheet of either cellulose shade fabric framed with wire (Figures 4A, B), rusty metal (Figures 4C, D), or metal with Rust-Oleum® stone texture (Vernon Hills, IL) sprayed on (Figure 4E). These were placed as pairs either with a 30 x 30 cm piece of burlap hanging or the trap alone. Another set of traps used two pieces of 18-gauge stainless steel wrapped with landscaping fabric and held together with spacers to create a sandwich (Figure 4F). Traps that incorporated rust used 18-gauge galvanized metal that had been sandblasted and then sprayed with an oxidizing solution (32:8:1 mixture of hydrogen peroxide: vinegar:salt). Rusty metal traps consisted of a series of 15 x 30 cm half pipe pieces attached to the tree at DBH either alone (Figure 5A) or with a piece of burlap hanging over it (Figure 5B). A final metal trap was in a "starfish" configuration that encompassed the tree trunk but had no burlap (Figure 5C). We placed a total of 200 traps using 11 configurations and evenly distributed them at eight study sites. Traps with and without burlap included half pipe and sheet metal (24 each). There were two types of tube hotels and sandwich traps (24 each) and single traps of the following were placed at each site: metal sprayed with Rust-Oleum®, shade, shade with burlap and starfish.

In 2020 we used a common design and focused on finding suitable oviposition materials. We constructed triangular traps out of 30 x 60 cm panels of black corrugated plastic (Uline, Pleasant Prairie, WI). One side was attached to the tree with staples or zip ties and the two outward facing surfaces were affixed with either ½" cork (Natural cork; Manton Cork, Hauppauge, NY), roofing material (Quick Start shingle roll; GAF, Parsippany, NJ) or gaffing tape (Lockport, Inc., Great Neck, NY). Materials were placed on the panels as either a single layer or using three over-hanging horizontal strips (Figures 6A-C). Each of these trap types was paired with an identical trap that was covered on the top with a 30 x 30 cm piece of the corrugated plastic. Traps were placed at a height of 3 m, 1.4 m and at the base of the tree. There were 156 traps placed at three study sites with eight or nine traps in each group.

Trapping designs for 2021 used the same triangular traps but the oviposition substrate consisted of a single layer of roofing or cork material applied to the inside surface of the panels. Traps were tested at 1.4 m and at the base of TOH and had covered and uncovered pairings as in the previous year. There was a second trap type tested that used a 30 x 60 cm panel of corrugated plastic overlaid with a second panel that had six large hexagons cut into it, backed with cork and roofing material (Figure 7). We deployed 144 traps at three study sites. There were 3 traps tested of each type (2 materials covered or not, hexagon) at two heights for each site.

2.3 Trapping approach 2022

The final trap design tested only roofing material as an oviposition substrate. In previous trap designs SLF females had to move off the tree trunk to encounter the substrate. This time we affixed the roofing material directly on TOH by wrapping it around the trunk at a 1.4 m height and we selected a range of tree diameters (± SE) for both vertical (Ave = 19.7 ± 0.8 cm; range = 9.7 to 35.1 cm; n = 73) and horizontal trap orientations (Ave = 11.4 ± 0.8 cm; range = 7.6 to 22.9 cm; n = 32). The trap material is overlapped slightly and stapled, and a zip tie is used to cinch the lower portion to the tree. At the top, batting material (9 cm wide, 2.5 cm thick) is attached, folded in half, and secured with a zip tie. The batting prevents SLF from passing through the trap while also holding the second layer of the trap away from the tree, creating a gap and a lamp shade appearance. The second layer of roofing material is inverted, and the top edge stapled to the tree right above the ring of batting fiber. The roofing material is positioned such that the asphalt sides of the two layers face each other with enough space between them to provide a protected area for the SLF females to interact with the trap substrate. Figure 8 provides a picture of a lamp shade trap (LST). Detailed instructions, step by step pictures during construction and a supply list is available in the supplemental materials (pdf file name: LST_Construction).

In the fall of 2022 we deployed 105 LSTs on TOH at infested sites at six locations in multiple counties in southeastern

FIGURE 3
Tube hotels placed at ground level next to the base of a TOH, **(A)** cardboard tubes, **(B)** metal duct tubes.

Pennsylvania and in northern Delaware (Figure 1). Installation of the traps began 21 September (Blandon) and two additional trap sites were set up in the two weeks that followed (Route 422 and Delaware). The other three sites were set up the week of 16 October. Trap removal and assessments were done between early December of 2022 and early January of 2023, well after SLF oviposition had ceased. SLF egg masses laid on the traps were counted during trap take down and additional egg masses were noted that were present on the trunk above and below the trap to a height of 3 m. Additionally a nearby TOH control tree of similar diameter to each trap tree was selected (n = 105) and all SLF egg masses on that tree trunk were counted to a height of 3 m.

2.4 Data analysis

Statistical comparisons were not conducted in 2018 to 2020 due to the small numbers of egg masses laid on traps not allowing for robust statistical comparisons. Trapping data from the 2021 and 2022 field seasons were analyzed using Statistix 10 software (Analytical Software, Tallahassee, FL). The data from the six study sites were not normally distributed and data were not normalized by transformation. Trapping data were not grouped across sites but were analyzed independently for each study site. Egg mass data comparisons for where oviposition occurred (trap, above and below the trap, control tree) were analyzed for each site with a Kruskal-Wallis one-way ANOVA with an *a priori* significance level of $\alpha = 0.05$. A similar approach was used for the comparisons of the area surveyed (m^2) of where oviposition occurred.

For each study site, Wilcoxon Rank Sum tests were used to assess differences between the number of egg masses laid due to the vertical or horizontal orientation of the traps. Variance in the data for the DBH groupings were not normally distributed and raw data were used to perform a one-way ANOVA for comparisons of both the egg mass counts and the trapping surface area comparisons.

3 Results

3.1 Trapping results 2018-2021

Our initial approach in 2018 using fake bark, burlap and fabric materials resulted in 34 total egg masses on the 75 objects deployed and no material or configuration was preferred for oviposition. During assessments we did note that one horizontal tree trunk draped with burlap had around a dozen egg masses laid in a row where the fabric and tree bark intersected. We also came across a single rusty metal lid leaning against a tree that had 25 egg masses on the protected side. The following year we set out a total of 200 objects, many of them focusing on rusty metal as well as various materials draped over the traps. These were set up at eight sites but when traps were checked only 31 egg masses had been laid on the traps in a random manner with no noted preferences.

Trapping in 2020 focused on suitable oviposition materials and height placement of the traps. There were 156 triangle traps set up at three study sites. However, only seven egg masses were observed on the trap surfaces. Notably, all egg masses were laid on traps that had a top or cover placed on them, but trap catch was so low that robust statistical comparisons could not be performed. In 2021 the same triangle traps were used but oviposition substrate was positioned on the interior portion of the traps. Covered and uncovered traps were paired and placed at the base of the trees

FIGURE 4
Set of cellulose traps, **(A)** without burlap, **(B)** with burlap, **(C)** rusty metal without burlap, **(D)** rusty metal with burlap, **(E)** metal with Rust-Oleum® spray, **(F)** metal sandwich wrapped with landscaping fabric with spacers in between.

and at DBH. Of the 144 traps deployed there were 326 egg masses, most of which were laid on traps that were covered. One site had only two egg masses laid on the traps and was excluded from the analysis. Trapping data for the remaining sites were pooled and a Wilcoxon Rank Sum test showed a significant difference. Covered traps averaged (± SE) 5.5 ± 1.4 egg masses and traps without covers averaged 1.3 ± 0.4 (W = 2.20, $P = 0.028$), a four-fold increase. Trap height and substrate material comparisons were not significant (W = 0.44, $P = 0.66$ and W = 1.16, $P = 0.25$).

3.2 Trapping results in 2022

The 105 LSTs deployed in 2022 were very attractive to SLF females for oviposition and 1,943 egg masses were laid upon the trap surfaces. The vertical orientation of the trap was highly preferred for egg laying. High variance in the pooled data across study sites did not allow a combined analysis and trapping parameters (tree DBH, trap orientation and egg mass location) were analyzed independently for each study site. Numbers of vertical traps deployed, tree DBH information, average number of egg masses and average egg masses calculated by surface area on the vertical traps, masses observed above and below vertical traps as well as on control trees from the base to 3 m are given in Table 1, summarized by study site. The average number of egg masses laid on vertical traps varied from 9.6 to 54.4 masses for the six sites with a mean value of 25.4 ± 2.9 (SE) egg masses per trap and three individual traps captured 98, 102 and 111 SLF egg masses. The average number of egg masses laid in the traps was compared at each site with the number of masses above and below the traps. Egg masses from the base up to 3 m on control trees of similar size without a trap were also recorded and compared with trap tree data. All Kruskal-Wallis ANOVA comparisons were significantly different ($P < 0.001$), with the traps being highly preferred for oviposition by SLF females (Table 1). This preference is even more pronounced when the surface area of each trap is calculated and

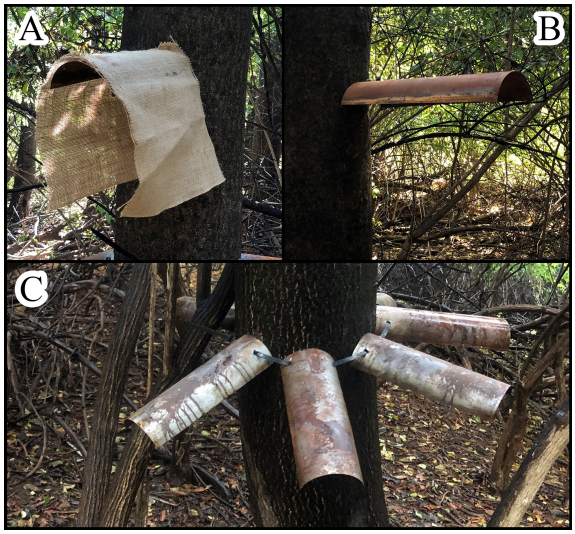

FIGURE 5
Half pipe rusty metal traps, **(A)** with burlap, **(B)** without burlap, **(C)** starfish formation around the trunk of the tree.

compared to the surface areas surveyed for SLF egg masses present above and below the traps and the egg masses present within the survey area of the control trees (last three columns, Table 1). Average number of egg masses by trap area (m^2) ranged from 0.203 to 1.080 while egg masses found adjacent to the traps and on control trees averaged far fewer, between 0.0 to 0.023 egg masses per m^2.

Comparisons of the vertical and horizontal orientation of the traps for each study site were significantly different (range $P < 0.027$ to $P < 0.001$; Wilcoxon Rank Sum Test) for all comparisons (horizontal stems were not present at the Easton site). The average number of egg masses (± SE) per site for vertical and horizontal trap orientation are displayed in Figure 9. Horizontal trap catch averaged 0.6 to 7.3 egg masses per trap compared to 14.5 to 54.4 egg masses for the paired site comparisons. A vertical trap orientation increased trap catch by an average of 13.2 times (range 6-24) across study sites when using horizontal trap catch as a baseline. Trap catch on the horizontal traps was not statistically different from surveys of the number of egg masses present on horizontal surfaces to either side of the traps and on nearby control trees (Kruskal-Wallis ANOVA; F=0.99, df = 4,95, $P = 0.38$).

The vertical trap trees were grouped into three diameter classes as follows: 10-13 cm (n = 22); 15-20 cm (n = 26); 23-33 cm (n = 25). Traps on trees of the larger two size classes averaged (± SE) significantly more egg masses (29.3 ± 5.6 and 33.5 ± 4.9) than traps in the smallest size class (11.7 ± 1.6) (Kruskal-Wallis ANOVA; F = 7.66, df = 2,72, $P < 0.001$). However, when the number of egg masses per trap was adjusted for the diameter of the tree used for the trap and hence trap surface area, there is no statistical difference among the three diameter classes (Figure 10), although the 15-20 cm size group trended as being the most efficient for collecting SLF egg masses (Kruskal-Wallis ANOVA; F = 0.41, df = 2,72, $P = 0.66$).

4 Discussion

Our trapping data show that the LST is an effective oviposition trap for SLF egg masses. These traps are durable, low-cost, simple to construct and can be set up and left in the field until harvest. The trap is made of roofing material affixed around the trunk of the tree with a second layer of material inverted and held away from the tree such that the appearance is that of a lamp shade (Figure 8). A two-page document on how to construct the traps is provided in the supplementary materials. Lamp shade traps oriented vertically stimulated SLF females to focus oviposition on the trap substrate and very few egg masses were noted above or below the traps or on the trunks of the paired control trees. LSTs provide an environment and a material on which SLF females will greatly concentrate their egg masses.

Initial attempts at an SLF egg mass trap design began in 2018 following our field observations that egg mass placement did not seem to be totally random. SLF oviposition behavior resulted in egg masses being laid on a wide variety of objects, but large concentrations were also observed, often on the underside of limbs. Egg mass clusters also appeared to be laid in areas that stayed dry, so we incorporated a covered and uncovered design to test for this. We made incremental progress in designing a successful trap and deployed over 560 traps over four years before a significant number of egg masses were laid on a subset of the 2021 traps; traps that had a top or covering yielded four times more egg masses than open traps. However, covered traps averaged only 5.5 masses per trap, not sufficient to serve as a survey tool or practical for even collection of SLF eggs masses for research purposes. We decided to pursue this trapping effort an additional season, focusing on the suitable oviposition substrate we and other researchers had identified (roofing material) (personal communication: Dr. Leskey, USDA-ARS, Kearneysville, WV) using a single trap style that enhanced the protected environment SLF females respond to, which the 2021 testing had indicated.

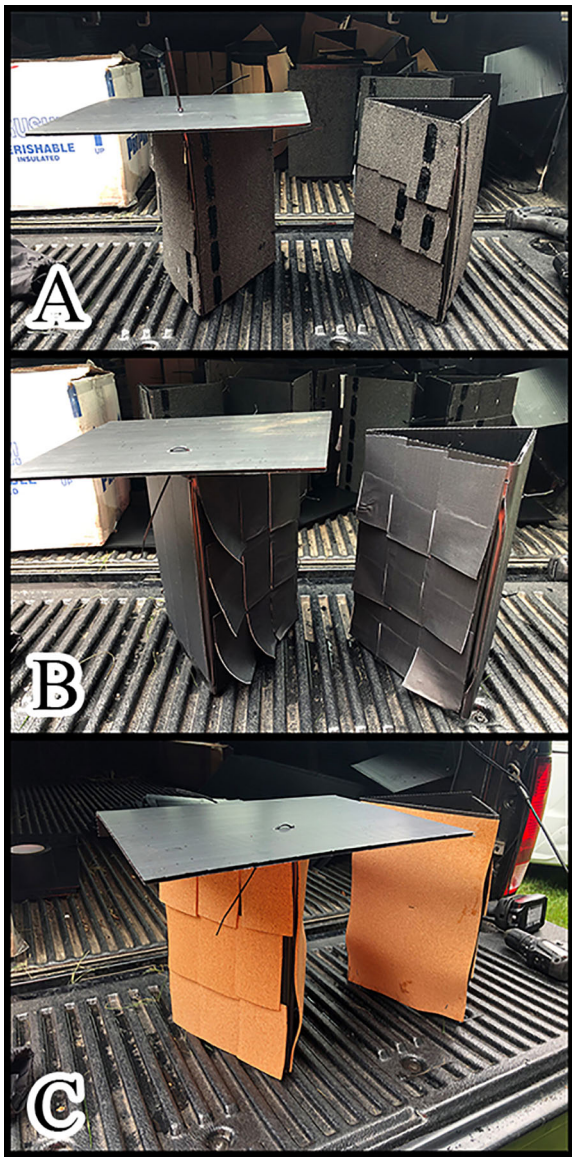

FIGURE 6
Triangle traps, **(A)** covered and uncovered with roofing, **(B)** covered and uncovered with gaffing tape, **(C)** covered and uncovered with cork. Each trap had one side with slightly overlapping strips of material and another side with a single smooth surface.

Testing in 2021 had also shown that trap height placement did not impact trap catch, so traps in 2022 were placed at the convenient working height of about 1.4 m. To create a better environment, we considered that the triangle traps placed the oviposition substrate away from the tree trunk and SLF had to move off the tree to interact with it. For the 2022 trap design, we wrapped roofing material around and directly to the tree trunk and chose the LST design which funneled SLF into a sheltered environment as they moved up the tree. SLF nymphs and adults are very active and are readily caught in traps that take advantage of their propensity for positive upward movement (16). The success of this trap for SLF oviposition was that it combined the two factors we had identified into a single trap design. SLF females walk up and onto a suitable oviposition substrate, and then encounter an environment where oviposition behavior is stimulated.

It was unexpected that trap orientation was such a significant effect and that SLF females did not interact with the trap when placed horizontally. The vertical traps on living trees were compared to horizontal stems that had partially fallen and could accommodate a trap. Only 4.5% of the total egg masses in the traps were laid on horizontal traps, not statistically different from egg mass counts on either side of the horizontal traps and nearby control trees. Perhaps not as many SLF enter the horizontal traps or that this trap orientation fails to stimulate oviposition behaviors to the extent that a vertical trap orientation does. The number of egg

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FIGURE 7
Hexagonal trap with roofing material and cork as egg laying substrates attached to *A. altissima*.

FIGURE 8
Lamp shade egg mass trap constructed on *A. altissima*.

masses found in horizontal traps reflected oviposition levels of SLF females in that immediate environment.

Vertical traps were deployed at each site on a range of tree diameters. This allowed us to identify the most efficient tree diameter on which to place LSTs, both in terms of efficiency (egg masses laid per trapping area) and for cost considerations of the amount of material required to build the traps. Although not statistically significant, traps placed on trees 15-20 cm in diameter averaged the most SLF egg masses per trapping area. Trees of this

TABLE 1 Trapping information and abundance of SLF egg masses found on and around vertically oriented traps, by site.

Study Site	No. Traps Deployed/ Date	Ave. DBH in cm (range)	Ave. No. Egg Masses per Trap	Ave. No. Egg Masses Above/Below	Ave. No. Egg Masses Control Tree	Ave. Egg Masses/m^2 of Trap Area	Ave. Egg Masses/m^2 Above/Below	Ave. Egg Masses/m^2 Control Tree
Blandon	8; 9/21	18.5 ± 3.8 (9.7-33.0)	28.63 ± 6.05a	0.75 ± 0.37b	0.38 ± 0.18b	0.667 ± 0.095a	0.003 ± 0.001b	0.019 ± 0.001b
Route 422	8; 9/28	20.8 ± 3.8 (10.2-35.1)	54.38 ± 12.70a	5.38 ± 3.31b	8.50 ± 5.81b	1.080 ± 0.288a	0.019 ± 0.008b	0.023 ± 0.013b
Easton	8; 10/20	17.3 ± 2.0 (9.9-28.2)	9.63 ± 3.12a	0.00 ± 0.00b	0.25 ± 0.25b	0.203 ± 0.059a	0.0 ± 0.0b	0.001 ± 0.001b
Delaware	8; 10/5	19.6 ± 0.8 (17.3-22.6)	47.13 ± 12.11a	0.63 ± 0.26b	1.00 ± 0.46b	0.957 ± 0.276a	0.003 ± 0.001b	0.004 ± 0.019b
Keystone	20; 10/18	20.1 ± 1.8 (10.9-33.0)	14.45 ± 1.92a	5.20 ± 1.69b	2.25 ± 0.79b	0.296 ± 0.041a	0.018 ± 0.005b	0.007 ± 0.002b
Route 30	21; 10/19	19.1 ± 1.0 (11.7-25.4)	21.38 ± 3.86a	4.81 ± 1.45b	4.57 ± 1.11b	0.426 ± 0.068a	0.023 ± 0.008b	0.019 ± 0.005b

Egg mass counts were tabulated for each trap, above and below the traps up to a 3 m height and from the trunk of a nearby tree that had no trap up to a 3 m height. Average values are followed by the standard error; different letters denote statistical significance ($P < 0.001$; Kruskal-Wallis ANOVA) at each site and for each data grouping (average egg masses and average masses by area).

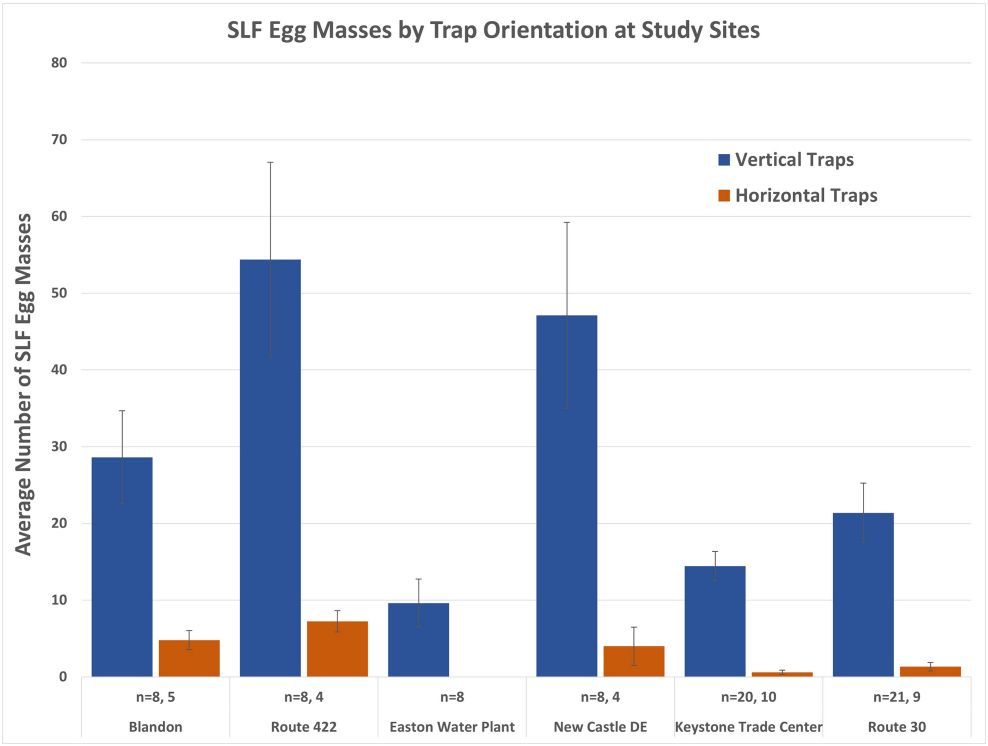

FIGURE 9
Average SLF egg masses laid in lamp shade traps oriented vertically and horizontally, by site. Vertically oriented traps were statistically different at each study site (range $P < 0.027$ to $P < 0.001$; Wilcoxon Rank Sum Test) except at the Easton site where no horizontal traps were set up.

size can be selected for routine trapping of egg masses with the assurance that egg mass yield will be high and trapping materials can be kept to a minimum. LSTs can certainly be placed on larger diameter trees, but a greater effort and cost will result.

LSTs placed on trees other than TOH should still be attractive. Although we did not test other tree species, if SLF females are active and feeding on a different host tree (e.g., silver maple) and a trap is placed on that tree, there is no reason they would not interact with it as they do when the trap is placed on TOH. If a tree species has rough or uneven bark, we suggest first attaching a strip of batting material around the trunk at the bottom of where the LST will be installed. This will fill in any gaps between the bark and the first layer of roofing material, so SLF do not get under the trap as they travel upward. We did note a significant amount of mold present on the egg masses at most of the study sites. If egg masses are to be used for research purposes this can probably be mitigated by keeping rainwater from entering the top of the trap by stapling and draping a small tarp above and over the LST. There are also mold inhibitors that might help if sprayed up and into the trap every few weeks when wet and humid conditions are present.

LSTs are low-cost, easy to set up and take down and were very efficient at concentrating SLF egg masses. These traps have the potential to be a valuable tool not only to aid in the collection of egg masses that are needed for the active biological control and research efforts against this invasive, destructive insect but also as a trapping tool that can be used to accumulate and destroy egg masses while monitoring SLF populations in a woodlot. This trap has great potential for detecting SLF in new areas, for monitoring SLF in areas of concern and potentially for estimating and predicting SLF population levels in an infested area.

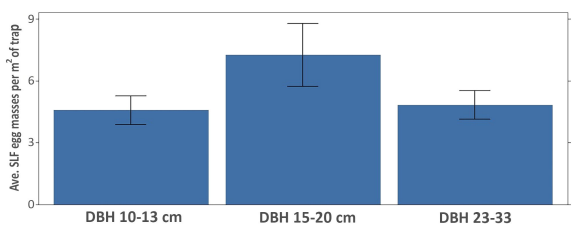

FIGURE 10
Average SLF egg masses (± SE) laid per m^2 of trapping area on all vertically oriented traps, grouped by tree diameter. DBH classes were not statistically different ($P = 0.66$; Kruskal-Wallis ANOVA).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PL and AD-F conceived the project. PL acquired funding. All authors participated in the design of the experiments. EW and AD-F oversaw the experiments, coordinated and participated in field work including trap set up, sampling and data collection and summary. PL

conducted data analysis. PL drafted the manuscript with contributions from AD-F and EW. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1154510/full#supplementary-material

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Assessing the host range of *Anastatus orientalis*, an egg parasitoid of spotted lanternfly (*Lycorma delicatula)* using Eastern U.S. non-target species

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The spotted lanternfly, *Lycorma delicatula* (Hemiptera: Fulgoridae), an invasive planthopper discovered in Pennsylvania, U.S. in 2014, has spread to many surrounding states despite quarantines and control efforts, and further spread is anticipated. A classical (importation) biological control program would contribute to the long-term management of *L. delicatula* in the eastern U.S. In its native range of China, *Anastatus orientalis* (Hymenoptera: Eupelmidae), an egg parasitoid, causes significant mortality. *Anastatus orientalis* consists of multiple haplotypes that differ in important biological parameters. To delineate the physiological host range of *A. orientalis* Haplotype C, we completed no-choice and choice testing. No-choice testing of non-target eggs from 36 insect species spanning six orders and 18 families showed that physiologically this haplotype of *A. orientalis* can develop in a variety of host species eggs from the families Coreidae, Fulgoridae, Pentatomidae, and Saturniidae. Ten of the 16 species that were attacked in the no-choice tests were also attacked in the choice tests. The production of progeny on non-target egg masses was significantly lower than on the controls (*L. delicatula* egg masses run simultaneously) in the no-choice and choice tests. For the non-target species that were attacked and resulted in female wasp progeny, these females were able to produce their own progeny at the same rate as control females that were reared from the *L. delicatula* eggs. Larger host eggs corresponded to an increased female-biased sex ratio of the progeny, suggesting that gravid females select them for fertilized eggs. Results from these studies suggest that *A. orientalis* Haplotype C prefers to parasitize *L. delicatula* egg masses but is capable of developing in some non-target species.

KEYWORDS

biological control, Eupelmidae, Fulgoridae, invasive species, natural enemy

Introduction

The spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is an invasive planthopper first detected in Berks County Pennsylvania in the fall of 2014 (1). It has since spread extensively within Pennsylvania and neighboring states where it threatens the grape, hops, tree fruit, plant nursery, and timber industries (2). Quarantines have been put in place to restrict the movement of plant, wood, and stone products, but because egg masses are cryptic and it is difficult to regulate the movement of all items potentially harboring them (3, 4), the pest has continued to spread and now has established populations in 14 states plus reports in two more (5). Research on host plant utilization indicates that *L. delicatula* can develop to the adult stage on several host plant species in addition to its favored host, tree-of-heaven (*Ailanthus altissima* (Mill.) Swin) (6), which further confounds the eradication efforts. Some mortality from resident fungal pathogens (7), egg parasitoids (8), and predation (9) have been noted but the incidences are rare and so far have resulted in inconsequential mortality rates. However, in its native range in China, several parasitoid wasps attack *L. delicatula* eggs and nymphs and cause significant mortality (10, 11). A classical (or importation) biological control program could contribute to management efforts against *L. delicatula*.

Anastatus orientalis Yang & Choi (Hymenoptera: Eupelmidae) is a small parasitic wasp that attacks *L. delicatula* eggs in its native range in China (10), most commonly in northeastern China (11). In Chinese field collections, *A. orientalis* parasitizes at a relatively high rate, ranging from 20 to 80% of egg masses attacked and up to 40% of eggs parasitized within individual egg masses (11, 12). Additionally, while suitable host plants are abundant for *L. delicatula* in these same locations in China, populations of *L. delicatula* are low compared to those of the invasive population in the U.S (11). This suggests that *A. orientalis* and other natural enemies are having a strong effect on *L. delicatula* populations in China. *Anastatus orientalis* was selected as a candidate biological control agent for helping to manage *L. delicatula* in South Korea where it is also invasive (13, 14). In 2011, the South Korean Rural Development Administration National Institute of Agricultural Sciences initiated a collaboration with the Chinese Academy of Forestry to collect and evaluate *A. orientalis* as a candidate biological control agent for invasive *L. delicatula* in Korea (10, 15, 16) and introductions of the parasitoids were made soon afterwards. *Anastatus orientalis* is being considered as a candidate biological control agent for the invasive populations of *L. delicatula* in the eastern United States.

Evaluation of the physiological host range is an essential first step to determine whether a natural enemy will be deemed sufficiently host-specific (17, 18) to be suitable for release as a biological control agent. We now know that multiple haplotypes of *A. orientalis* are present in China (19). Therefore, while initial testing work conducted for the releases in Korea suggested that the haplotype of *A. orientalis* released in Korea did not attack their species of concern (15), it is essential to test the strain of *A. orientalis* maintained in U.S. quarantine cultures (20) against the eggs of selected non-target species present in the United States.

Here we outline our results from no-choice and choice tests in which we tested eggs of planthoppers, stink bugs, other hemipterans, silk moths and selected other species that are native to or resident in the United States as potential hosts of *A. orientalis*. This manuscript describes testing of species of concern present in the eastern United States where *L. delicatula* currently is invasive. Due to concern about the threat of invasion by *L. delicatula* to the western United States, simultaneous coordinated testing of species resident to the western coast of the United States was conducted at the University of California Riverside Insectary and Quarantine Facility (21). To evaluate the physiological host range, we first exposed non-target eggs to mated female *A. orientalis* wasps in no-choice tests where the wasps had access to the non-target species eggs for a full week of exposure. For any species that were parasitized in the no-choice tests, we then conducted choice tests. For choice tests, the parasitoid was offered the target host, *L. delicatula*, together with the non-target egg masses so that the wasps could choose which (or both) host(s) to parasitize. This provided information on behavior and showed whether a parasitoid would choose to use a non-target when the primary host was also available. We present results of testing more than 30 different non-target species. Testing included *Poblicia fuliginosa* (Olivier) (Hemiptera: Fulgoridae), which is a closely related native species present in the current invasive range of *L. delicatula*. Initial priority species also included other species corresponding to taxonomic relatedness, the morphological similarity of egg masses, and occurrence in the same microhabitat as *L. delicatula* in the landscape.

Materials and methods

Parasitoid colony

The parasitoid colony we tested is maintained in the containment facility at the USDA APHIS Forest Pest Methods Laboratory, Buzzards Bay, MA, and all studies were completed in this facility. Laboratory colonies of *A. orientalis* were established from parasitized egg masses collected annually from the field in Beijing, China (N39.9925, E116.2109) from 2016 to 2019. To confirm the species and haplotype, we extracted genomic DNA from a single leg pulled from five randomly selected colony specimens using the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD). Each specimen was genotyped for two mitochondrial fragments of the COI gene. We aligned the newly generated DNA sequences with reference sequences from the six haplotype groups identified in Wu et al. (19). Two months later, we conducted a second round of genotyping with 40 specimens to ensure colony homogeneity. After another eight months, we conducted the final round of genotyping including eight more specimens from the C colony and another 60 from the other haplotype colonies to ensure no contamination between strains.

We reared the wasps using *L. delicatula* egg masses collected each year from December to March in Pennsylvania. We collected egg masses whole and intact by cutting them from bark (primarily from *A. altissima*) and held them in a growth chamber at a constant 5°C with no light. We maintained the *A. orientalis* laboratory

colony by setting up groups of three to five females with one or two males in a medium-sized rearing container (473-ml plastic deli cup, AD16 GenPak, Charlotte, NC) with the following temperature and light conditions: daily high of 25°C and low of 14°C, lights on 5:55 AM to 6:23 PM, 65% RH. These conditions were chosen to emulate environmental conditions in mid-September in Beijing, China, and hereafter will be referred to as 'Beijing-fall conditions' and work well for continuous rearing of this haplotype of *A. orientalis* (20). We provided each set of wasps with a streak of honey as a food source. The wasps were held without access to egg masses for one week (corresponding with their preoviposition period), and then given one *L. delicatula* egg mass for a period of one week. We held the developing progeny under Beijing-fall conditions for another month then moved to a 25°C constant temperature and a light cycle of 16 hours light and 8 hours dark to promote emergence. Further rearing details are provided in Broadley et al. (20).

Non-target selection, collection, and rearing

We selected non-target species for testing based on relatedness to *L. delicatula* (prioritizing *Poblicia fuliginosa*, which is in the same family, Fulgoridae) and other planthoppers, morphological similarity of egg clusters, and based on prior information on species utilized by other *Anastatus* species (22–25). Potential eastern U.S. non-target species spanning six insect orders and 18 families were field collected or acquired from laboratory colonies for no-choice and choice testing (Table 1). We saved voucher specimens of all species. We acquired species not already established in laboratory colonies as nymphs and adults through field collection in natural habitats, using visual inspection of host plant material, sweep netting, or beating of host plant material. We then moved field collected insects into rearing conditions designed for each species' needs to produce egg masses. We kept phytophagous species on whole potted host plants or provided plant material such as vegetables. Typical laboratory rearing conditions were set at a temperature of 25°C and a light cycle of 16 hours light and 8 hours dark. We maintained entomophagous species in enclosures mimicking their natural habitat and provisioned them with insects that met their dietary requirements.

No-choice host testing experiments

To prepare for no-choice testing, we gently aspirated recently emerged *A. orientalis* wasps (24 hours old or less) from their plastic rearing containers. We placed up to five female and five male wasps together in a 1:1 ratio in a small glass rearing container (8 oz wide mouth mason jar 00500, Kerr, Newell, Atlanta, GA). The jars were streaked with honey for food and covered with mesh (no-see-um polyester netting 7250NSW, Bioquip, Rancho Dominguez, CA) to provide ventilation. Before use in experiments, the glass containers were autoclaved, and together with the metal lid rings and mesh, were washed with a one percent Citronox (Alcanox, White Plains, NY) solution, rinsed with DI water, then acetone, and air dried. This cleaning removed chemical traces that might affect the olfactory cues presented to the wasp because previous work found that *A. orientalis* responded to chemical traces left behind by *L. delicatula* (25). We placed the rearing containers in secondary plastic containers (6 Quart Storage Box 1642, Sterilite, Townsend, MA) to further isolate the tests from any outside olfactory cues. Before use, the secondary containers were also washed in a 1% Citronox solution and rinsed with DI water, then rinsed with 95% ethanol and left to air dry. The wasps were given a one-week preoviposition period at Beijing-fall conditions (described above).

Following the one-week preoviposition period, we removed all male wasps, and the female wasps were moved individually to a new glass rearing container and given either a non-target egg mass or a *L. delicatula* egg mass as a control to parasitize. The non-target eggs were put into testing as young as possible with the aim of testing them within less than a week. If they were not run within a five-day window, they were ramped down by moving them into 10°C for half a week then into 5°C until they could be put into testing. When multiple non-target species were tested during the same day, a single set of controls for that day was used for all the simultaneously-tested species, with sufficient numbers of control replicates to match the number of replicates of the non-target species having the most replication on that day. We tested non-target and control host species in separate secondary plastic containers to avoid mixing kairomones and other chemical cues. All wasps were allowed one week of oviposition under Beijing-fall conditions. After the one week, we removed the female wasps from the egg masses and preserved them in 95% ethanol. We placed the egg masses individually in plastic rearing containers (6oz, Clear Hinged Deli Cup, AD06, GenPak, Charlotte, NC, modified to include a mesh lid) and held them in Beijing-fall conditions for one month and then subsequently placed them under 25°C long day conditions for emergence. Our goal was to test 30 replicates for each non-target species, but some of the species were challenging to obtain so less replication was possible. We recorded host nymphal and F1 parasitoid emergence daily (Monday-Friday) until there was no further emergence for one month. We noted the sex of the parasitoids that emerged. Non-target nymphs were saved in 95% ethanol for vouchers.

Egg size measurements

We recorded egg size measurements for each species, including *L. delicatula*, to determine the mean volume. We measured ten eggs from three of the egg masses being dissected. For circular eggs, we measured the diameter; for ellipsoid and cube-shaped eggs, we measured height, width, and length; for cylindrical and oblate spheroid eggs, we measured width and height; and lastly for pentagonal frustum-shaped eggs, we measured the egg height, the width at the top of the pentagon, and width at the base of the pentagon. The volume of the eggs was calculated from these measurements.

We dissected a subset of available replicates (Table 2) for each non-target species and a comparative subset of *L. delicatula* egg masses. We waited at least two weeks after the last parasitoid emergence before dissecting the egg masses. For egg masses with

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TABLE 1 Non-target species collection information.

Order	Family	Species	Collection location/Commercially obtained	Acquisition date
Blattodea	Blaberidae	*Nauphoera cinerea*	Reared by Alex Baranowski in colony	Jul. 26, 2020
Coleoptera	Coccinellidae	*Harmonia axyridis*	Kingston, Charlestown, Exeter, Hopkinton, RI/Bristol & Southbury, CT	Nov. 2019
Coleoptera	Coccinellidae	*Hippodamia convergens*	Purchased live adults from Natures Good Guys	Feb. 9, 2021
Hemiptera	Acanaloniidae	*Acanalonia bivittata*	Exeter, RI and University of Delaware Farm, Newark, DE	Aug.-Sep. 2020 (RI); Jul. and Aug. 2017 - 2022 (DE)
Hemiptera	Acanaloniidae	*Acanalonia conica*	Field-caught individuals University of Delaware Farm, Newark, DE added to colony	Jul. and Aug. 2017 - 2022
Hemiptera	Coreidae	*Anasa armigera*	Kingston, RI	Aug. 2019
Hemiptera	Coreidae	*Anasa tristis*	Kingston, RI	Aug. 2020
Hemiptera	Dictyopharidae	*Rhynchomitra microrhina*	Field-caught from the University of Delaware Farm, Newark, DE added to colony	Jul. and Aug. 2017 - 2022
Hemiptera	Flatidae	*Flatormenis proxima*	Field-caught from the University of Delaware Farm, Newark, DE added to colony	Jul. and Aug. 2017 - 2022
Hemiptera	Fulgoridae	*Poblicia fuliginosa*	Field-caught from Jones Lake, Bladen Co., NC	Aug. 2018 - 2022
Hemiptera	Lygaeidae	*Oncopeltus fasciatus*	Kingston, RI & Bristol, CT	Aug. 2020
Hemiptera	Membracidae	*Thelia bimaculata*	Newark, New Castle Co., DE	Jun. and Jul. 2020-2021
Hemiptera	Pentatomidae	*Chinavia hilaris*	Kingston, North Kingstown, RI/Windsor, Falls Village, CT; and <50 km from Newark, DE	Jun.-Sep. 2018 and 2020
Hemiptera	Pentatomidae	*Edessa florida*	<50 km from Newark, DE	Jun.-Sep. 2018 and 2020
Hemiptera	Pentatomidae	*Euschistus servus*	Kingston & Exeter, RI	Jun.-Sep. 2020
Hemiptera	Pentatomidae	*Euschistus tristigmus*	<50 km from Newark, DE	Jun.-Sep. 2019 - 2021
Hemiptera	Pentatomidae	*Halyomorpha halys*	NJ Dept. of Agriculture egg masses reared in Newark, DE	Continuously reared
Hemiptera	Pentatomidae	*Murgantia histrionica*	<50 km from Newark, DE	Jun.-Sep. 2019 - 2021
Hemiptera	Pentatomidae	*Oebalus pugnax*	<50 km from Newark, DE	Jun.-Sep. 2020
Hemiptera	Pentatomidae	*Podisus maculiventris*	<50 km from Newark, DE	Jun.-Sep. 2019 and 2020
Hemiptera	Pentatomidae	*Thyanta custator*	<50 km from Newark, DE	Jun.-Sep. 2020
Hemiptera	Reduviidae	*Phymata pennsylvanica*	Exeter, RI/Southbury and Bristol, CT	Aug. 2020
Hemiptera	Reduviidae	*Zelus luridus*	Kingston, RI/Bristol, CT	Jun.-Sep. 2020
Lepidoptera	Bombycidae	*Bombyx mori*	Reared by Alex Baranowski in colony	Jan. 8, 2020
Lepidoptera	Erebidae	*Lymantria dispar dispar*	From the Forest Pest Methods Laboratory, Buzzards Bay, MA continuous colony	Sep. 2019
Lepidoptera	Lasiocampidae	*Malacosoma americanum*	New Shoreham, RI; Exeter, RI: Bristol, CT	Dec. 2019 and Mar. 2020
Lepidoptera	Nymphalidae	*Danaus plexippus*	Kingston, Charlestown, Exeter, RI	Jul. and Aug. 2020
Lepidoptera	Saturniidae	*Actias luna*	Purchased cocoons from Carolina Biological and Magic Wings Butterflies	Dec. 2020; Feb. 2021; Feb 2022
Lepidoptera	Saturniidae	*Antheraea polyphemus*	Purchased cocoons from Carolina Biological and Magic Wings Butterflies	Dec. 7-14, 2020

(Continued)

TABLE 1 Continued

Order	Family	Species	Collection location/Commercially obtained	Acquisition date
Lepidoptera	Saturniidae	*Callosamia promethea*	Reared by Kathrine Straley	Aug. 2020
Lepidoptera	Saturniidae	*Hyalophora cecropia*	Purchased cocoons from Carolina Biological and Magic Wings Butterflies	Dec. 7-14, 2020
Mantodea	Mantidae	*Mantis religiosa*	Kingston and Exeter, RI	Jul. and Aug. 2020
Mantodea	Mantidae	*Stagmomantis carolina*	Brooklyn, NY	Jan. 7, 2020
Mantodea	Mantidae	*Stagmomantis limbata**	Davis, CA	Jan. 2020
Mantodea	Mantidae	*Tenodera sinensis*	Kingston and W. Greenwich, RI/Southington, CT	Sep. and Oct. 2019
Phasmatodea	Diapheromeridae	*Manomera blatchleyi*	Kingston, RI	Jul. 2020

*Not resident to the eastern United States.

no parasitoid emergence, we dissected them after one month. For those dissected, we recorded the fate of each egg.

Fitness of *A. orientalis* reared from non-target eggs

For each non-target egg mass that had female wasp emergence, we evaluated the fecundity of a randomized subset of these progeny (F1 generation). We placed up to five female wasps in a plastic rearing cup. Five male wasps from a different egg mass of the same species host were added to the rearing cup for a 1:1 ratio. If five males were not available, as many as possible were added, and in the event of no male wasps being present, five male wasps produced from a *L. delicatula* egg mass were added to ensure that the females could mate. We provisioned them with honey and held them under Beijing-fall conditions for a one-week preoviposition period. Following preoviposition, we removed all male wasps and all except two female wasps. The remaining two female wasps were each placed in a container and given a single *L. delicatula* egg mass. After one week of oviposition under Beijing-fall conditions, we removed the female wasps and saved them in 95% ethanol. We allowed the egg masses to develop for one month under Beijing-fall conditions before we moved them to 25°C long day conditions for emergence. We recorded nymphal and parasitoid emergence (F2 generation) daily (Monday-Friday) and saved all female wasps in 95% ethanol.

We compared the size of female progeny reared out of non-target hosts (F1 from non-targets) as compared to the simultaneously run controls reared from *L. delicatula* (F1 from controls) and to progeny produced when these non-target reared females (F1 from non-targets) were given *L. delicatula* to parasitize (F2 from the non-target females). We measured both hind tibiae from up to 10 F1 and F2 generation female parasitoids from each category from the testing of the non-targets *Actias luna* (L.) and *Halyomorpha halys* (Stål). Hind tibia measurements can be a useful proxy for fecundity, mating ability, and longevity (26), which together can suggest greater fitness (27–29). We chose progeny reared from these two non-target species to capture a wide range of egg volumes because *A. luna* had the largest non-target egg, and *H. halys* was among the smallest non-target species to produce female progeny. For each replicate, we removed both hind tibiae from female wasps and took measurements using Leica Microsystems model M125 C dissecting microscope with LASX software Version 3.7.2.22383.

Choice host testing experiments

For non-target species that produced wasps in the no-choice tests, we then conducted choice testing. We followed the same procedure as for the no-choice testing except that larger (16 oz wide mouth mason jar 1440061180, Ball, Newell, Atlanta, GA) glass rearing jars were used, and both the non-target and target (*L. delicatula*) egg mass were provided for oviposition. Following wasp exposure, we placed each egg mass into individual plastic rearing cups so that emergence could be recorded separately. We conducted host specificity testing between September 2018 and June 2021, with no-choice testing starting in September 2018 and choice testing starting in August 2020.

Statistical analyses

To test for the effect of non-target as compared to the controls, we used a Wilcoxon paired-sample test with the no-choice and choice tests analyzed separately. To test the effect of egg size on the resulting sex of wasp progeny produced, we ran a generalized linear model with a logit link function and a binomial distribution. To test for a difference in the number of F2 progeny produced from each F1 female, we used a one-way ANOVA. To compare the mean tibia measurements of female *A. orientalis* wasps (F1 generation) reared from a large non-target host (*A. luna*) and a small non-target host (*H. halys*) as compared to the controls (*L. delicatula*) and the size of the female progeny of these females, we averaged the measurements of both hind tibia for each individual, then ran a one-way ANOVA

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TABLE 2 Egg masses dissected from no-choice testing.

Order	Family	Species	Number of replicates dissected	Detection of any unsuccessful *A. orientalis* emergence	Mean (± SE) unsuccessful *A. orientalis* emergence per egg mass	Mean (± SE) number of eggs per egg mass	Proportion of unsuccessful *A. orientalis* emergence
Coleoptera	Coccinellidae	*Harmonia axyridis*	1	No	0	0	0
Hemiptera	Acanaloniidae	*Acanalonia bivittata*	29	No	0	0	0
Hemiptera	Acanaloniidae	*Acanalonia conica*	56	No	0	0	0
Hemiptera	Coreidae	*Anasa armigera*	28	Yes	0.07 ± 0.05	10.46 ± 0.93	0.01
Hemiptera	Coreidae	*Anasa tristis*	40	Yes	5.35 ± 1.56	25.90 ± 2.03	0.21
Hemiptera	Flatidae	*Flatormenis proxima*	8	No	0	0	0
Hemiptera	Fulgoridae	*Poblicia fuliginosa*	22	Yes	4.45 ± 1.31	32.32 ± 1.65	0.14
Hemiptera	Fulgoridae	*Lycorma delicatula*	66	Yes	3.05 ± 0.60	39.79 ± 3.05	0.08
Hemiptera	Lygaeidae	*Oncopeltus fasciatus*	11	No	0	0	0
Hemiptera	Pentatomidae	*Chinavia hilaris*	20	Yes	0.45 ± 0.30	22.55 ± 2.04	0.02
Hemiptera	Pentatomidae	*Euschistus servus*	39	Yes	0.59 ± 0.26	20.15 ± 1.70	0.03
Hemiptera	Pentatomidae	*Euschistus tristigmus*	16	No	0	0	0
Hemiptera	Pentatomidae	*Halyomorpha halys*	36	Yes	0.64 ± 0.24	25.14 ± 0.64	0.03
Hemiptera	Pentatomidae	*Murgantia histrionica*	43	Yes	0.21 ± 0.10	12.49 ± 0.84	0.02
Hemiptera	Pentatomidae	*Oebalus pugnax*	2	No	0	0	0
Hemiptera	Pentatomidae	*Podisus maculiventris*	15	No	0	0	0
Hemiptera	Pentatomidae	*Thyanta custator*	19	Yes	0.21 ± 0.21	28.10 ± 2.60	0.01
Hemiptera	Reduviidae	*Phymata pennsylvanica*	2	No	0	0	0
Hemiptera	Reduviidae	*Zelus luridus*	10	No	0	0	0
Lepidoptera	Bombycidae	*Bombyx mori*	10	No	0	0	0
Lepidoptera	Lasiocampidae	*Malacosoma americanum*	6	No	0	0	0
Lepidoptera	Nymphalidae	*Danaus plexippus*	12	No	0	0	0
Lepidoptera	Saturniidae	*Actias luna*	40	Yes	0.93 ± 0.49	30.30 ± 1.17	0.03
Lepidoptera	Saturniidae	*Antheraea polyphemus*	19	Yes	0.16 ± 0.09	23.89 ± 1.34	0.01

(Continued)

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TABLE 2 Continued

Order	Family	Species	Number of replicates dissected	Detection of any unsuccessful *A. orientalis* emergence	Mean (± SE) unsuccessful *A. orientalis* emergence per egg mass	Mean (± SE) number of eggs per egg mass	Proportion of unsuccessful *A. orientalis* emergence
Lepidoptera	Saturniidae	*Hyalophora cecropia*	10	Yes	2.70 ± 2.59	27.00 ± 1.18	0.10
Mantodea	Mantidae	*Mantis religiosa*	10	No	0	0	0
Mantodea	Mantidae	*Stagmomantis carolina*	11	No	0	0	0
Mantodea	Mantidae	*Stagmomantis limbata*	11	No	0	0	0
Mantodea	Mantidae	*Tenodera sinensis*	10	No	0	0	0
Phasmatodea	Diapheromeridae	*Manomera blatchleyi*	10	No	0	0	0

The species that contained unsuccessfully emerged *A. orientalis* are indicated in gray.

and a Tukey-Kramer's test. All statistics were run using JMP 13.1.0 (SAS Institute Inc.) and figures were constructed using JMP 13.1.0 and R version 4.1.1 (The R Foundation for Statistical Computing).

Results

Parasitoid haplotype

Three rounds of genotyping on 53 A. *orientalis* specimens from the wasp colony used for this study over a 10-month period showed that the colony was composed of a homogenous population all representing the same species and the same haplotype. All sequences were identical and matched with *A. orientalis* Haplotype C.

No-choice and choice host-testing experiments

We completed no-choice testing of non-target eggs from 36 insect species spanning six orders and 18 families (Table 3). These tests included planthoppers (including a Fulgoridae) but also tests of non-targets from more distantly related species. Of these 36 species, *A. orientalis* was able to parasitize the eggs of 16 species and produce F1 progeny. No progeny were produced from any egg masses tested from species in the order Blattodea (cockroaches), Coleoptera (beetles), Mantodea (mantises) or Phasmatodea (stickbugs). However, every species tested in no-choice testing in the families Coreidae (leaf-footed bugs), Fulgoridae (lanternflies), Pentatomidae (stinkbugs), and Saturniidae (giant silk moths) was attacked to some degree.

The production of progeny on non-target egg masses was significantly lower than the production of progeny on the controls (*L. delicatula* egg masses run simultaneously) in the no-choice tests (Figure 1; $\chi^2 = 1172.97$, df = 1, $p<0.0001$) as well as in the choice tests (Figure 2; $\chi^2 = 481.07$, df = 1, $p<0.0001$). Ten of the 16 species that were attacked in the no-choice tests were also attacked in the choice tests (Table 3), although two of these (*Euschistus tristigmus* (Say) and *Thyanta custator* (Fabricius)) showed negligible attack rates. The non-target hosts that experienced the highest attack and the closest attack rates to that of the *L. delicatula* controls were the giant silk moths (Saturniidae) eggs, followed by stink bugs (Pentatomidae). For *Edessa florida* Barber, *Oebalus pugnax* (Fabricius), and *Callosamia promethea* (Drury), three species that were attacked in the no-choice tests, we were not able to acquire additional eggs for the subsequent choice testing. However, we conclude from the other tests of stink bug and silk moth eggs that these likely would have been utilized. For four species (*Acanalonia bivittata* (Say), *Acanalonia conica* (Say), *Zelus luridus* Stål, and *Stagmomantis carolina* (Johannson)) that were not attacked in the no-choice testing, we had extra available eggs and so we conducted a small number of choice tests with them as well, and no progeny were produced in the choice tests either. The number of progeny produced in the choice testing was overall lower than in the no-choice testing. This was true for the non-target eggs tested as well as the corresponding *L. delicatula* controls, and when more attack on non-targets was evident there was also less attack on the *L. delicatula* controls (as evident in results from testing the saturniids and *H. halys*).

For 29 non-target species, a subset of egg masses was dissected (Table 2). We found no unemerged *A. orientalis* in 18 of the species and very little in the rest. In 11 species (*A. luna, Anasa armigera* (Say), *Anasa tristis* (DeGeer), *Antheraea polyphemus* (Cramer), *Chinavia hilaris* (Say), *Euschistus servus* (Say), *H. halys, Hyalophora cecropia* (L.), *Murgantia histrionica* (Hahn), *P. fuliginosa, T. custator*), all of which also successfully reared some *A. orientalis*, we found low numbers of unemerged *A. orientalis* present in the host eggs, either in a diapause state or as desiccated adults. Due to the scope of these dissections, we did not quantify

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TABLE 3 Non-target species tested in no-choice and choice tests.

Order	Family	Species	Common name	No-Choice Tests			Choice Tests		
				Number of replicates	*A. orientalis* Progeny	Prop. of progeny female*	Number of replicates	*A. orientalis* Progeny	Prop. of progeny female*
Blattodea	Blaberidae	*Nauphoeta cinerea*	speckled cockroach	30	no	N/A	N/A	N/A	N/A
Coleoptera	Coccinellidae	*Harmonia axyridis*	harlequin ladybird	3	no	N/A	N/A	N/A	N/A
Coleoptera	Coccinellidae	*Hippodamia convergens*	convergent ladybeetle	30	no	N/A	N/A	N/A	N/A
Hemiptera	Acanaloniidae	*Acanalonia bivittata*	two-striped planthopper	33	no	N/A	16	no	N/A
Hemiptera	Acanaloniidae	*Acanalonia conica*	green conehead planthopper	60	no	N/A	15	no	N/A
Hemiptera	Coreidae	*Anasa armigera*	horned squash bug	60	yes	0	30	yes	0
Hemiptera	Coreidae	*Anasa tristis*	squash bug	55	yes	0	30	no	N/A
Hemiptera	Dictyopharidae	*Rhynchomitra microrhina*	planthopper	1	no	N/A	N/A	N/A	N/A
Hemiptera	Flatidae	*Flatormenis proxima*	northern flatid planthopper	30	no	N/A	N/A	N/A	N/A
Hemiptera	Fulgoridae	*Poblicia fuliginosa*	sooty planthopper	33	yes	0.22	30	no	N/A
Hemiptera	Lygaeidae	*Oncopeltus fasciatus*	large milkweed bug	22	no	N/A	N/A	N/A	N/A
Hemiptera	Membracidae	*Thelia bimaculata*	locust treehopper	8	no	N/A	N/A	N/A	N/A
Hemiptera	Pentatomidae	*Chinavia hilaris*	green stink bug	15	yes	0.02	11	yes	0
Hemiptera	Pentatomidae	*Edessa florida*	Edessa stink bug	2	yes	0	0	No data	No data
Hemiptera	Pentatomidae	*Euschistus servus*	brown stink bug	30	yes	0	30	yes	0
Hemiptera	Pentatomidae	*Euschistus tristigmus*	dusky stink bug	30	yes	0	30	yes	0
Hemiptera	Pentatomidae	*Halyomorpha halys*	brown marmorated stink bug	30	yes	0.01	30	yes	0.02
Hemiptera	Pentatomidae	*Murgantia histrionica*	harlequin bug	48	yes	0	12	yes	0
Hemiptera	Pentatomidae	*Oebalus pugnax*	rice stink bug	2	yes	0	0	No data	No data
Hemiptera	Pentatomidae	*Podisus maculiventris*	spined soldier bug	35	yes	0	30	no	N/A
Hemiptera	Pentatomidae	*Thyanta custator*	red shouldered stink bug	30	yes	0	27	yes	0
Hemiptera	Reduviidae	*Phymata pennsylvanica*	Pennsylvania ambush bug	2	no	N/A	N/A	N/A	N/A

(Continued)

TABLE 3 Continued

Order	Family	Species	Common name	No-Choice Tests			Choice Tests		
				Number of replicates	*A. orientalis* Progeny	Prop. of progeny female*	Number of replicates	*A. orientalis* Progeny	Prop. of progeny female*
Hemiptera	Reduviidae	*Zelus luridus*	pale green assassin bug	30	no	N/A	11	no	N/A
Lepidoptera	Bombycidae	*Bombyx mori*	domestic silk moth	30	no	N/A	N/A	N/A	N/A
Lepidoptera	Erebidae	*Lymantria dispar dispar*	spongy moth	30	no	N/A	N/A	N/A	N/A
Lepidoptera	Lasiocampidae	*Malacosoma americanum*	eastern tent caterpillar	10	no	N/A	N/A	N/A	N/A
Lepidoptera	Nymphalidae	*Danaus plexippus*	monarch butterfly	30	no	N/A	N/A	N/A	N/A
Lepidoptera	Saturniidae	*Actias luna*	luna moth	36	yes	0.41	30	yes	0.61
Lepidoptera	Saturniidae	*Antheraea polyphemus*	polyphemus moth	36	yes	0.75	29	yes	0.74
Lepidoptera	Saturniidae	*Callosamia promethea*	promethea silk moth	9	yes	0	0	No data	No data
Lepidoptera	Saturniidae	*Hyalophora cecropia*	cecropia moth	30	yes	0.67	31	yes	0.6
Mantodea	Mantidae	*Mantis religiosa*	European mantis	27	no	N/A	N/A	N/A	N/A
Mantodea	Mantidae	*Stagmomantis carolina*	Carolina mantis	23	no	N/A	6	no	N/A
Mantodea	Mantidae	*Stagmomantis limbata*	bordered mantis	28	no	N/A	N/A	N/A	N/A
Mantodea	Mantidae	*Tenodera sinensis*	Chinese mantis	30	no	N/A	N/A	N/A	N/A
Phasmatodea	Diapheromeridae	*Manomera blatchleyi*	Blatchley walking stick	30	no	N/A	N/A	N/A	N/A

*The corresponding proportion of the progeny that were female when testing *L. delicatula* in the no-choice tests was 0.75 and for the choice testing was 0.78.
The egg species that produced *A. orientalis* progeny from no-choice and choice tests are indicated in gray.
N/A, Not applicable.

dead early immature or encapsulated wasp larvae. Proportions of dissected eggs with unemerged *A. orientalis* were low, ranging from 0.01 to 0.21 (i.e., 1 to 21%) of egg masses with *A. armigera* and *T. custator* showing the lowest rates and *A. tristis* the highest rates. For all non-target species egg masses with some unemerged wasps, on average 1.44 ± 0.26 wasps per egg masses did not emerge. Parasitized *L. delicatula* egg masses reared under the same conditions had a similar rate of unsuccessful emergence, a proportion of 0.08 (or 8%) or a mean of 3.05 ± 0.60 wasps per egg mass that did not successfully emerge.

Effect of egg size

The size of the non-target egg presented to the *A. orientalis* wasps was important not only as a factor in whether the egg was successfully used but also in the resulting sex bias. Egg size had a significant effect on the sex of progeny produced (df = 1; χ^2 = 9.26, p = 0.0023; Figure 3A). There was a significant relationship between egg volume and the sex ratio of the progeny produced with larger eggs having a female skewed sex ratio (df = 1; χ^2 = 8.25, p = 0.0041; Figure 3B). The giant silk moth eggs overall showed the highest proportion of female progeny and were the only non-target species that resulted in female progeny in both the no-choice tests and the choice tests (Table 3). The giant silk moths also had some of the largest eggs that were put into testing. The species with the next highest ratio of female to male progeny was *P. fuliginosa*, followed by species of stink bugs.

Fitness of *A. orientalis* reared from non-target eggs

For the non-target species that were attacked, and which resulted in female wasp progeny (F1 generation), the F1 females were able to produce their own progeny (F2 generation) at the

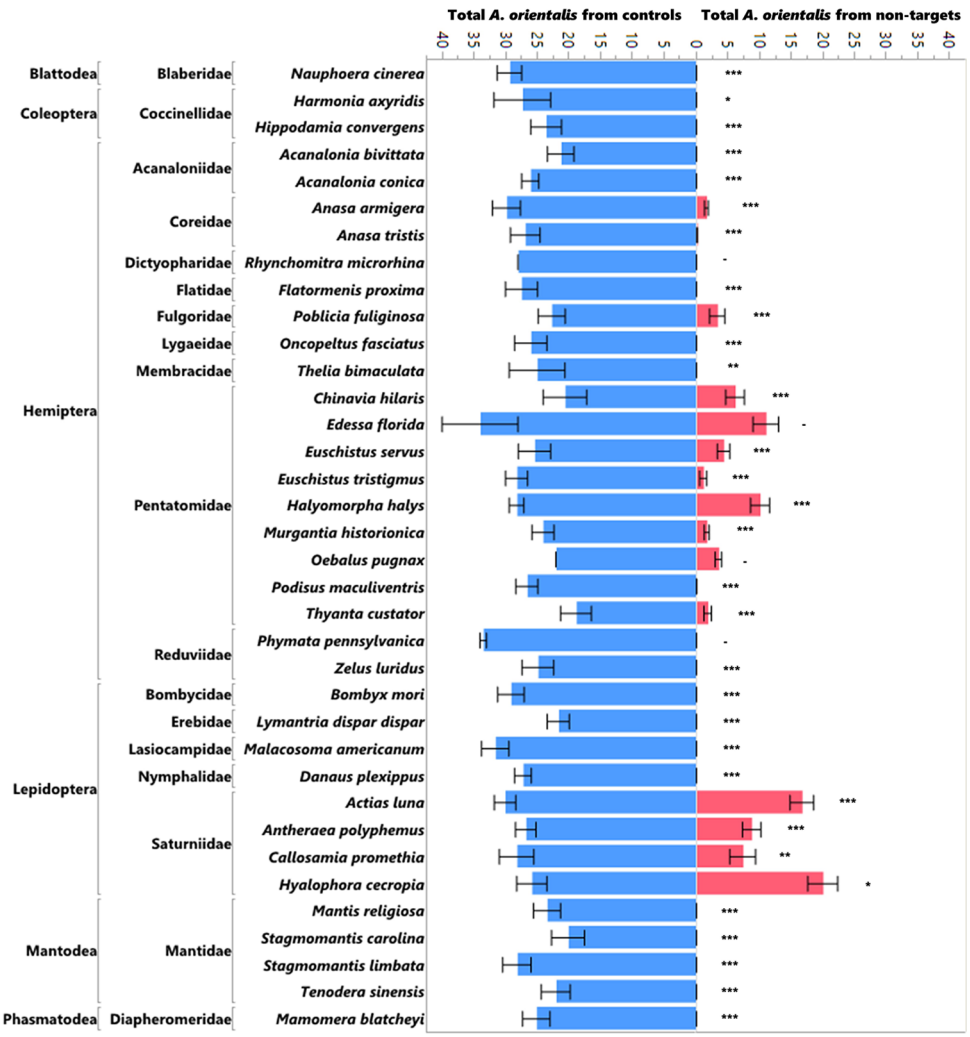

FIGURE 1
Mean number of *A. orientalis* produced in non-targets (pink) as compared to simultaneously run controls (blue) in no-choice tests organized by insect order and family. The error bars represent standard errors. Wilcoxon paired-sample test; *P < 0.05; **P < 0.01; ***P < 0.001; and a dash when the sample sizes were too small to run the test.

same rate as the F1 females that were reared from the control *L. delicatula* eggs. There was no significant difference (df = 5, 387, F = 1.6728, p = 0.1401) between the number of F2 wasp progeny produced by the F1 females reared out from the non-targets as from the F1 females reared out of *L. delicatula* (Figure 4). By comparing the mean tibia measurements of female *A. orientalis* wasps (F1 generation) reared on a large non-target host (*A. luna*) and a small non-target host (*H. halys*) compared to controls (*L. delicatula*), we found that the females reared from *H. halys* eggs were significantly smaller (0.59 mm ± 0.02 SE) than those reared from *A. luna* (0.88 mm ± 0.01 SE) and *L. delicatula* (0.94 mm ± 0.01 SE; df = 2, 22, F-ratio = 131.068, p < 0.0001). When these differently sized females, reared from different hosts, were all provided *L. delicatula* egg masses, their female progeny did not show any significant differences in tibia size (df = 2,27, F-ratio = 0.0655, p = 0.9368). Smaller progeny from non-target hosts were able to attack *L. delicatula* at the same rate as larger females and their progeny were of normal size.

Discussion

Evaluation of the physiological host range is one of the first steps in assessing the risk-benefit potential of a biological control agent to determine if it is sufficiently host-specific (16, 17). The results from this study demonstrate that *A. orientalis* Haplotype C is physiologically able to develop in non-target egg masses of coreids, other fulgorids, pentatomids, and saturniids. However, the number of progeny produced from the non-targets is consistently lower than from the *L. delicatula* controls and the attack rates were particularly low when the wasps had a choice of using the non-target egg masses or the *L. delicatula* egg masses. The fact that we did not see attack on *P. fuliginosa* in the choice tests, even though the no-choice testing tells us that it is physiologically possible is interesting because, of the species tested, *P. fuliginosa* is the one most closely related (and biologically similar) to *L. delicatula*. Additional host range testing of western fulgorid planthoppers, as well as other potential non-targets that are resident to the western

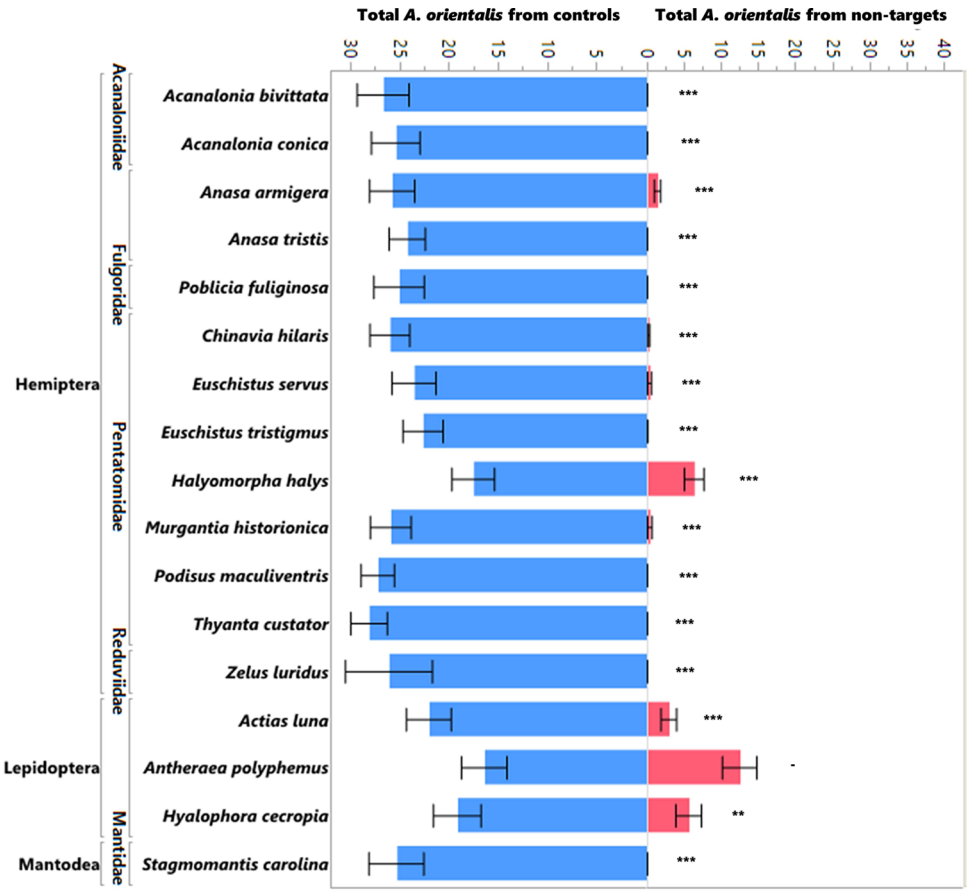

FIGURE 2
Mean number of *A. orientalis* produced in non-targets (pink) as compared to simultaneously run controls (blue) in choice tests organized by insect order and family. The figure includes all the species that showed attack in the no-choice tests as well as four additional species (*A. bivittata, A. conica, Z. luridus*, and *S. carolina*) that were put into testing. The error bars represent standard errors. Wilcoxon paired-sample test; **P < 0.01; ***P < 0.001; and a dash when the sample sizes were too small to run the test.

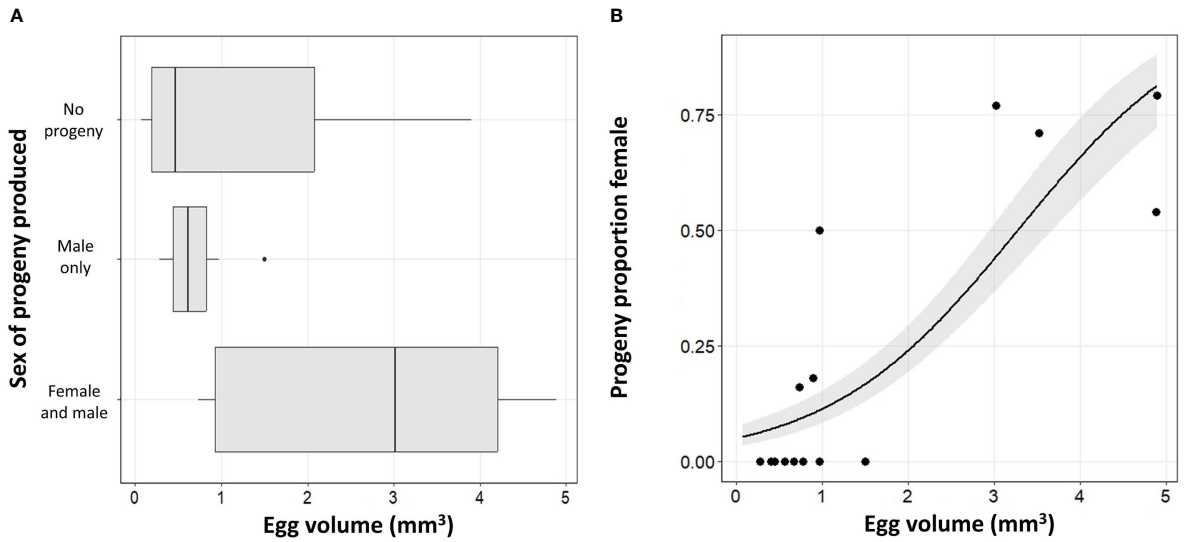

FIGURE 3
Mean egg volume (mm^2) and the resulting wasp emergence patterns of the progeny showing **(A)** the sex of the progeny that emerged and **(B)** the proportion female.

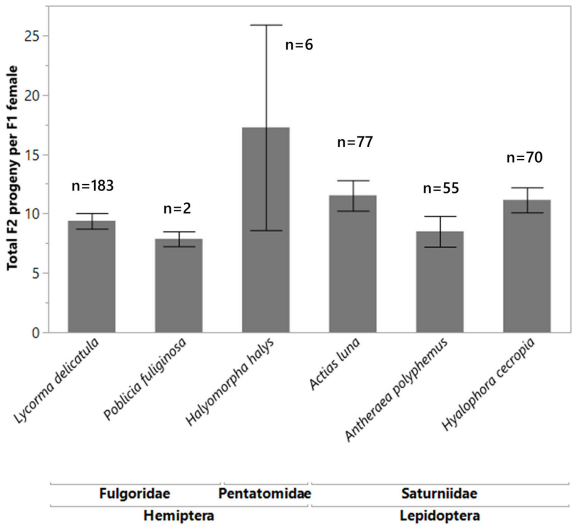

FIGURE 4
Mean number of F2 progeny wasps produced from spotted lanternfly eggs masses for each F1 female, which was reared from non-target species eggs. There was no significant difference (df = 5, 387, F = 1.6728, p = 0.1401) between the number of progeny produced by females reared out from the non-targets as compared to those reared from *L. delicatula*. The sample sizes are written above each species.

United States, was conducted by collaborators at the University of California Riverside (21). Together these studies show that *A. orientalis* Haplotype C prefers to parasitize *L. delicatula* egg masses but is willing to attack and is physiologically able to develop in various non-target species suggesting that it is facultatively oligophagous or polyphagous.

We found that *A. orientalis* Haplotype C is more likely to use large eggs, and more often used these large eggs to produce female progeny, and the progeny from large eggs are larger than those reared from smaller eggs. The three largest eggs included in the study were those of saturniid (giant silk moth) eggs—*A. luna*, *A. polyphemus*, and *H. cecropia*. Along with the two fulgorids—*L. delicatula* and *P. fuliginosa*—these showed the highest levels of attack and the highest proportion of female progeny. This is commonly found with other species of egg parasitoids (30, 31). We did not find any notable differences in the number of developing wasps that did not successfully emerge from the egg masses and the rate of this occurrence was very similar in the non-targets as it was for the controls. While *H. halys* was previously found to not be parasitized by *A. orientalis* (15), our tests and those of our colleagues (21) found that it could be parasitized. Also, Seo et al. (15) found that the saturniid *Antheraea pernyi* was only parasitized if the eggs were immature and dissected out of a gravid female. However, we found that older saturniid eggs were readily parasitized by *A. orientalis*. It may be that this difference in host range is due to differences in the haplotype or strain of *A. orientalis* tested in these studies. Future studies are planned to test the host range of other detected *A. orientalis* haplotypes.

Various factors may have affected our results. One factor could be the number of eggs presented to the wasps for each test. Wasps were given enough eggs to satisfy their oviposition needs. On average there were 42.9 ± 3.0 (mean ± SE, n = 84) *L. delicatula* eggs available in each egg mass tested, from which were produced on average 23.0 ± 0.43 wasp progeny during the one-week exposure period. The non-target replicates had an average of 27.4 ± 0.4 (mean ± SE, n = 725) eggs per replicate. This is a lower number of eggs per replicate than the control *L. delicatula* because they either did not lay their eggs in large masses or were challenging to rear in numbers. Considering that the mean number of adult wasps produced from the *L. delicatula* replicates was 23 wasps/egg mass, the wasps were provisioned with enough eggs such they were unlikely to use all available host eggs by the end of the oviposition period. Contamination by resident *Anastatus* species at the site of *L. delicatula* egg mass collection is another potential factor. However, parasitism of field-collected eggs by *Anastatus* species native or resident to the U.S. has been shown to be extremely rare (unpublished data), and no or at least inconsequential numbers would have been present in egg masses used for this study. The rearing temperatures, Beijing-fall conditions, were selected to optimize *A. orientalis* fitness (20) and are not necessarily the optimal rearing condition for the non-target species. However, for the most conservative tests possible here, we felt it was important to prioritize conditions for the parasitoid rather than for the various hosts. And lastly, when put into testing there was some variability of the age of eggs. Most non-target eggs were tested when they were less than a week old (median age = 6 days old, n=991) however there was variation around this median. The mean age was higher (12.5 ± 0.52 days). When exposed to wasps, the eggs of *A. bivittata*, *A. conica*, *B. mori*, *Flatormenis proxima* (Walker), *P. fuliginosa*, and *Tenodera sinensis* Saussure all had a median age that was greater than a week (13, 20, 49, 21, 30, and 18.5 days, respectively). The eggs of all other species were exposed when less than a week old. The fact that the eggs of these species were overall older may have artificially decreased their attractiveness or viability for *A. orientalis* as has been seen with other egg parasitoids (32–34). Additionally, the control eggs (of *L. delicatula*) were collected over the winter, stored in chill as described above, and used throughout the year whenever any of the non-targets were available for testing. However, based on prior research, *L. delicatula* eggs even up to a year old were viable hosts for *A. orientalis* with no discernable effect on parasitism rate (20).

While no-choice and choice host range testing are essential steps in assessing the risk-benefit of a candidate classical (or importation) biological control agent, it is important to keep in mind that these tests determine the ability of a parasitoid to physiologically use the hosts. By design, these tests are conducted in a controlled laboratory setting, which purposely limit the complexities of environmental conditions. In addition, any research conducted with *A. orientalis* in the U.S. as a candidate agent must be done in quarantine containment. Thus, these tests can overestimate ecologically relative host usage due to the limited ecological and environmental aspects of the tests (35, 36). In a field setting many additional factors influence a natural enemy's ability to locate and utilize a potential host. A parasitoid must first be able to locate the host within a complex habitat, and once found, it may either accept it or decide to continue searching. For example, *Trissolcus japonicus* (Ashmead), a parasitoid of *H. halys*, displayed an oligophagous physiological host range in laboratory

choice and no-choice assays (37–39) but a more restricted behavior in laboratory behavioral assays and field tests (40, 41).

Studies that focus on a natural enemy's ability to find hosts include, but are not limited to, testing for attraction response to kairomones left by the non-targets and target pest species or large arena studies where the natural enemy is provided a larger and more complex space to search for the target and non-target hosts. Prior studies that tested the foraging behaviors of *A. orientalis* when in the presence of residues left by *L. delicatula* and the oothecal covering of *L. delicatula* eggs found that wasps detected chemical traces left by the *L. delicatula*, eliciting a strong arrestment response (42). Subsequent work evaluating ecological host range found that *A. orientalis* spent significantly more time interacting with chemical traces left behind by *L. delicatula* than the controls or than with the chemical traces left by *P. fuliginosa* (unpublished). This provides additional evidence that the preferred host of *A. orientalis* is *L. delicatula*. Tests evaluating the host range of *A. orientalis* in the field in China are planned using sentinel egg masses.

The population of *A. orientalis* tested in this study was Haplotype C. However, several other haplotypes of *A. orientalis* have been identified and these have now been separated into isofemale lines (19). Laboratory studies evaluating the rearing of the haplotypes shows that they respond differently to rearing conditions (19). This suggests that these haplotypes are genetically distinct but also that their biologies are distinct. Previous host range testing of *A. orientalis* conducted before release of it as a biocontrol agent in South Korea showed that it was highly host specific to *L. delicatula* and that *H. halys* eggs did not support development of *A. orientalis* (15). This is different than the testing results we obtained with Haplotype C, suggesting that the haplotype tested for release in South Korea may have been a different haplotype, potentially Haplotype D (19). Unlike our colony of Haplotype C wasps, the line tested for release in South Korea was successfully reared using constant 25°C temperature and long day light conditions, adding further evidence that what was released in Korea and the strain we are studying have different biological characteristics. Additional host range testing will be conducted with the additional genetic lines we have in colony. However, this study, along with the work of Gómez Marco et al. (21), shows that *A. orientalis* Haplotype C is willing and able to develop in various non-target species but prefers to parasitize *L. delicatula*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

HB, SS, DP, KH, SD, JK, and JG designed the study. X-YW and L-MC provided the parasitoids for the study. HB, SS, DP, YW, and SD ran the studies. HB, SS, DP, KH, LT, TH, CB, AR and JK provided non-target insects to test. HB, SS, DP, and YW summarized the results. HB, SS, DP, TH, AR, JK, YW, and JG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Cryptic diversity and virulence of *Beauveria bassiana* recovered from *Lycorma delicatula* (spotted lanternfly) in eastern Pennsylvania

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The entomopathogenic fungus *Beauveria bassiana* is cosmopolitan and known to infect a variety of sap-sucking pests like aphids, mealybugs, and scales in the order of Hemiptera. In Fall 2017, spotted lanternfly (SLF) adults killed by the fungal entomopathogen *B. bassiana* were found in Berks County, Pennsylvania. In 2018-2020 we collected SLF and nearby non-target insects killed by *Beauveria* spp. from 18 field sites in southeastern Pennsylvania. We identified 159 *Beauveria* isolates from SLF and six isolates from non-targets. Five isolates of *B. bassiana* and one isolate of *B. brongniartii* were identified from the non-targets. Based on sequence data from the nuclear B locus (Bloc) intergenic region, all the isolates from SLF were identified as *B. bassiana*, but there were 20 different strains within this species, grouped into two clades. Three *B. bassiana* strains (A, B, and L) were found in most field sites and were the most prevalent. Representative isolates for these three strains were used in laboratory bioassays and were compared to a commercial *B. bassiana* strain (GHA). Strain B was inferior to A, L, and GHA against nymphs; strains A and L had greater efficacy than B and GHA against adults. We also quantified conidial production on SLF cadavers. This paper discusses the diversity of these *B. bassiana* strains in SLF populations and implications for biological control of this abundant invasive.

KEYWORDS

Beauveria, Lycorma delicatula, entomopathogenic fungi, planthopper, invasive insect

Introduction

Entomopathogenic fungi infect a diversity of insects but are well known as acute pathogens of hemipterans, including aphids, leafhoppers and planthoppers (1, 2). *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) is a well-studied entomopathogen (3, 4) and has been reported causing epizootics (sometimes as part of a complex of entomopathogens) in hemipteran pests, including the chinch bug (*Blissus*

leucopterus (Say) [Hemiptera: Blissidae]) (5), elongate hemlock scale (*Fiorinia externa* (Ferris) [Hemiptera: Diaspididae]) (6), and kudzu bug (*Megacopta cribraria (Fabricius)* [Hemiptera: Plataspidae]) (7). In 2019, a co-epizootic caused by two native fungal pathogens, *B. bassiana* and *Batkoa major* (Thaxt.) Humber (Entomophthorales: Entomophthoraceae), was reported in two populations of the new invasive planthopper, the spotted lanternfly (SLF), *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), in southeastern Pennsylvania (8). Clifton et al. (9) discovered two more species of hypocrealean fungi infecting SLF that are assumed to be native: *Metarhizium pemphigi* (Driver & R.J. Milner) (Hypocreales: Clavicipitaceae) and the new species *Ophiocordyceps delicatula* (Hypocreales: Ophiocordycipitaceae).

The genus *Beauveria* includes more than 12 cryptic species that cannot be identified with morphological characters alone (10–12). *Beauveria* isolates can be identified to species by sequencing the nuclear B locus (Bloc) intergenic region and other genes (12, 13). Previous studies describing *Beauveria* isolates that naturally infected invasives like the emerald ash borer (*Agrilus planipennis* Fairmaire [Coleoptera: Buprestidae]) (14) and coffee berry borer (*Hypothenemus hampei* (Ferrari) [Coleoptera: Curculionidae]) (13, 15) discovered multiple cryptic species and a wide assemblage of *B. bassiana* strains. Additional studies on these invasives involved bioassays and demonstrated how some native isolates of *B. bassiana* had greater virulence than a commercialized strain (GHA) and produced more conidia on their cadavers, which is indicative of greater epizootic potential (14, 16).

SLF is a new invasive, univoltine planthopper that was first discovered in Berks County, Pennsylvania in 2014 (17) and has spread to 13 additional US states (18). The native range of SLF includes China, Taiwan, and Vietnam, and SLF can be a sporadic pest in China feeding on tree of heaven, *Ailanthus altissima* (Mill.) Swingle (Sapindales: Simaroubaceae) (19, 20). SLF prefers tree of heaven which is invasive in North America, but will feed on wild and cultivated grapes (*Vitis* spp. [Vitales: Vitaceae]) as well as other woody plants; this species is now infamous for its voracious feeding, which has caused reduced productivity and mortality in grapevines (21–23). These impacts, as well as others on trees such as black walnut, *Juglans nigra* L. (Fagales: Juglandaceae) and red maple, *Acer rubrum* L. (Sapindales: Sapindaceae) (24–26), have resulted in the need for means of controlling this harmful insect. Eradication of SLF seems unlikely and long-term management tools, including natural enemies like entomopathogenic microorganisms, could help control this invasive species (27–29).

Mycoinsecticides containing different *B. bassiana* strains are available for commercial use in the United States (30). In field trial applications, BoteGHA (Certis USA; containing *B. bassiana* strain GHA) killed 43-48% of SLF nymphs and adults infesting *A. altissima* in a public park (31). Laboratory bioassays testing mycoinsecticides found that *Cordyceps javanica* (Frieder. & Bally) Kepler, B. Shrestha & Spatafora (Hypocreales: Cordycipitaceae) was less effective than *B. bassiana*, but all *B. bassiana*-based products had similar efficacy against SLF of different ages (32).

In 2018-2020 we conducted parallel studies on the genetic diversity of naturally occurring *B. bassiana* (this study) and *B. major* (33) that infect SLF. The goals of the current study were to (1) isolate and identify *Beauveria* spp. infecting SLF and non-targets, predominantly collected in natural forested areas and edge habitats (e.g., tree lines in a neighborhood), in southeastern Pennsylvania, and (2) describe the prevalence and distribution of indigenous *B. bassiana* strains in these areas invaded by SLF. This study was used to select indigenous *B. bassiana* strains for laboratory bioassays carried out in 2021 with potential for use in biological control of this new invasive pest.

Materials and methods

Sample collection

We collected fungal isolates from dead SLF or non-targets, mostly on the ground and at bases of trees, in Pennsylvania, USA. The first *B. bassiana* sample in this study was sent to our laboratory in August 2017 (site a, Table 1; Figure 2). In May 2018, we found four SLF adult cadavers with *B. bassiana* outgrowth beneath leaf litter in site b (Table 1; Figure 2). Although these SLF adults died in Fall 2017, we isolated these *B. bassiana* samples in 2018. In 2018-2020, we opportunistically sampled *Beauveria* spp. associated with SLF and non-target insects in 17 more sites among 5 counties in southeastern Pennsylvania (Table 1; Figure 2). Most of the sampling occurred in Berks County, Pennsylvania. Dense populations of SLF were mostly restricted to municipalities in and around eastern Berks County in 2014-2016. We only sampled sites around Lancaster and Philadelphia in 2020, mainly because SLF populations had only recently established there, and the Pennsylvania counties with these sites were added to the SLF quarantine in 2018 (34).

For most of the *Beauveria*-infected SLF and non-target insects, we collected cadavers that already had fungal outgrowth that is characteristic for *Beauveria* spp. We identified non-target insects to family, genus, or species with a dissecting microscope following dichotomous keys for morphological characters. In some cases, we obtained isolates from SLF or non-target cadavers that had no fungal outgrowth at the time of sampling but later produced conidia after incubation on 1.5% water agar for 5-10 days in the laboratory. For non-target insects that already had fungal outgrowth at the time of sampling, we first isolated the fungus on selective media (see next section) before cleaning the cadaver with ethanol for identification. We found most of the cadavers in 2018-2020 near the bases of *A. altissima* trees (Figure 1A). In rare instances we found SLF adults with fungal outgrowth still attached to host trees by their mouthparts and/or legs (Figure 1B). In some sampling sites (e.g., Leesport and Sinking Spring) we collected live SLF and reared individuals on potted plants in a quarantine lab (for other studies), and some of these SLF later died from naturally occurring infections (i.e., these individuals were already infected with fungi before the time of sampling and rearing in the laboratory; see Supplementary Materials). Collaborators from USDA-APHIS (Kelly Murman, Stefani Cannon, Miriam Cooperband, John Baker, Regina Whitfield), Pennsylvania Department of Agriculture (Emily Fricke, Albert Ciccarone, Betsy Myers, Sandie Conway), and Penn State University (Emelie Swackhamer & Julie Urban) also helped

TABLE 1 Sampling sites in southeastern Pennsylvania from 2017 to 2020.

Map code	Site name	Pennsylvania County	Coordinates[a]	Elevation (meters)	Sampling year (# isolates)[b]
a	Boyertown Reservoir	Berks	40°20'25.2"N 75°41'00.9"W	184	2017 (1)
b	Fleetwood residence[a]	Berks	40°27'14.5"N 75°49'05.7"W	170	2017 (4); 2018 (1)
c	Angora Fruit Farm	Berks	40°21'30.9"N 75°52'59.9"W	214	2018 (31); 2019 (3); 2020 (6)
d	Blandon	Berks	40°26'31.4"N 75°52'54.3"W	114	2018 (2); 2019 (5); 2020 (4)
e	Conrad Road residence[a]	Berks	40°26'50.7"N 75°37'20.3"W	317	2018 (13); 2020 (8)
f	Kutztown University	Berks	40°30'32.8"N 75°46'29.9"W	138	2018 (9)
g	Lilitz residence[a]	Lancaster	40°09'26.0"N 76°18'26.0"W	117	2018 (1)
h	Penn State Berks	Berks	40°21'35.6"N 75°58'34.2"W	82	2018 (1)
i	Pottstown Quarry	Montgomery	40°14'07.9"N 75°35'25.8"W	70	2018 (12)
j	Schuler Road[a]	Berks	40°29'37.9"N 75°49'00.2"W	116	2018 (18); 2020 (1)
k	Leesport[a]	Berks	40°26'55.7"N 75°57'51.3"W	84	2019 (5)
l	Sinking Spring	Berks	40°19'36.0"N 76°02'21.9"W	116	2019 (14); 2020 (5)
m	Graffa Pond[a]	Lancaster	40°01'36.3"N 76°14'28.9"W	110	2020 (7)
n	Hill road	Berks	40°21'11.8"N 75°52'32.9"W	225	2020 (3)
o	Lancaster Central Park	Lancaster	40°01'13.5"N 76°16'42.5"W	114	2020 (2)
p	Overlook Park	Lancaster	40°05'00.1"N 76°19'11.2"W	108	2020 (6)
q	Schuylkill Center for Environmental Education	Philadelphia	40°03'38.1"N 75°14'44.5"W	113	2020 (1)
r	Treichlers Bridge	Lehigh	40°44'03.2"N 75°32'21.5"W	102	2020 (1)

[a]For sites located on private properties, coordinates are provided for nearby towns and intersections. Elevations for the areas of sampling are based on USGS data (https://apps.nationalmap.gov/viewer/). Sampling sites are marked on the map (see Figure 1).
[b]The number of isolates includes Beauveria spp. from non-targets.

with sampling SLF cadavers with fungal outgrowth in some sites outside of Berks County (e.g., Lancaster and Philadelphia).

Isolation of fungi, DNA extraction, PCR, and sequencing

We swabbed conidia from SLF cadavers with fungal outgrowth with a sterile cotton-tip applicator and transferred to 6-cm Petri dishes containing selective media for *Beauveria*. The medium was adapted from Chase et al. (35), with 30 g wheat germ liter^{-1}, autoclaved and then filtered through cheesecloth, before adding 0.25 g liter^{-1} chloramphenicol, 0.20 g dodine liter^{-1}, 0.01 g crystal violet liter^{-1}, and 15 g agar liter^{-1} before autoclaving again. We sealed selective media plates with Parafilm strips and placed in an incubator (20°C, 0:24 (L:D) h). After 10-14 days, we stored cultures of *Beauveria* isolates at 4°C until further use. Additional cultures of each *Beauveria* isolate were stored in 10% sterile glycerol at -80 °C in 2 mL cryovials (Nalgene, Thermo Fisher Scientific, Rochester, NY, USA).

We produced mycelium for DNA extraction from each *Beauveria* isolate following the protocol described in Clifton et al. (31) using potato dextrose broth. Bidirectional nucleotide sequences were determined for the nuclear Bloc intergenic region. A region of Bloc was amplified and sequenced using the primer pair B22-deg × B3.3R, following PCR conditions described by Rehner et al. (12). We used the SAP/EXO protocol to clean up reaction mixtures prior to sequencing (33). Sequencing was done by Cornell University Institute of Biotechnology on an ABI 3730x1 or through submissions to GeneWiz (https://www.genewiz.com/).

Beauveria phylogenetics and prevalence of strains

We edited/trimmed, assembled, and aligned chromatograms and sequences with Geneious Prime software (2021.1.1; Biomatters Ltd.), resulting in contigs with 931-934 base pairs for the Bloc region. Sequence data were checked with the National Center for Biotechnology Information (NCBI) nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). After we identified unique strains of *B. bassiana*, we included the sequence data of each representative strain in the analysis, with one strain of *B. brongniartii* as an outgroup (Table 2). Maximum Likelihood (ML) analysis was performed using the rapid bootstrap algorithm in RAxML-HPC2 on XEDE version 8.1.11 using the default GTR+G in CIPRES Science Gateway online system (36). The Best Tree from ML analysis was drawn using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). We deposited representative isolates of each strain with the USDA ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY). ARSEF accession numbers and Genbank accession numbers are listed in Table 2.

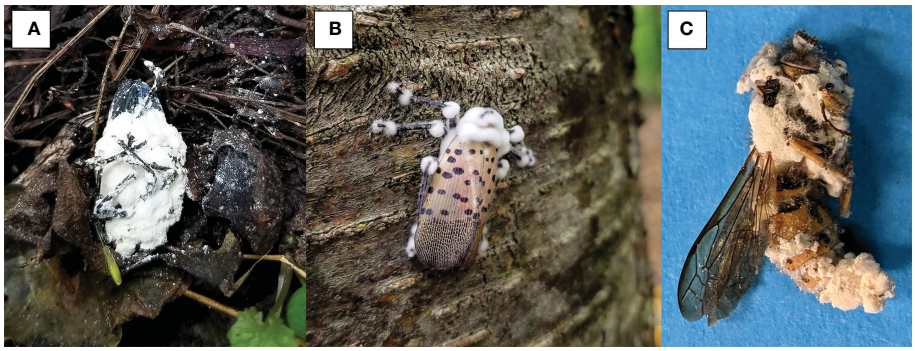

FIGURE 1
Examples of spotted lanternfly and a non-target with *Beauveria* fungal outgrowth. **(A)** Spotted lanternfly adult with profuse *Beauveria* outgrowth and conidia (infective spores). The white powdery spores are visible on the nearby ground and debris. **(B)** Spotted lanternfly adult killed by *Beauveria* and still attached to tree bark. **(C)** Non-target yellowjacket wasp with *Beauveria* outgrowth (later identified as *B brongniartii*).

Beauveria bassiana bioassays for *L. delicatula* nymphs and adults

Selection of *B. bassiana* isolates and inoculum production

Based on results from the field studies with *B. bassiana* strains, in early 2021 we chose six isolates to evaluate conidial production using biphasic-solid fermentation on flaked barley following the methods described by Jaronski & Jackson (37) (Table 3). These six isolates were representative of the prevalent strains (A, B, and L based on Bloc sequence data) in field sites with epizootics that occurred in 2018 (Tables 1; 3).

We inoculated 50 ml of a liquid medium (20 g L^{-1} glucose, 1g L^{-1} yeast extract, 5 g L^{-1} KNO_3, 2.5g L^{-1} KH_2PO_4, 150 mg L^{-1} chloramphenicol) with conidia from each agar culture in 100 ml Wheaton bottles, which, loosely capped, were then incubated for 4 days at 26° C. Agitation was provided by a magnetic stir bar, rotating at moderate speed in each bottle. This culture medium

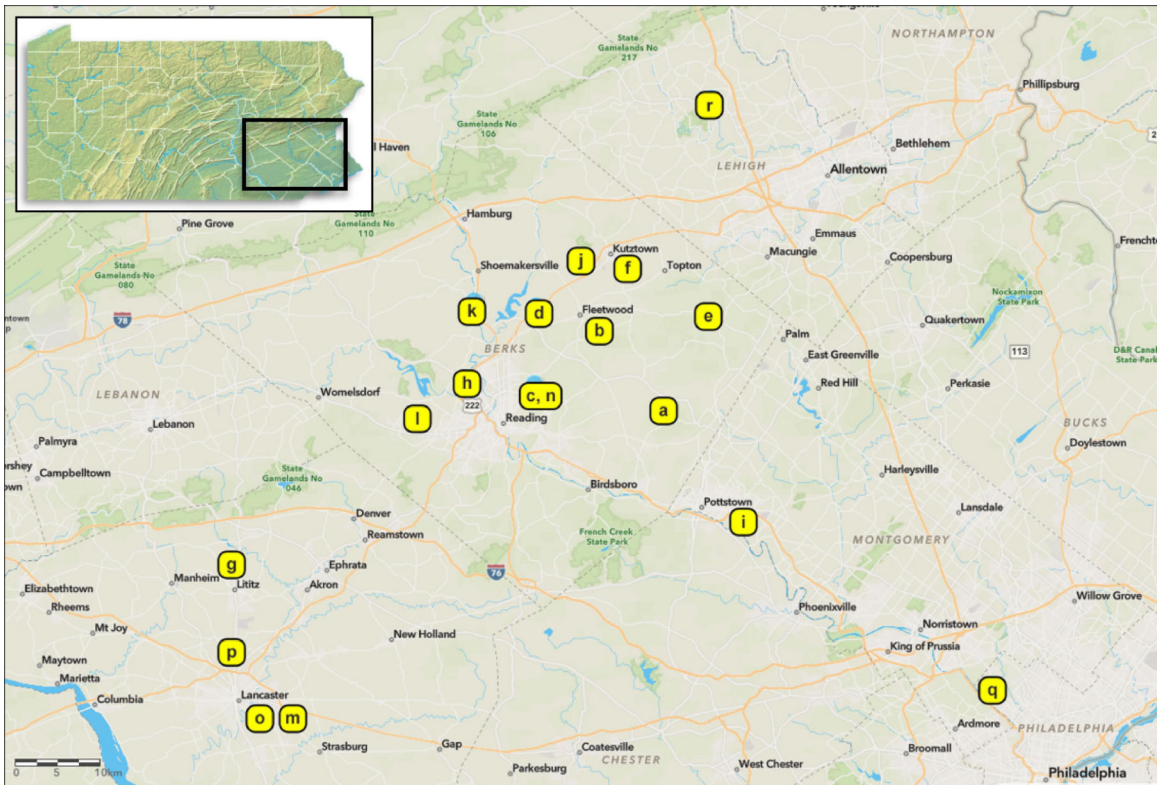

FIGURE 2
Sampling sites in southeastern Pennsylvania, USA. Inset image on the top left highlights the region. Isolates of *Beauveria* spp. were obtained from spotted lanternfly and non-targets in 2017 to 2020 (see Table 1 for sampling site information). All sampling sites were infested with tree-of-heaven (*Ailanthus altissima*) and other host plants. More information about isolates and sampling methods are provided in the Supplementary Materials.

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TABLE 2 GenBank accession numbers of *Beauveria* spp. strains from spotted lanternfly (SLF) and other insect hosts analyzed in this study.

Representative strain (lab code; ARSEF accession no.)	Host(s) and location	B locus Genbank accession no.	Reference
B. bassiana			
A (18-02); ARSEF 14462	SLF (Pennsylvania, USA)	OP897311	This study
B (18-58); ARSEF 14463	SLF (Pennsylvania, USA)	OP897312	This study
C (18-57); ARSEF 14464	SLF (Pennsylvania, USA)	OP897313	This study
D (18-376); ARSEF 14465	Nitidulid beetle*	OP897314	This study
	(Coleoptera: Nitidulidae) (Pennsylvania, USA)		
E (18-56); ARSEF 14466	SLF (Pennsylvania, USA)	OP897315	This study
F (18-391); ARSEF 14467	SLF (Pennsylvania, USA)	OP897316	This study
G (18-328); ARSEF 14468	SLF (Pennsylvania, USA)	OP897317	This study
H (18-432); ARSEF 14469	SLF (Pennsylvania, USA)	OP897318	This study
I (18-427); ARSEF 14470	SLF (Pennsylvania, USA)	OP897319	This study
J (18-285); ARSEF 14471	SLF (Pennsylvania, USA)	OP897320	This study
K (18-333); ARSEF 14472	SLF (Pennsylvania, USA)	OP897321	This study
L (18-21); ARSEF 14473	SLF (Pennsylvania, USA)	OP897322	This study
M (18-65); ARSEF 14474	SLF (Pennsylvania, USA)	OP897323	This study
N (19-503); ARSEF 14475	SLF (Pennsylvania, USA)	OP897324	This study
O (18-63); ARSEF 14476	SLF (Pennsylvania, USA)	OP897325	This study
Q (18-393); ARSEF 14477	SLF (Pennsylvania, USA)	OP897326	This study
S (18-93); ARSEF 14478	SLF (Pennsylvania, USA)	OP897327	This study
T (18-329); ARSEF 14479	SLF (Pennsylvania, USA)	OP897328	This study
V (18-356); ARSEF 14481	Acalypterate fly* (Diptera) (Pennsylvania, USA)	OP897330	This study
Y (19-483); ARSEF 14482	SLF (Pennsylvania, USA)	OP897331	This study
GHA (commercial strain)	N/A (not applicable)	MN551319	Castrillo et al., (13)
Naturalis (commercial strain)	N/A (not applicable)	KM031766	N/A
ARSEF 1831	*Atta* sp.	DQ384380	Rehner et al., (11)
	(Hymenoptera) (Brazil)		
ARSEF 1853	*Dendroctonus ponderosae*	KM031773	Johny et al., (14)
	(Coleoptera) (Canada)		
ARSEF 4093	*Nezara viridula*	DQ384387	Rehner et al., (11)
	(Hemiptera) (Brazil)		
ARSEF 7972	(Coleoptera – unknown beetle)	KM031774	Johny et al., (14)
	(British Columbia, Canada)		
ARSEF 8170	*Agrilus planipennis*	KM031776	Johny et al., (14)
	(Coleoptera) (Michigan, USA)		
B. brongniartii (outgroup)			
(19-508); ARSEF 14483	*Vespula* sp.	OP897332	This study

(Continued)

TABLE 2 Continued

Representative strain (lab code; ARSEF accession no.)	Host(s) and location	B locus Genbank accession no.	Reference
	(Pennsylvania, USA)		
ARSEF 7376	*Magicicada septendecim*	HQ880701	Rehner et al., (12)

*The same B. bassiana strains recovered from these hosts have also been recovered from spotted lanternfly in the current study.

produces almost pure blastospore cultures. Duplicate (triplicate in the case of strain GHA) amounts of 100 g flaked barley (Grain Millers, Eden Prairie MN), hydrated to 50% V:W with reverse osmosis water, were autoclaved in vented, plastic, 30 x 20 cm mushroom spawn bags (Mycolabs, Crystal Lake IL) at 122° C for 25 minutes. After cooling, each bag was inoculated with 10 ml liquid culture and sealed. Blastospore concentrations in the inocula ranged from 4-9 x10^7 ml^{-1}. We incubated these bags of solid substrate at 25-26° C for 14 days after which the sporulated barley was transferred to aluminum trays and air dried with gentle laminar air flow over the trays. Drying was complete within 4 days with a final water activity of 0.42-0.45. Conidia were mechanically harvested through stacked 12-mesh and 80-mesh sieves using a table-top automatic powder sifter (Sidasu, Amazon.com). The harvested conidia were then dried to a final water activity of 0.25-0.30 by exposure to silica dessicant in a sealed chamber for 3-4 days. The field-derived strains and commercial strain GHA were grown simultaneously to harvest conidia for bioassays. Yields were calculated based on hemocytometer counts of appropriately diluted spore suspensions in 0.01% Silwet L77 of 100 mg harvested conidial powder and 1 g samples of the spent substrate after conidia had been harvested. Yields were adjusted to conidia Kg^{-1} dry flaked barley. After analyzing the data on conidial production on flaked barley, conidia of each of the higher-yielding isolates of each strain (02-A, 21-L, 58-B) and GHA were used in laboratory bioassays against SLF in June – August 2021.

One to two days before bioassays, we measured the viability of strains by spreading a dilute aqueous conidial suspension on Sabouraud dextrose agar. Germinated conidia were counted at 400× magnification 14–18 h after incubation at 25°C. All strains had 90% or higher viability before the bioassays on SLF.

Collecting and rearing *L. delicatula*

For all bioassays, we collected SLF in Pennsylvania and reared them at the Sarkaria Arthropod Research Laboratory at Cornell University, under USDA-APHIS permits (P526-18-02512 and P526P-21-02895). We collected SLF nymphs and adults from the same field site in Sinking Spring, Pennsylvania, as described by Clifton & Hajek (32). Additional details regarding growing *A. altissima* plants, SLF collection, and rearing in the laboratory are also provided in the Supplementary Materials for Clifton & Hajek (32). Before bioassays, we reared SLF in 91 cm mesh cages (61 cm L × 61 cm W× 91 cm H; ASIN #B07GN4BWZ7, RESTCLOUD) containing potted *A. altissima*. Cages were held on shelving units in walk-in environmental chambers (10.2 m^2; 22.5°C:15°C day:night) with a photoperiod of 16:8 [L:D] h and 65% RH. Light ballasts (New Wave T5 48, Sunlight Supply Inc., Vancouver, Washington) with 4 bulbs (F54T5/840, colour temperature 4000 K, Philips Lighting, Eindhoven, Netherlands) were suspended from the tops of shelving units for illumination.

Direct spray applications and daily mortality checks

We conducted bioassays with *B. bassiana* against 3rd instar, 4th instar, and adult SLF. We applied field-derived strains and *B. bassiana* strain GHA at the same concentration of 1.0 × 10^7 conidia mL^{-1} suspended in 0.05% sterile Silwet. We cold anesthetized SLF at 4°C for 8–10 min and transferred to 355 mL

TABLE 3 *Beauveria bassiana* isolates from adult spotted lanternfly that were used for solid substrate fermentation on barley flake.

Isolate #	Bloc strain	Collection site (2018)	Conidia yield (conidia/Kg substrate)	Standard deviation	Viability
02	A	Conrad Road residence	1.80×10^{13}	4.39×10^{12}	95%
45	A	Angora Fruit Farm	1.33×10^{13}	2.00×10^{12}	94%
03	B	Conrad Road residence	7.10×10^{12}	1.81×10^{11}	95%
58	B	Angora Fruit Farm	9.13×10^{12}	2.02×10^{12}	91%
21	L	Conrad Road residence	2.18×10^{13}	4.05×10^{12}	92%
67	L	Angora Fruit Farm	1.12×10^{13}	1.92×10^{12}	94%
GHA*	N/A	N/A	1.29×10^{13}	1.43×10^{12}	98%

*GHA, commercialized strain used in mycoinsecticides.
The two sites in Berks County, Pennsylvania had epizootics in 2018 (same sites marked as *c* and *e* on Table 1 and Figure 2). Strains are based on Bloc sequence data (see Figures 3, 4).

cardboard cups (diameter: 9.2 cm, height: 6.4 cm) before spray applications. The middle of the corresponding lid for each cardboard cup was punched out and a 15 ×15 cm piece of polyester tulle fabric (bridal veil mesh; pore size = 4 mm2 (2 mm × 2 mm; W× L); ASIN #B01NAU9OD5) was tightly secured between the lid's rim and the cup to contain SLF. Cardboard containers were only used once for each replicate spray application and disposed. 1.0 mL of each suspension was applied *via* airbrush. The control treatment was 0.05% Silwet with no *B. bassiana* spores. Containers held 20 nymphs (3rd and 4th instars) or 15 adults (either male or female) during spray applications. After spray applications, we inverted containers on top of newspaper to dry for 10 minutes. Nymphs were transferred to 63 cm mesh cages (24.5 L × 24.5 W× 63 cm H; BugDorm 4M2260, MegaView Science Co., Ltd., Taiwan) containing one potted *A. altissima* plant. We sprayed one container of male adults and one container of female adults separately before they were transferred to same mesh enclosure with potted *A. altissima*. We used larger plants with a 100-cm mesh bag and bamboo support for the adult SLF, which is also described in Clifton & Hajek (32). We sprayed five containers for each life stage and treatment (n = 100 nymphs; 75 males; 75 females).

Before spraying SLF with *B. bassiana* suspensions, we prepared 1 cm^2 squares of water agar that were cut from 150 mm Petri dishes that were prepared in the laboratory. Each agar square was transferred to a smaller 60 mm Petri dish. As we started to spray SLF in each cardboard container, we briefly stopped and sprayed a water agar square for one second before the remainder of the 1.0 ml suspension was applied to SLF. A similar method was used by Poprawski et al. (38) to measure conidia coverage. The lid was placed on the 60 mm Petri dish with the sprayed water agar squares, labelled with the treatment and replicate, and then placed in the refrigerator. Within 48 h of spray applications, we stained the agar squares with lactophenol cotton blue to count conidia and confirm that spray coverage was consistent among treatments. For each water agar square, we scanned 20 random microscope fields at 400×. Counts were averaged and expressed as dosages applied per square millimeter.

We checked SLF daily for 14 days after treatment. We removed SLF that died within 24 h of treatment from cages and excluded them from analysis. Mortality within 24 h was low; for example, 4th instar SLF sprayed with *B. bassiana* averaged 0.65 ± 0.22 dead nymphs 1 day after spraying. During daily mortality checks, we transferred SLF cadavers from each replicate cage to plastic well plates (24 wells per plate; 1.9 cm^2 surface area per well) held inside sealed plastic food containers (13 × 13 × 5 cm), lined with moistened filter paper, to promote fungal outgrowth and confirm mortality from fungal infections (39). We kept SLF cadavers in these sealed containers for 10–14 days after the time of death to allow for fungal outgrowth. After fungal outgrowth, we covered these well plates with respective lids and stored them in a refrigerator for 20-30 days. The well plates allowed for easy separation of cadavers before we removed *B. bassiana* conidia for quantification (described in next section).

Quantification of conidial production on *L. delicatula* cadavers

While carrying out the bioassays and handling SLF that were killed by *B. bassiana* strains, we found apparent differences among strains for conidial production on cadavers (Supplementary Figure 1). Previous studies have also quantified *B. bassiana* conidial production on host cadavers as one measurement of epizootic potential (14, 16). For each life stage and treatment, we randomly selected 20 cadavers with *B. bassiana* fungal outgrowth to quantify *B. bassiana* conidial production. Detailed methods for removing and quantifying conidia with 70% ethanol are described in the Supplementary Materials. After removing *B. bassiana* conidia from SLF, we dried these cadavers under a fan in a biosafety cabinet for one hour before weighing them on a precision scale for dry body mass (mg). We divided the numbers of total conidia for each cadaver by its dry body mass. While there is less variation in body mass for nymphs, adult SLF can vary a great deal, and adult females are usually heavier than adult males, as noted by Clifton & Hajek (32).

Data analysis

We calculated mean survival times and standard errors for SLF receiving each treatment based on Kaplan-Meier survival distribution functions using PROC LIFETEST in SAS 9.4 (40). For multiple comparisons of survival times among different treatments, we used the Cox proportional hazards model with PROC PHREG. Contrasts between treatments were conducted using least-square means, adjusted with the Bonferroni correction. For 3-4 instar nymphs, we combined data before we compared survival times (n = 200 per treatment). For adult SLF, we combined data for males and females and compared mycoinsecticide treatments. (n = 150 per treatment).

For each spray trial on 3rd instars, 4th instars, adult males, and adult females, we compared mean conidial coverage (# per mm^2 on water agar squares) using a one-way ANOVA with Fisher's least-significant-difference test, using PROC ANOVA. For counts of conidial production on SLF cadavers, we also used one-way ANOVA and compared total conidia per dry mg of body mass. We checked these data with the Shapiro-Wilk Test (α=0.05) and they did have normal distribution before analysis (41).

Results

Identification and diversity

Sequencing of the Bloc region resulted in 931-934 bp of reliable data for 165 isolates, with 812 constant sites and 119 variable characters. One strain (F) had an extra 3 bp (CCC) between positions 527-528 in the Bloc sequence. All *Beauveria* isolates from SLF were identified as *B. bassiana* (Figure 3). One isolate from a nontarget yellowjacket wasp was identified as *B. brongniartii*

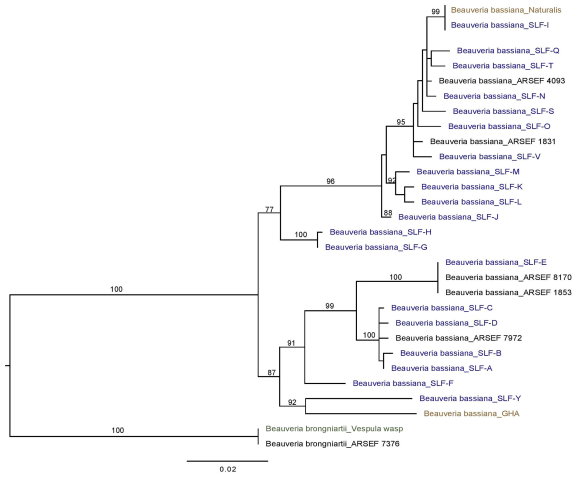

FIGURE 3
Phylogeny of *B bassiana* associated with the spotted lanternfly (SLF; in blue text), *L. delicatula*. Best tree from Bayesian analysis of the B locus sequence data (alignments of 934 bp). The analysis includes 20 *B bassiana* strains from SLF, the commercial strains GHA and Naturalis (in brown text) and *B brongniartii* as outgroup, including one strain obtained from a yellowjacket (*Vespula* sp.; in green text).

(Figure 1C). Bloc sequence data revealed 20 distinct strains among the 164 *B. bassiana* isolates from SLF and non-targets in Pennsylvania, and these strains were grouped into two clades (Figure 3).

Non-targets killed by *Beauveria* spp.

Seven non-target cadavers with fungal outgrowth were collected in 2018-2020. Two of these seven non-targets were yellowjacket wasps infected by *Beauveria* spp. Six non-targets (Table 4) were killed by *B. bassiana* strains A, B, D, and V, which were also isolated from SLF. *B. bassiana* strains A, B, and D were placed in one clade while strain V was placed in a separate clade. One isolate of *B. brongniartii* was recovered from a yellowjacket wasp in 2019, but at this time we have not yet recovered this species from SLF.

Prevalence of *B. bassiana* strains

Strains A, B, C, L were the only strains recovered from both SLF nymphs and adults. In total, ten *B. bassiana* isolates were recovered from nymphs. Strains A, B, and L were the most prevalent, accounting for 114 isolates out of the total 164 isolates (70%) that were analyzed in this study (Figure 4). These dominant strains were found in multiple years for some sites, including Angora Fruit Farm, Blandon, Conrad Road, and Sinking Spring (Table 5). Many of the *B. bassiana* isolates sampled from Angora Fruit Farm and Conrad Road in October 2018 came from SLF adults during an epizootic that caused localized population collapses (8).

Bioassays with *B. bassiana* and *L. delicatula*

For all the spray experiments with *B. bassiana* strains, there was no significant difference among treatments for conidia coverage on squares of water agar. Third instar nymphs: no significant differences ($F = 1.28$, $df = 3, 16$, $P = 0.3156$), with averages that ranged from 467 to 573 conidia per mm^2. Fourth instar nymphs: no significant differences ($F = 2.39$, $df = 3, 16$, $P = 0.1068$), with averages that ranged from 472 to 563 conidia per mm^2. Adult males: no significant differences ($F = 2.28$, $df = 3, 16$, $P = 0.1182$), with averages that ranged from 525 to 571 conidia per mm^2. Adult females: no significant differences ($F = 0.78$, $df = 3, 16$, $P = 0.5215$), with averages that ranged from 538 to 572 conidia per mm^2. These data provided confidence that SLF exposed to the *B. bassiana* strains had received similar doses.

The survival times of SLF nymphs were significantly different among treatments, (Log-rank $\chi^2 = 306.41$; $df = 4$; $P < 0.0001$); those exposed to *B. bassiana* died significantly faster than controls (Figure 5). Additionally, *B. bassiana* strain B caused significantly less mortality to SLF nymphs compared to *B. bassiana* strains A, L, and GHA.

The survival times of SLF adults were significantly different among treatments, (Log-rank $\chi^2 = 216.68$; $df = 4$; $P < 0.0001$); those exposed to *B. bassiana* died significantly faster than controls (Figure 6). Additionally, SLF adults exposed to *B. bassiana* strains A and L died significantly faster than those exposed to strain B and GHA.

TABLE 4 Summary of non-target insects killed by *Beauveria* spp.

Site	Date sampled	Non-target	Isolate #	Entomopathogen species	*B. bassiana* Bloc strain
Fleetwood	5/23/2018	Ant	18-434	*Beauveria bassiana*	A
Kutztown University	10/04/2018	Nitidulid beetle	18-376	*Beauveria bassiana*	D
Angora Fruit Farm	10/09/2018	Acalypterate fly	18-356	*Beauveria bassiana*	V
Conrad Road	10/09/2018	Stonefly	18-357	*Beauveria bassiana*	B
Angora Fruit Farm	10/01/2019	Vespid (yellowjacket wasp)	19-508	*Beauveria brongniartii*	N/A (not applicable)
Hill Road	9/15/2020	Pyralid moth	20-030	*Beauveria bassiana*	B
Blandon	10/01/2020	Vespid (yellowjacket wasp)	20-069	*Beauveria bassiana*	B

2018: 4 non-target cadavers infected by *B. bassiana*.
2019: 1 non-target cadaver infected by *B. brongniartii*.
2020: 2 non-target cadavers infected by *B. bassiana*.

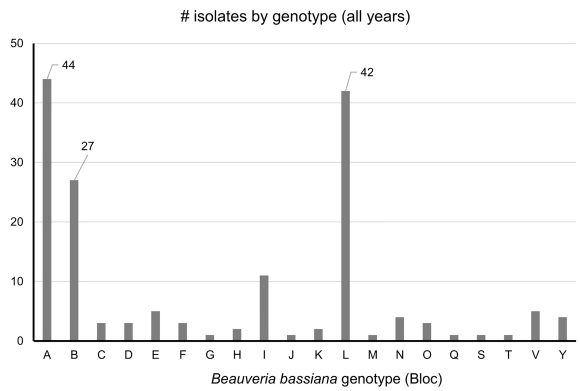

FIGURE 4
Prevalence of *B bassiana* strains associated with spotted lanternfly adult and nymphs collected in 2017-2020 from Pennsylvania, USA. The most common strains (A, B, and L) have data labels. *n* = 164.

Conidial production for *B. bassiana* strains A, B, and L was significantly higher than strain GHA for both the 3rd instar nymphs ($F = 44.87$, df = 3, 75, $P < 0.0001$; Figure 7A) and 4th instar nymphs ($F = 29.67$, df = 3, 76, $P < 0.0001$; Figure 7B). Conidial production for *B. bassiana* strains B and L was significantly higher than strain A for adult males, which was significantly higher than GHA ($F = 72.94$, df = 3, 76, $P < 0.0001$; Figure 7C). For adult females, conidial production for strain A was significantly higher than strain L, but strain B was not significantly different compared to both A and L ($F = 40.78$, df = 3, 76, $P < 0.0001$; Figure 7D). All three field-derived *B. bassiana* strains had significantly higher conidial production than GHA for adult females.

Discussion

All *Beauveria* isolates infecting SLF collected in Pennsylvania from 2017 to 2020 were identified as *B. bassiana*. *Beauveria bassiana* is also known to infect SLF in China (42), where this invasive host is native. *Beauveria bassiana* was also a major

TABLE 5 The most common *B. bassiana* strains (A, B, and L) based on B locus sequences, associated with spotted lanternfly (SLF) and other hosts in sites in Pennsylvania, USA.

Strain	Sampling Site	County	Year (# samples/total)	Host(s)
A	Angora Fruit Farm	Berks	2018 (12/30); 2019 (1/3)	SLF
	Blandon	Berks	2018 (1/2); 2019 (1/5)	SLF
	Boyertown reservoir	Berks	2017 (1/1)	SLF
	Conrad road residence	Berks	2018 (6/13)	SLF
	Fleetwood residence	Berks	2017 (3/4); 2018 (1/1)	2017: SLF; 2018: ant (1)
	Kutztown University	Berks	2018 (1/7)	SLF
	Leesport	Berks	2019 (3/5)	SLF
	Lilitz residence	Lancaster	2018 (1/1)	SLF
	Penn State Berks	Berks	2018 (1/1)	SLF
	Pottstown Quarry	Montgomery	2018 (2/12)	SLF
	Schuler Road	Berks	2018 (5/18)	SLF
	Sinking Spring	Berks	2019 (4/14)	SLF
B	Angora Fruit Farm	Berks	2018 (1/30); 2020 (1/6)	SLF
	Blandon	Berks	2019 (1/5); 2020 (2/5)	2019: SLF; 2020: SLF (1), yellowjacket wasp (1)
	Conrad road residence	Berks	2018 (4/13); 2020 (6/8)	2018: SLF (3), stonefly (1); 2020: SLF
	Hill road	Berks	2020 (2/3)	SLF (1), pyralid moth (1)
	Kutztown University	Berks	2018 (4/7)	SLF
	Leesport	Berks	2019 (1/5)	SLF
	Schuler Road	Berks	2018 (2/18); 2020 (1/1)	SLF
	Sinking Spring	Berks	2020 (3/6)	SLF
	Treichlers Bridge	Lehigh	2020 (1/1)	SLF

(Continued)

TABLE 5 Continued

Strain	Sampling Site	County	Year (# samples/total)	Host(s)
L	Angora Fruit Farm	Berks	2018 (7/30); 2019 (1/3); 2020 (1/6)	SLF
	Blandon	Berks	2018 (1/2); 2019 (2/5); 2020 (2/5)	SLF
	Conrad road residence	Berks	2018 (1/13)	SLF
	Graffa Pond	Lancaster	2020 (6/7)	SLF
	Lancaster Central Park	Lancaster	2020 (1/2)	SLF
	Overlook Park	Lancaster	2020 (5/6)	SLF
	Pottstown Quarry	Montgomery	2018 (3/12)	SLF
	Schuler Road	Berks	2018 (5/18)	SLF
	Schuylkill Center for Environmental Education	Philadelphia	2020 (1/1)	SLF
	Sinking Spring	Berks	2019 (6/14); 2020 (3/6)	SLF

entomopathogen infecting invasive scolytines attacking coffee berries in Hawaii (13) and infecting emerald ash borers in Michigan and southwestern Canada (14). This fungal species acts as an entomopathogen but also persists in ecosystems as a plant endophyte, or a saprophyte in the soil (43). As an entomopathogen *B. bassiana* is a generalist, occurring worldwide and known to infect > 200 species of insects across many insect orders (3), although host range is more limited for individual strains (44). Perhaps the breadth in host range of *B. bassiana* as well as life as a saprophyte and endophyte help to explain the natural occurrence of abundant genetic diversity in *B. bassiana*. In our study alone, among *B. bassiana* isolates from SLF in southeastern Pennsylvania we found 20 different strains based on Bloc sequence data. Other studies, each based on one host species, have also documented genetic diversity in *B. bassiana* isolates (13, 14). Our genetic analysis is based on only one locus commonly used for identification of *Beauveria* species (12), but sequencing additional loci or genes could show more genetic differences among these 20 strains.

We hypothesize that the *B. bassiana* isolates from our study are native and their ability as generalists facilitates their switching over to use the relatively new resource constituted by abundant SLF populations. Some of the same *B. bassiana* strains that infected SLF also infected non-targets in this study (Table 4), suggesting that these native hosts could constitute some of the sources for the naturally occurring strains. Infection levels by *B. bassiana* will vary over time and space, depending on population densities of susceptible hosts, the fungal titers in the soil and on phylloplanes, and weather (45, 46). Additional sampling methods, e.g., the "*Galleria* bait method" (47) and serial dilutions of soil samples on selective media, could help to better describe the communities of native entomopathogens in these sites that were invaded by SLF.

Three *B. bassiana* strains (A, B, and L) were most prevalent and widespread among field sites in Pennsylvania. *Beauveria bassiana* strain A did not have any 100% matches to *B. bassiana* isolates on GenBank, but it did have high similarity (99-99.8% matches) to Bloc sequences from *B. bassiana* isolates recovered from emerald ash borer

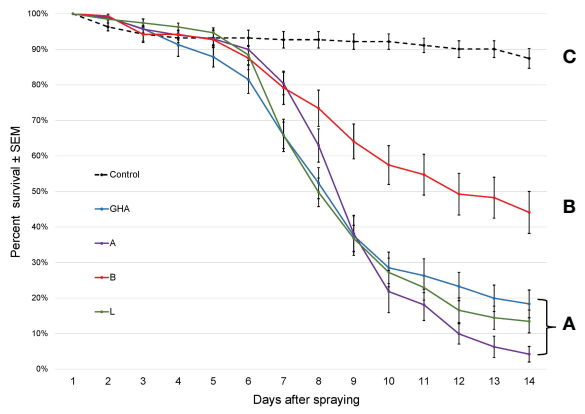

FIGURE 5
Survival of *L. delicatula* nymphs on potted *A altissima* plants. Data are combined for 3rd and 4th instar nymphs. Lines represent different *B bassiana* strains (control = dotted line). Letters represent significant differences among treatments for survival curves based on Cox proportional hazards.

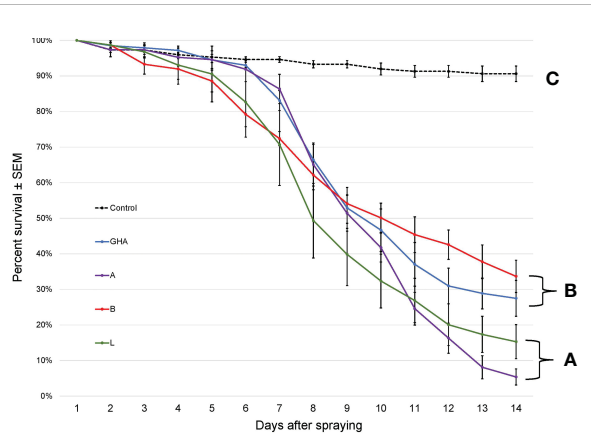

FIGURE 6
Survival of *L. delicatula* adults on potted *A altissima* plants. Lines represent different *B bassiana* strains (control = dotted line). Letters represent significant differences among treatments for survival curves based on Cox proportional hazards.

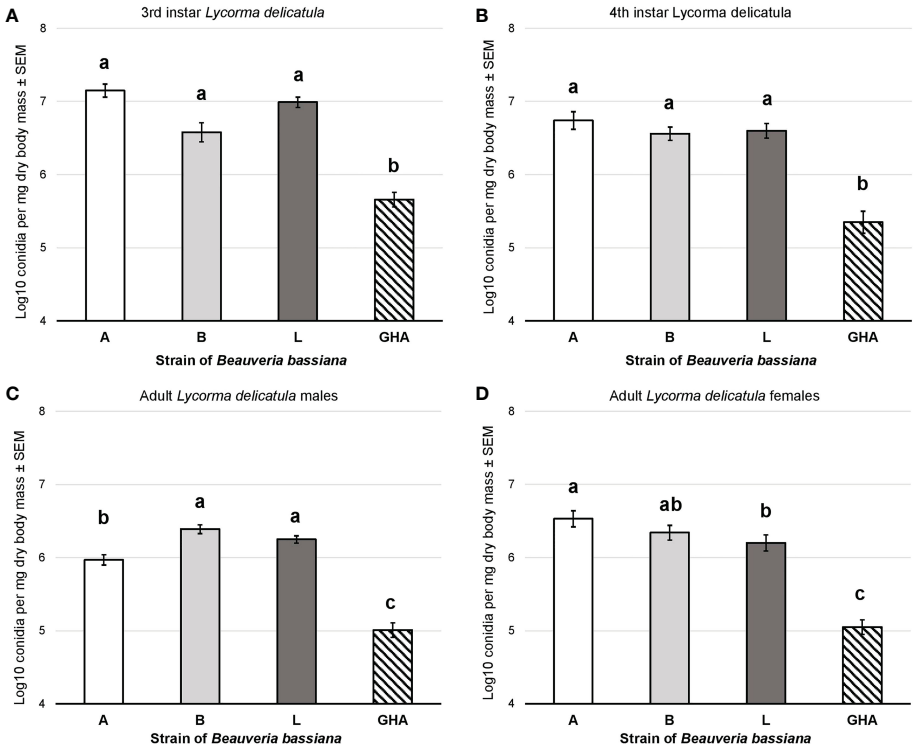

FIGURE 7
Conidial production of *B bassiana* strains on *L. delicatula* cadavers from experiments on **(A)** third instar nymphs, **(B)** fourth instar nymphs, **(C)** adult males, and **(D)** adult females. Bar shading represent different *B bassiana* strains used in bioassays. Lowercase letters above bars represent significant differences among treatments based on Fisher's least-significant-difference test.

populations in Canada (GenBank JN849673.1 and others in PopSet 379045698). Strain L and other strains in the top clade (Figure 3) had high similarity to Brazilian strains of *B. bassiana* (ARSEF 4093 and 1831; Table 2), and the *B. bassiana* strains in this top clade already have a wide distribution in mainland North America and South America. According to the Bloc sequence data, strain I has a 100% match to *B. bassiana* strain ATCC 74040, which is used in the mycoinsecticide Naturalis® (GenBank KM031766.1), but other loci would need to be sequenced to confirm that strain's similarity. *Beauveria bassiana* strain ATCC 74040 was originally isolated from the cotton boll weevil, *Anthonomus grandis* (Boheman) (Coleoptera: Curculionidae), in Texas and is known to naturally occur throughout the United States (48). Genetic analysis of *B. bassiana* isolates infecting SLF in other countries is needed to further assess the specificity of these fungal entomopathogens and insect hosts.

In the field studies, we found *B. bassiana* strain A infecting one ant and strain B infecting 3 different non-target insects (Table 4). Among the three most collected *B. bassiana* strains, strain L was the only one not found infecting non-targets. Two of the seven non-targets that we found infected by *Beauveria* were yellowjacket wasps (*Vespula* sp. [Hymenoptera: Vespdae]), which were regularly observed foraging for honeydew produced by SLF feeding. We isolated and identified one isolate of *B. brongniartii* from a yellowjacket wasp in 2019 (Table 4). *Beauveria brongniartii* has a more restricted host range than *B. bassiana*, principally infecting Coleoptera but also known to infect other insect hosts (12), but to our knowledge this fungus is not associated with SLF or other fulgorids, even though it was collected in the same areas as SLF. It is likely that there is horizontal transmission of *Beauveria* spores between SLF and these hymenopterans, especially since the other vespid had been infected by strain B, one of the most common strains infecting SLF. It should be noted that our sampling of non-targets may not capture the full range of potential non-targets killed by *Beauveria* spp. in these sites. We primarily sampled near *A. altissima* trees, collecting any dead and mycosed non-targets that we could find, often in proximity to dead SLF; otherwise, we did not spend additional time in field sites exclusively looking for non-targets. A variety of insect traps, nets, and other sampling tools are needed to thoroughly describe potential infections of these native non-targets and to find any possible fungus-infected hosts before they are lost to scavengers and weathering.

The laboratory bioassays revealed differences in efficacy, with *B. bassiana* strain B killing significantly fewer nymphs than strains A, L, and GHA, but the bioassays against adults showed that strains A and L had greater efficacy than strains B and GHA. All the field-derived *B. bassiana* strains had significantly greater conidial production on cadavers compared to commercial strain GHA on all SLF life stages that were tested. Wraight et al. (16) found that *B. bassiana* strain HI-25 produced >2.5 times greater numbers of conidia than strain GHA on coffee berry borer cadavers. In our study, we found that strains A, B, and L produced >15 times more conidia than strain GHA on 4th instar SLF (Figure 7B), and these results on conidial production suggest these strains have greater epizootic potential than strain GHA. A similar pattern of conidial production was observed for the same *B. bassiana* strains growing on selective medium, with strain

GHA having smaller colony growth compared to the field-derived strains (Supplementary Figure 2).

More work is needed on these field-derived *B. bassiana* strains to discern any potential for commercialization, in particular relative to the ability to economically mass produce them. If the fungal growth process were scaled up to the larger spawn bags used by industry, it is not yet clear how cost effective it would be to grow these different *B. bassiana* strains even though some strains produced similar if not higher yields than strain GHA on a small scale (Table 3). Aside from considerations regarding feasibility of mass production of new *B. bassiana* strains, there would also need to be evaluations of shelf-life, toxicity testing, and other criteria (49). Additional studies are needed to further characterize these *B. bassiana* strains, which would include non-target testing, nutritional requirements for large scale fermentation, and more bioassays to determine virulence to SLF (LC_{50} tests).

In summary, aside from epizootics driven by *B. major* in 2018, *B. bassiana* is the most common entomopathogenic fungus that naturally infects SLF each year in the areas we studied. Surprisingly, among *Beauveria* spp. isolated from SLF, we only found the species *B. bassiana*. Twenty-one *B. bassiana* strains infected SLF, with three being more common. Based on bioassay data, strains A and L are more promising candidates for biological control of SLF, exhibiting similar efficacy to a commercialized strain (GHA) and high epizootic potential due to more abundant conidial production.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. Additional raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

All authors designed the study. EC and SJ conducted the experiments. All authors analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author SJ was employed by company Jaronski Mycological Consulting LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1127682/full#supplementary-material

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Effects of transgenerational photoperiod experience on the reproduction and development of *Anastatus orientalis*, an egg parasitoid of the spotted lanternfly

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Transgenerational experience can affect a range of natural enemies' life-history traits and can be involved in the control of developmental plasticity. As a major egg parasitoid of the spotted lanternfly, *Lycorma delicatula* (Hemiptera: Fulgoridae), the wasp *Anastatus orientalis* (Hymenoptera: Eupelmidae) is effective at suppressing its host populations. The reproductive and developmental traits of *A. orientalis* is known to depend on photoperiod conditions, but transgenerational photoperiodic effects have yet to be evaluated. To evaluate the transgenerational photoperiodic effects on *A. orientalis*, we assessed wasp adult longevity, female fecundity, sex ratio, and diapause rate over three consecutive generations under different experimental photoperiods (L16:D8, L12:D12, and L8:D16), using *Antheraea pernyi* (Lepidoptera: Saturniidae) eggs as hosts. The results suggest that transgenerational experience significantly impacts several biological parameters of progeny. All parasitoids entered a diapause under the long photoperiod condition (i.e., L16:D8), after which the number of female parasitoids and fecundity of the 2nd and 3rd generations increased significantly as compared to the 1st generation. With the long photoperiod conditions, the female ratio rose from 68.1% (1st generation) to 86.0% (3rd generation) and the progeny per females increased from 35.8 to 75.7. However, adult longevity of females and males were shortened significantly. With the intermediate photoperiod (L12:D12) conditions, fecundity and sex ratio of the 2nd and 3rd generations increased significantly as compared to the 1st generation. With the short photoperiod (L8:D16) conditions, there were no significant differences in fecundity among three generations, but sex ratio of

the 2nd and 3rd generations increased significantly as compared to the 1st generation. These results on transgenerational photoperiodic effects can be applied to improve laboratory rearing efficiency of parasitoids and to better understand population dynamics in the field across a latitudinal gradient.

KEYWORDS

Anastatus orientalis, diapause, *Lycorma delicatula*, maternal effect, parasitoid, photoperiod, transgenerational effect

1 Introduction

Extensive phenotypic plasticity can allow the populations of natural enemies to better adapt to changes in local environmental conditions and thereby gain greater efficiency in attacking their target pests. Phenotypic plasticity includes transgenerational effects, which are known to affect natural enemies' reproductive and developmental traits, such as diapause, survival rate, development time, and oviposition (1–4). Although not all plastic responses of natural enemies are optimal, phenotypic plasticity is particularly advantageous when the environmental conditions the progeny will face can be better predicted by the parents than by the progeny themselves. For example, cues experienced by parents can provide progeny with additional environmental information beyond their own and the parents can induce or inhibit diapause in their progeny and affect other traits depending on the environmental signals they perceive (5–8). Transgenerational effects can be broader than simply maternal effects, and mounting evidence suggests that a grandmaternal effect can also alter the fecundity and development of natural enemies (9–12). Yet controlled evaluations of grandmaternal effects are limited and our understanding of how grandmaternal effects enable the progeny to better adapt to changes in their environmental conditions is still not well explained.

The spotted lanternfly, *Lycorma delicatula* White (Hemiptera: Fulgoridae), is a recent invasive pest in South Korea, Japan, and North America (13–16), where it threatens commercially grown grapevines and tree fruits, in addition to plant nurseries and timber industries (17, 18). Despite intensive efforts by federal, state, and local stakeholders to stop the spread of spotted lanternfly in the USA, the populations continue to spread. Since initial detection of spotted lanternfly in Berks County, Pennsylvania, USA, in 2014, spotted lanternfly infestations have been detected in 130 counties (87 under quarantine) within Connecticut, Delaware, Indiana, Maryland, New Jersey, New York, Ohio, Virginia, and West Virginia (19). Additional control methods are needed to help in the management of spotted lanternfly populations in the USA, and we are evaluating important parasitoids of spotted lanternfly in its native range as candidate biological control agents.

The insect of the current study, the wasp *Anastatus orientalis* (Hymenoptera: Eupelmidae), is the most widespread native egg parasitoid of spotted lanternfly, and has been used as a biological control agent in South Korea and is being considered a candidate for biological control in the USA (20–23). Large numbers of wasps are needed for testing candidate biological control agents or implementing a biocontrol programme, but artificial rearing spotted lanternfly is difficult, and the methods are not well developed (24). Thus, for laboratory research in South Korea on *A. orientalis*, eggs of a substitute host *Antheraea pernyi* (Lepidoptera: Saturniidae) were used for rearing the parasitoid (25). For this study, owing to the challenges of rearing or acquiring non-parasitized spotted lanternfly egg masses, we are also using *A. pernyi* as the host for rearing. However, when using substitute hosts to rear a parasitoid, changes in the biological characteristics of the insects under different rearing conditions should be considered because non-target hosts could influence the parasitoids' response to the target host. They could influence offspring sex or vitality and poor-quality parasitoids may yield low efficiency in reared colonies or biological control programmes (26–28). Suitable environmental conditions for both the development and fecundity of natural enemies are imperative for their mass rearing in laboratory settings. Photoperiod and temperature are widely considered the most influential external factors for the development and fertility of natural enemies (29, 30).

Evaluations of the life cycle of *A. orientalis* in the field in China indicate that adults had two emergence periods per year. Some individuals emerged in May and others in September, with a summer diapause in between (21). Hou (31) found that, in a laboratory setting, *A. orientalis* could complete seven or eight generations over a span of 7 months (reared from April to December) when reared at 25°C with a L12:D12 photoperiod, and the emergence rate could reach up to 78%. In contracts, Broadley et al. (23) observed a low emergence rate of *A. orientalis* exposed to 16L:8D hour long-day conditions at 25°C, with most parasitoids entering a diapause. This suggests that summer diapause in *A. orientalis* is induced by a long photoperiod. In another study, Seo et al. (22) reported the longevity, oviposition, and sex ratio of *A. orientalis* under a 16L:8D photoperiod at four different temperatures (15°C, 20°C, 25°C, and 30°C). This led those authors to speculate that photoperiod influences the reproductive and developmental traits of *A. orientalis*. Thus, understanding the relationship between photoperiod and natural enemies' biological characteristics is crucial for optimizing rearing methods (32, 33).

A transgenerational effect has been observed in other *Anastatus* species. In addition to environmental variables, the maternal oviposition experience and age of *Anastatus disparis* (Hymenoptera: Eupelmidae), an egg parasitoid of *Lymantria dispar* (Lepidoptera:

Lymantriidae), significantly influences sex allocation in their progeny (34, 35). To our knowledge, however, no study has yet reported on whether or not transgenerational experience affects the biological characteristics of *A. orientalis*. Moreover, the maternal photoperiodic response has not been sufficiently investigated. Hence, in the present study, we investigated the effects of transgenerational photoperiod experience on the fertility and development of *A. orientalis* by using *A. pernyi* eggs as a host. The empirical data on the effects of transgenerational photoperiod experience on insect fertility and development acquired by our study could also be used for optimizing rearing methods and storage of natural enemy parasitoids, and for better understanding *A. orientalis* population dynamics in the field across a geographic and climatic range.

2 Materials and methods

2.1 Insect rearing

A population of *A. orientalis* was collected from overwintering spotted lanternfly egg masses in Haidian (40°00′34″N, 116°23′32″E), Beijing, China. The spotted lanternfly egg masses were kept under laboratory conditions at 25 ± 1°C and 60% ± 5% relative humidity (RH), with a L12:D12 photoperiod until parasitoid emergence. The parasitoid species were confirmed using scanning electron microscopy (SEM) micrographs by examining the morphology of adult specimens (21). The *A. orientalis* were reared for five generations in 25°C with 12 h of light using *A. pernyi* eggs as their host (25), and then they were reared another three generations on *A. pernyi* in 25°C with 14 h of light (22). These conditions were identified as effective rearing conditions for *A. orientalis*. Within the first 48 h after emergence, five females and one male of *A. orientalis* (i.e., 'Generation 0', G0) were placed in ventilated insect rearing cages (20 cm × 20 cm × 20 cm) for 36 h for mating, with access to honey and water.

For each experiment, a kraft paper card with 100 A. *pernyi* eggs glued to it (with a polyvinyl acetate suspension) were prepared. We selected one female parasitoid (G0), which was released into a transparent plastic container (a 482-ml plastic deli cup) with one *A. pernyi* egg card each to obtain the first progeny generation ('Generation 1', G1) of *A. orientalis*. Then, the containers were randomly separated into three treatment groups that were placed in three photoperiodic regimes: L:D = 8:16, 12:12, or 16:8 at 60%–75% RH, at 25°C. After a 48-h exposure to the egg card, female adult parasitoids were removed, while the parasitized eggs were maintained at different photoperiods conditions until emergence from the eggs.

To obtain the second and third generations ('Generation 2', G2; 'Generation 3', G3) of *A. orientalis* in three photoperiodic regimes, five females and one male less than 48 h old were placed in a ventilated insect rearing cage (20 cm × 20 cm × 20 cm container, described above) under each rearing condition, these were following generations all reared in one of the three photoperiod treatments the same as their parent generations. In the meantime, honey and water were provided in each condition for 36 h for mating success. Then, one mated female *A. orientalis* was selected randomly and placed in a transparent plastic container (a 482-ml container, described above) with one new paper card with 100 fresh eggs of *A. pernyi*. After a 48-h exposure to fresh eggs of *A. pernyi*, the female *A. orientalis* adult was removed, while the parasitized eggs were kept in the same rearing condition until emergence from eggs (Figure 1).

2.2 Observation of fecundity, sex ratio, adult longevity, and diapause pattern of the parasitoid wasp

To determine the fecundity, sex ratio, and diapause of the parasitoid wasps of different generations under three photoperiods, 40 days after the mass emergence of the non-diapausing fraction of the progeny generation, all parasitized host eggs were dissected and examined, respectively. If none of the parasitoid progeny emerged under a certain treatment, they were considered to be in a diapause state. Therefore, to obtain parasitoid progeny, the host eggs were not dissected until wasp emergence after approximately 3 months. The diapausing larvae (each living larva that was assumed to be in diapause) and non-diapausing individuals (mostly emerged adults, few dead adults inside the host, and sporadic pupae) were identified and counted, and the sex of the progeny that successfully emerged from each egg mass was recorded. Because typically only one *A. orientalis* is produced out of each *A. pernyi* egg, the number of emerged adults was estimated as the number of parasitized eggs with emergence holes. The few (less than 1%–2%) individuals that died during the larval or prepupal stages were excluded. Next, the percentage of diapausing individuals was calculated for each generation in each photoperiod condition. To determine the percentage diapausing, a random sample of 100 host eggs that were parasitized during a 48-h window by one female were dissected. Overall, the experiment included 10 replicates for each of the three photoperiod treatments conducted with each of the three generations of *A. orientalis* (for a total of 90 cards and 9,000 parasitized host eggs). To estimate the average longevity of *A. orientalis* adults of different generations under three photoperiods, newly emerged (< 24 h old) *A. orientalis* of each treatment were collected. Each adult was kept individually in a rearing container (a 482 ml-container, described above) in each photoperiod condition, with 10% honey. The wasps were checked daily, and the date of death was recorded until all individuals died.

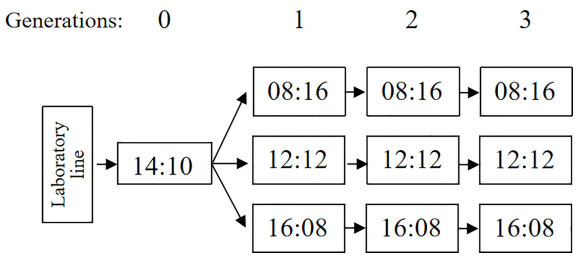

FIGURE 1
The general design of the experiment. Generations are indicated at the top of the figure, photoperiods are shown in the figure as number of hours light:dark, and the arrows indicate the succession of generations.

2.3 Statistical analyses

Excel 2007 and SPSS 19.0 software programs were used to statistically analyze the data. Two-way ANOVA followed by least significant difference tests was used to compare longevity of adult and female fecundity among photoperiods, generation, and their interaction (Tukey's *post-hoc* test, $p < 0.05$). To further evaluate the effects of photoperiod and generation on daily survival of *A. orientalis*, effects of different treatments on the adult longevity of *A. orientalis* were represented by Kaplan–Meier survival curves. Survival time was measured in days from the date of experiment beginning to the date of wasp death. Each adult was used as one replicate. A mixed-effects Cox regression model was fitted to analyze the death risk of *A. orientalis* reared from different treatments, including generations, photoperiods, and longevity as covariates, using the package "coxme" in SPSS software. We present a hazard ratio with corresponding 95% CI. The differences in the sex ratio and diapause between different photoperiods or generations were compared using chi-squared tests. We also examined potential trade-offs between life history traits by performing linear regression analyses between diapause trait and other traits.

3 Results

3.1 Female fecundity

We found that generation significantly affected (df = 2, 81; $F = 18.914$; $p < 0.01$) female fecundity, which increased in later generations under the same photoperiod (Figure 2). Under the photoperiod L16:D8, the mean number of *A. orientalis* progeny produced was larger in the second and third generations than in the first generation (74.40, 75.70, and 35.80, respectively), and the differences were significant (df = 2, 27; $F = 26.015$; $p < 0.01$). Under the photoperiods L12:D12 (df = 2, 27; $F = 3.253$; $p = 0.054$) and L8:D16 (df = 2, 27; $F = 1.883$; $p = 0.172$), the progeny produced were similar among the first, second, and third generations.

Fecundity outcomes of *A. orientalis* females of the same generation under each of the three photoperiods are plotted in Figure 2. The photoperiod also affected (df = 2, 81; $F = 6.442$; $p < 0.05$) female fecundity, and the interaction between generational and photoperiod was significant (df = 4, 89; $F = 2.818$; $p < 0.01$). For the first generation, their mean number of progenies produced was 53.30 under the L8:D16 photoperiod, this was larger than that produced under the L12:D12 or L16:D8 photoperiods (df = 2, 27; $F = 2.984$; $p = 0.068$). For the second generation, there was a significant difference (df = 2, 27; $F = 3.685$; $p < 0.05$) in female fecundity between L16:D8 (74.40%), L12:D12 (53.4%), and L8:D16 (62.4%) photoperiods. In addition, for the third generation, female fecundity was significantly greater (df = 2, 27; $F = 5.932$, $p < 0.01$) under the L16:D8 (75.70) and L8:D16 (70.30) photoperiods than under the L12:D12 (54.10) photoperiod.

3.2 Sex ratio

Photoperiod and generation both had a significant effect on sex ratio (photoperiod: $\chi^2 = 87.752$, df = 2, $p < 0.001$; generation: $\chi^2 = 237.355$, df = 2, $p < 0.001$). The lowest female ratio was recorded in the first generation, averaging 62.80% across different photoperiods (Figure 3). The highest female ratio was recorded in the second generation, averaging 81.59% across different photoperiods. Considering just the third generation, the female ratio of *A. orientalis* was highest (85.96%) under a photoperiod of L16:D8.

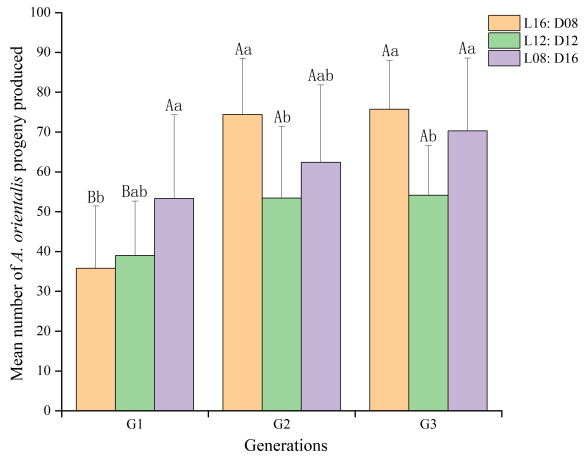

FIGURE 2
Effect of generation on the female fecundity of *Anastatus orientalis* under three photoperiods. Different upper-case letters within the same photoperiod indicate significant differences among the three generations, whereas different lower-case letters within the same generation represent significant differences among the three photoperiods (one-way ANOVA, least significant difference multiple comparison, $p < 0.05$). The bars show means and standard deviation.

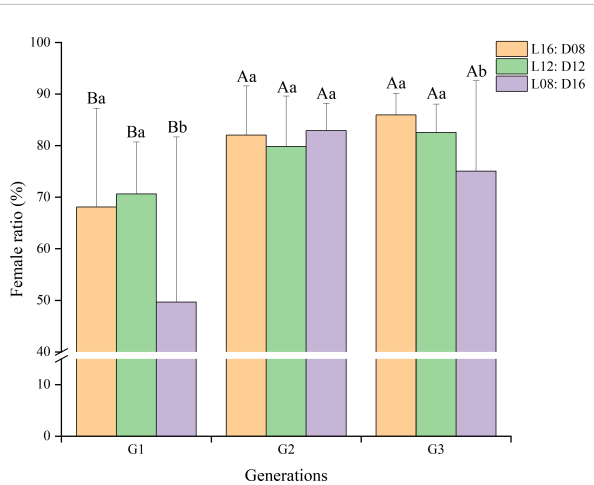

FIGURE 3
Effect of generation on sex ratio of *Anastatus orientalis* under three photoperiods. Different upper-case letters within the same photoperiod indicate significant differences among the three generations, whereas different lower-case letters within the same generation represent significant differences among the three photoperiods (chi-squared test, $p < 0.05$). The bars show means and standard deviation.

3.3 Adult longevity

The interaction between generation and photoperiod had a significant effect on female longevity (df = 4, 418; $F = 5.846$; $p < 0.01$; Figure 4A). However, there was no statistically significant interaction between generation and photoperiod on male longevity (df = 4, 381; $F = 1.111$; $p = 0.351$; Figure 4B), and the effects of generation and photoperiod on male longevity were additive rather than synergistic. Photoperiod had a significant effect on female (df = 2, 424; $F = 29.234$; $p < 0.01$) and male (df = 2, 387; $F = 4.897$; $p < 0.01$) longevity. Male and female longevity was longest under the L8:D16 photoperiod (average: 8.69 days for males and 54.50 days for female) and shortest under the L16:D8 photoperiod (average: 7.46 days for males and 39.66 days for females), indicating that a greater photoperiod reduced the longevity of *A. orientalis*. Generation significantly affected female (df = 2, 424; $F = 5.382$; $p < 0.01$) and male (df = 2, 387; $F = 6.382$; $p < 0.01$) longevity, which decreased by increasing generation in the second and third generations of progeny.

The longevity of surviving female wasps under different photoperiod conditions was significantly different (log-rank $p < 0.01$; Figure 5A), but male longevity was not significantly different (log-rank $p = 0.09$; Figure 5B). The Kaplan–Meier survival curve of female wasps with the photoperiod L16:D08 was consistently below the survival curve of those rearing under the photoperiods L12:D12 and L08:D16. Survival curves of female longevity also showed a significant difference between different generations (log-rank $p < 0.001$; Figure 5C), whereas male longevity was not different between different generations (log-rank $p = 0.05$; Figure 5D). The Kaplan–Meier survival curve of G3 females was consistently below the survival curve of G1 and G2 females. The hazard ratio of death of females and males for the different photoperiods were 1.20 (95% CI 0.95 to 1.51) and 1.30 (95% CI 1.02 to 1.66) in the mixed-effects Cox regression model, respectively. The hazard ratio of death of females and males for the different generations were 1.01 (95% CI 0.80 to 1.28) and 0.84 (95% CI 0.66 to 1.07), respectively.

3.4 Diapause in progeny

Photoperiod was the most important factor determining the proportion of diapausing progeny ($\chi^2 = 5820.070$, df = 2, $p < 0.001$; Figure 6), and the different generations were also significantly influenced ($\chi^2 = 93.602$, df = 2, $p < 0.001$). The progeny of *A. orientalis* females that developed under the long-day length entered diapause significantly more often than females that developed under the medium- and short-day lengths. All parasitoids entered a diapause under the long photoperiod condition (L:D = 16:8), irrespective of which generation they constituted. Under the L12:D12 photoperiod, diapause incidence was significantly increased by an increasing generation ($\chi^2 = 28.660$, df = 2, $p < 0.001$). Under the short photoperiod (L8:D16), no parasitoid from both the first and second generations entered diapause, but diapause incidence strongly increased for the third generation ($\chi^2 = 143.668$, df = 2, $p < 0.001$).

There was no significant relationship between diapause of *A. orientalis* and fecundity ($p = 0.510$, $R^2 = 0.064$; Figure 7A), sex ratio ($p = 0.528$, $R^2 = 0.059$; Figure 7B), or male longevity ($p = 0.259$, $R^2 = 0.177$; Figure 7C). There was a positive correlation between diapause and female longevity (linear regression using female longevity and diapause as a dependent and explanatory variable, respectively: $p < 0.05$, $R^2 = 0.509$; Figure 7C).

4 Discussion

Transgenerational photoperiod effects have been shown to influence reproductive and developmental traits of wasp progeny,

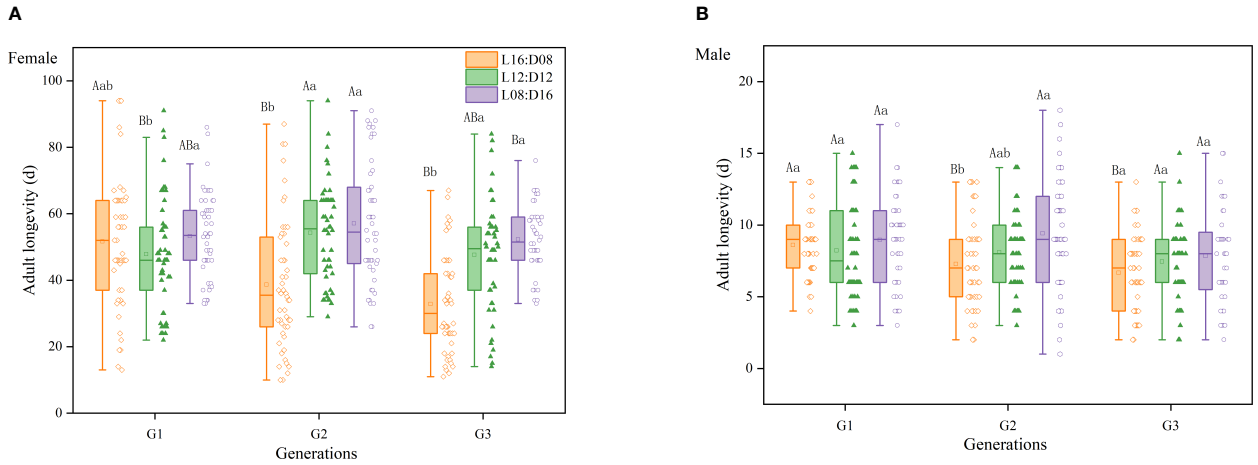

FIGURE 4
Female **(A)** and male **(B)** longevity of *Anastatus orientalis* wasps from different generations under three photoperiods. Different upper-case letters within the same photoperiod indicate significant differences among the three generations, whereas different lower-case letters within the same generation represent significant differences among the three photoperiods (one-way ANOVA, least significant difference multiple comparison, $p < 0.05$).

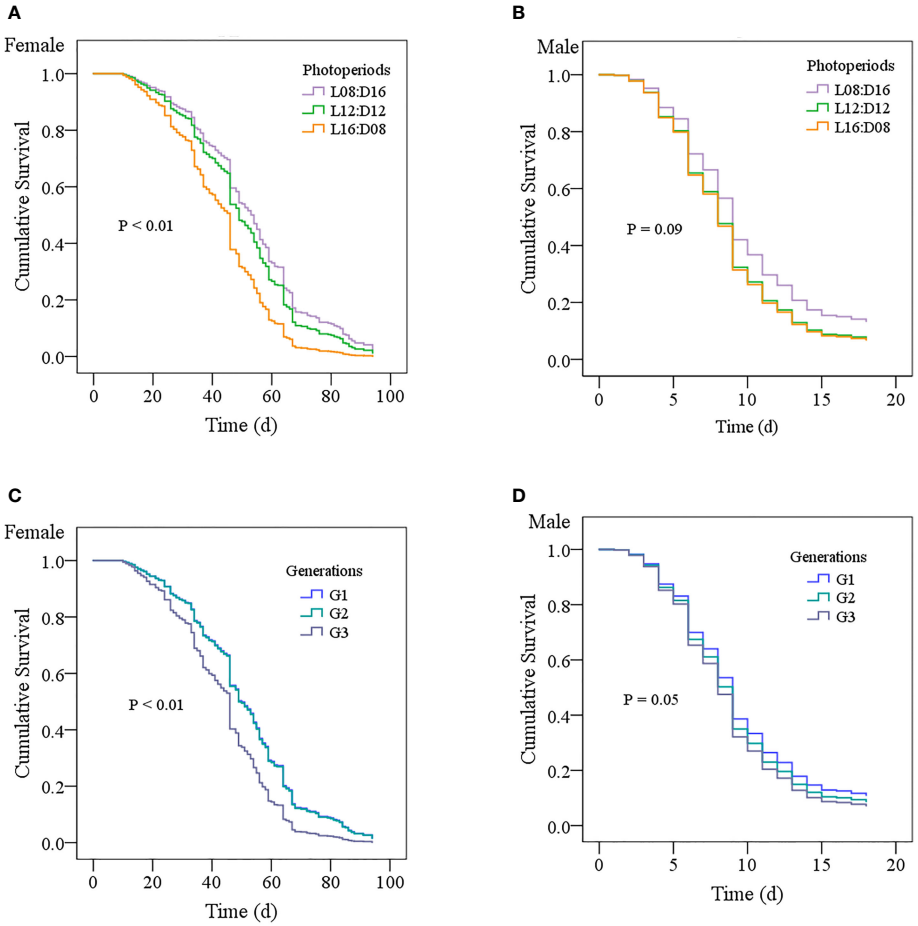

FIGURE 5
Kaplan–Meier survival curves show the female **(A)** and male **(B)** longevity of *Anastatus orientalis* wasp probabilities over time for the long photoperiod condition L16:D8 (N_{female} = 145, N_{male} = 133), middle photoperiod condition L12:D12 (N_{female} = 143, N_{male} = 133), and short photoperiod condition (N_{female} = 139, N_{male} = 124) lines, as well as by generation **(C, D)**.

thus achieving a cumulative photoperiodic effect (34). However, as far as we know, a transgenerational photoperiod influence on the fertility and development of *Anastatus* species progeny has not been clearly demonstrated. The present results show that photoperiod and generation significantly affect female fecundity, female ratio, adult longevity, and diapause of *A. orientalis* wasps. The interaction between generation and photoperiod had a significant effect on female longevity and female fecundity, but there was no significant interaction between generation and photoperiod on male longevity. When reared under the long photoperiod condition (L16:D8), the third generation of *A. orientalis* had greater fecundity, a larger number of progenies, and a higher proportion of females than the population of wasps reared under the other photoperiods or the other generations of wasps. These results show that transgenerational photoperiods affect the biological traits of *A. orientalis*.

Studies to date have shown that various factors are important in regulating the fertility and development of *Anastatus* species. The key role of the natal host, photoperiod, temperature, and other

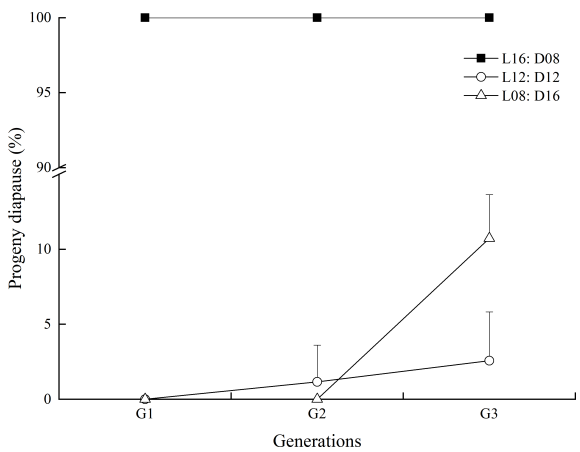

FIGURE 6
Influence of photoperiod conditions during the development of the transgenerational generations on the diapause of *Anastatus orientalis*. The bars show means and standard deviation.

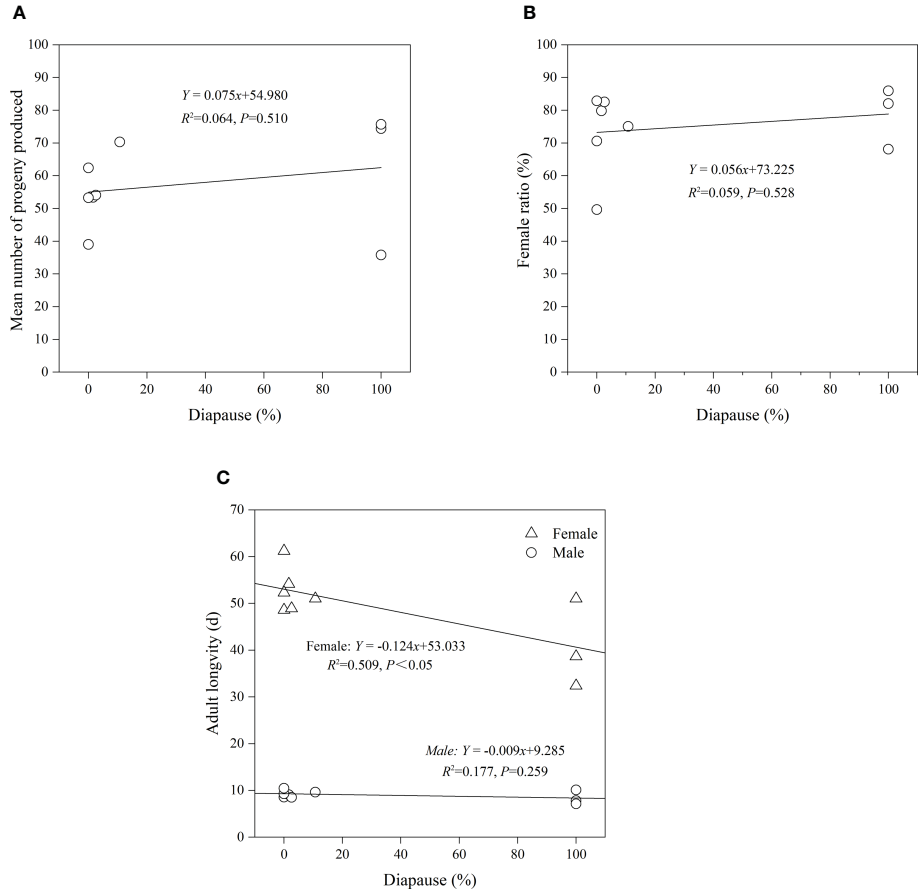

FIGURE 7
Regression relationship between fecundity **(A)**, female ratio **(B)**, adult longevity **(C)**, and diapause of *Anastatus orientalis*.

environmental factors in the regulation of sex allocation, fertility, and adult longevity of *Anastatus* has been clearly demonstrated in both field observations and experimental studies (36–38). In addition, endogenous factors, such as their maternal age, parental experience, food plant of the host prey, and individual size, can also influence the resulting biological characteristics in certain *Anastatus* species (39). Our results suggest that photoperiod conditions and parental experience significantly impact longevity, female fecundity, sex ratio, and diapause rate of *A. orientalis* progeny. Host egg nutrient contents can affect sex ratio, adult longevity, and fecundity of a parasitoid's progeny. Bai et al. (40) showed that females of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) from natural hosts were larger, more fecund, and lived longer than those from factitious hosts. Prior studies have found that *A. pernyi* eggs with higher nutrient contents are preferred and consumed, and this results in longevities reaching up to 64.3 days (25). Prior studies found that females can have a longevity of anywhere from 39 to 68 days in laboratory rearing settings (22, 23). In addition, findings show that larger parasitoids emerge when they develop in larger or more nutritious host eggs. Larger-sized parasitoid progenies show increased longevity, and larger-sized females are capable of higher oviposition rates. Together, these findings and this study show that *A. pernyi* is a suitable alternative host for rearing of the parasitoid.

Although temperature and photoperiod are known to induce diapause in most insect natural enemies, the results of this study indicate that the diapause in *A. orientalis* is induced by photoperiod alone. Seo et al. (22) tested the effect of temperature on biological characteristics of *A. orientalis*, but did not find any correlation between temperature and its diapause. Results by Broadley et al. (23) showed that a long photoperiod induces *A. orientalis* to enter diapause. Our experimental results show that diapause of *A. orientalis* is affected by its exposure to photoperiod, with wasps that experience a longer photoperiod showing a stronger prosperity for entering diapause. Therefore, we conclude that photoperiod is an important environmental factor for diapause induction in this parasitoid wasp. In addition, generation also had a significant effect on the diapause of *A. orientalis*; it increased in later generations. Only by surveying more successive generations can we verify this trend. These results provide additional information for viable long-term storage methods for *A. orientalis*, which in turn will improve rearing efficiency.

The interactive effect of temperature and photoperiod on diapause regulation has been documented in many insect species. For example, development of *Chrysocharis pubicornis* larvae (Hymenoptera: Eulophidae) showed that a short-day-type response affected by temperature and the percentage of individuals entering diapause increased with the temperature and photoperiod (41). In addition, results by Li et al. (42) suggest that the diapause response of

Microplitis mediator (Hymenoptera: Braconidae) is determined by photoperiod and mediated by temperature. When *M. mediator* were exposed to 16°C and 18°C combined with a photoperiod of L10:D14, the percentages of parasitoids that entered diapause was 97.9% and 87.8%, respectively, and there was no incidence of diapause at temperatures of 22°C, 24°C, and 26°C or photoperiods of L2:D22, L14:D10, L16:D8, L18:D6, L20:D4, or L22:D2. Therefore, we are now performing experiments to study the effects of photoperiod and temperature, as well as the interaction of the two, on induction of diapause of *A. orientalis*.

Similar to Broadley et al. (23), but in contrast to work done by other scientists (22, 25), we were unable to produce non-diapause *A. orientalis* adults under the long photoperiod. This may be due to the strain of *A. orientalis* used between these different studies. Wu et al. (43) used molecular tools to examine the genetic composition of *A. orientalis*, and the results suggest a genetic component in determining the diapause behaviors of *A. orientalis*. In addition, Broadley et al. (44) determined that the *A. orientalis* used previously in their study (23), which also went into diapause when exposed to long photoperiod conditions, was a homogenous colony composed of haplotype C. Because the wasps in this study responded similarly, it is highly likely that specimens from our study are composed primarily of haplotype C. However, the different results across studies also may be due to the differences in colony rearing protocol or potentially in how transgenerational effects were expressed. Hence, several generations preceding the experiment should be kept under strictly controlled constant conditions, as this helps to evaluate such discrepancies. Similar precautions to exclude multigenerational maternal effects should be considered in experimental studies with this species and other insect species.

Storage time plays a key role in the production of *Anastatus* species, in that a longer storage time can provide a flexible supply of parasitoids for their timely and urgent field release in biological control programmes. The most commonly used preservation method for an egg parasitoid is cold storage (45–48). However, this method not only fails to guarantee its shelf life but also affects the quality of natural enemy products (23, 49, 50). According to our study's results, diapause may be a better way to preserve *A. orientalis*, and rearing at long-day conditions can induce diapause. In addition, individuals that experienced diapause exhibited a higher sex ratio and greater fecundity, two traits that can augment its performance as a biological control agent. In other insect species, diapause has a positive effect on post-diapause adults' fertility and development (51, 52). However, although diapause allows insects to cope with adverse environmental conditions, it also poses substantial fitness costs. Ellers et al. (53) showed that an increase in diapause length not only led to higher mortality among diapausing pupae of *Asobara tabida* (Hymenoptera: Braconidae), but also caused a significant decrease in egg load, fat reserves, and dry weight of the emerging adult females. Carvalho (54) results suggested that individuals experiencing diapause of *Utetes anastrephae* (Hymenoptera: Braconidae) have lower fecundity. Therefore, the correlation between diapause and reproductive or developmental traits varies with insect species.

Our study clearly shows that transgenerational experience can have far-reaching effects in subsequent generations. Unfortunately, it is not yet known how such effects are mediated or how grandmaternal effects in isolate of material effects would affect the outcome, but future studies are planned. Temperature conditions experienced transgenerationally may alter fertility and development of *A. orientalis*, but further study is needed (55). The empirical data from this study on the effects of transgenerational photoperiod experience on insect fertility and development have potential applications for better understanding *A. orientalis* population dynamics in the field across a geographic and climatic range, and improving insect rearing and storage methods.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

All authors conceived, facilitated, and designed the research. K-XB conducted the experiments, analyzed the data, and conducted statistical analyses. X-YW and BX wrote the manuscript. X-YW, L-MC, HB, and JG secured funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1153723/full#supplementary-material

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Cryptic genetic diversity and associated ecological differences of *Anastatus orientalis*, an egg parasitoid of the spotted lanternfly

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Anastatus orientalis, native to northern China, is an egg parasitoid wasp of the spotted lanternfly (*Lycorma delicatula*) and is being tested as a potential biological control agent for invasive *L. delicatula* in the United States. As a component of these evaluations, live *A. orientalis* collected from Beijing and Yantai in China were reared in containment in the U.S. These specimens showed different responses in diapause behaviors to rearing conditions used previously by other researchers. To understand the primary mechanism potentially driving discrepancies in important life history traits, we used molecular tools to examine the genetic composition of *A. orientalis* from China and from South Korea, where the parasitoid has been introduced to aid in the population management of invasive *L. delicatula*. Molecular analysis of mitochondrial DNA recovered six haplotype groups, which exhibit biased frequency of abundance between collection sites. Some haplotypes are widespread, and others only occur in certain locations. No apparent pattern is observed between wasps collected from different years or emergence seasons. Uncorrected genetic distances between haplotype groups range from 0.44% to 1.44% after controlling for within-group variation. Genetic variance of *A. orientalis* is characterized by high levels of local diversity that contrasts with a lack of a broad-scale population structure. The introduced Korean population exhibits lower genetic

diversity compared to native populations. Additionally, we created iso-female lines for major haplotype groups through laboratory rearing. Differences in diapause behavior were correlated with mitochondrial haplotype. Our results indicate that the observed life history traits in *A. orientalis* have a genetic base.

KEYWORDS

spotted lanternfly, biological control, Eupelmidae, genetic diversity, life history
iso-female lines

Introduction

The spotted lanternfly, *Lycorma delicatula* White (Hemiptera: Fulgoridae), is a destructive invasive insect in North America. It is a highly polyphagous planthopper feeding on over 170 species of plants across 33 families (1). Its preferred host plant is the tree of heaven (*Ailanthus altissima*), which is also invasive in the United States, but *L. delicatula* is considered a high-risk pest of grapes and hops with the potential to impact fruit trees, walnuts, ornamentals, hardwood, forest, and shade tree species (2). *Lycorma delicatula* is native to China (3), where populations are typically relatively low in density, and it is not a significant pest (4). To control the invasive population of *L. delicatula* in the Unites States, efforts have been made to identify its natural enemies in the native range. An egg parasitoid wasp, *Anastatus orientalis* Yang & Choi (Hymenoptera: Eupelmidae), has been identified as a promising candidate for the biological control of *L. delicatula* (4). Previous work has shown that *A. orientalis* demonstrates strong attraction to *L. delicatula* (5) and has a high attack rate on *L. delicatula* in its native range (typically between 30%–40%, 4, 6; but as high as 80% in 7). *Anastatus orientalis* was introduced to South Korea where it is being used as a management strategy for the control of invasive *L. delicatula* (8), where population density seems to be suppressed as a result of the introduction. Extensive studies are currently being conducted with *A. orientalis* to consider it as a potential biological control agent for invasive *L. delicatula* in the U.S.

It has been reported that Chinese and South Korean researchers were able to continuously rear *A. orientalis* for at least eight generations under 25°C and long-day conditions (6, 9). However, a recent study that evaluated progeny production under the same reported conditions found that, in contrast, nearly all *A. orientalis* larvae went into apparent diapause (10). The parent generations of those larvae were collected in Beijing, China, as were those in the earlier studies. However, when reared under temperature and daylight conditions that mimicked Beijing in the fall, nearly all larvae emerged from the host eggs without diapause (10). The observed discrepancy leads to speculation that differences exists between the strains or lineages of *A. orientalis*.

It has been documented that genetic factors play an important role in the plasticity of life cycles in insects, particularly diapause, which is arguably the most important adaptative strategy to face seasonal environmental heterogeneity (11–13). For example, differential gene expression could regulate insect diapause at the transcriptional level (14, 15). Variable life cycle traits in insects can also be directly inherited by progeny from parents, which may lead to genetically differentiated strains maintained by selection (16). Some traits are mainly under the control of multiple genes through epistasis, such as the differential photoperiodic response of the pitcher-plant mosquito (*Wyemyia smithii*) (17). Other traits can be affected by a single segregating locus on the sex chromosome, like the voltinism and pheromone pattern in the European corn borer moth (*Ostrinia nubilalis*) (18). Furthermore, different genetic strains can exhibit physiological differences such as the level of virus-resistance in biotypes of coconut rhinoceros beetle (*Oryctes rhinoceros*), which has significant implications for pest management (19).

An initial survey of the standard mitochondrial COI barcode of *A. orientalis* in northern China suggested variable diapause behaviors among different geographic populations (20). However, using 48 specimens sampled from five locations, this study did not find a distinct population structure but did find that within-location variations dominated the overall genetic variance (20). In the current study, we aimed to further understand cryptic genetic differentiation within this parasitoid, which appears morphologically conserved across its range, and assess the association between genetic lineages and life cycle characteristics. We analyzed a larger number of *A. orientalis* specimens collected in multiple years along with additional samples from South Korea. We also designed new species-specific COI barcode primers and added a second mitochondrial DNA fragment downstream from the standard barcode sequence. The expanded sampling and sequence data in the current study provides new insights into the genetic diversity of *A. orientalis* both in its native and introduced range. In addition, we created iso-female lines based on the genetic results and showed the impact of genetics on diapause behaviors. We also evaluated rearing conditions to maximize insect production in the event that *A. orientalis* is selected as the biological control agent for spotted lanternfly.

Materials and methods

Insect material collection

Parasitized *Lycorma delicatula* egg masses were collected from China and South Korea between 2019–2021. In China, *L.*

delicatula egg masses were collected during the winter and spring from two locations: Beijing and Yantai (Shandong province), from areas where, based on prior knowledge, some parasitism was expected by *Anastatus orientalis*. Three collections were made in Beijing between 2019–2021 and two in Yantai from 2020–2021. In addition, we collected *L. delicatula* egg masses around three cities in South Korea (Nonsan, Anseong, and Buyeo) between March and April 2021. *Anastatus orientalis* that emerged from those egg masses were included in the molecular analysis. Egg masses were carved from the bark of tree trunks with a small knife, stored in locked food boxes by locations at room temperature, and shipped using appropriate permits to the Forest Pest Methods Laboratory (FPML) Insect Containment Facility, U.S. Department of Agriculture in Massachusetts. Voucher specimens were stored in 95% ethanol for molecular analysis (Table 1).

Genomic DNA extraction

We applied two DNA extraction methods to minimize damages to the morphology of each adult wasp, which can be used later for morphological comparison. The process started with a crude DNA extraction, where individual insect was submerged in 100 µl of the prepared extraction buffer that included proteinase K (the ProtK 21) and incubated at 37°C overnight. The reaction was deactivated the next morning by heating the buffer to 75°C for 30 minutes on a heat block. The intact wasp was removed from the buffer to be stored separately. The DNA extract was then cooled and stored at -20°C for subsequent use. When the crude DNA extract failed to yield any PCR product, we revisited the specimen and pulled a single leg, which was processed with the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD) following the manufacturer's protocol, except that purified DNA was eluted in 100 µl buffer. South Korean wasps were all processed with the QIAGEN kit.

Primer design, PCR, and sequencing

We first attempted to amplify the mitochondrial COI barcode for *A. orientalis* using the universal primers LCO1490/HCO2198 (22) as has previously been done (20). However, some specimens yielded no PCR product, and even among those with a successful PCR, the sequencing reaction resulted in mostly noisy data. Upon close examination, an 11-T (thymine) repetitive region near the 5′ end of the barcode was identified as the cause of frame shifts during the extension phase of DNA the sequencing that resulted in noisy data downstream. Therefore, we designed a new primer pair (forward 192F: 5′-TTGGGAATTATTTTGTTCCA-3′; reverse 720R: 5′-TGAGAAATCAATCCAAATCC-3′) to circumvent the repetitive region and maximize amplification/sequencing success for *A. orientalis*. The primer pair amplified a 529 bp fragment that overlapped with 70.1% of the full COI barcode. To further increase the amount of data, we amplified an additional 437 bp fragment immediately downstream from the COI barcode using a second pair of universal primers known as NJ/MD with minor modifications (forward NJ: 5′- TATATTTTAATTTTRCCTGGATTTGG-3′, modified 23; reverse MD: 5′- ATTGCAAATACTGCACCTAT-3′; 24), which demonstrated its usefulness in parasitoid wasps (25).

PCR amplification was conducted in a reaction mix (20 µl total volume) containing 9 µl of molecular grade water, 2 µl of 10X PCR buffer without $MgCl_2$, 2.8 µl of $MgCl_2$ (25 mM), 3.2 µl of dNTP solution (1.25 mM), 0.4 µl of forward and reverse primers (10 pmol/µl), 0.2 µl of JumpStart taq DNA Polymerase (2.5 units/µl), and 2 µl of DNA template. The primer pair 192F/720R was amplified under the following cycling condition: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Cycling condition for the primer pair NJ/MD consisted of initial denaturation at 93°C for 3 min, followed by 35 cycles of denaturation at 93°C for 15 s, annealing at 46°C for 45 s, extension at 68°C for 45 s, and a final extension at 68°C for 7 min. All PCRs included negative control for monitoring contamination. Amplified PCR products were examined on 3%

TABLE 1 Sampling locations in China and South Korea.

Country	City	Collection time	n	h	S	Hd	π
China	Beijing (N 39.9925°, E 116.2109°)	2019	33	6	14	0.703	0.0065
		2020	29	7	17	0.685	0.0061
		2021	30	4	12	0.561	0.0061
	Yantai (N 37.3570°, E 121.4028°)	2020 Spring	7	4	19	0.714	0.0079
		2020 Fall	15	4	17	0.667	0.0075
		2021 Spring	30	5	21	0.692	0.0079
		2021 Fall	16	4	18	0.442	0.0052
South Korea	Buyeo (N 36.3184°, E 126.8451°)	2021	80	4	11	0.601	0.0060
	Anseong (N 36.9373°, E 127.2670°)	2021	42	3	12	0.180	0.0022
	Nonsan (N 36.1662°, E 127.1914°)	2021	12	2	11	0.303	0.0037

Population genetic statistics include the number of sequences (n), number of unique haplotypes (h), number of polymorphic sites (S), haplotype diversity (Hd), and nucleotide diversity (π).

agarose gels, purified by ExoSAP-IT (Affymetrix, Cleveland, OH) following the manufacturer's protocol, and then sequenced on an ABI 3730XL (ACGT, Inc., Wheeling, IL).

Phylogenetic analysis

We used Geneious Prime 2021.1.1 (https://www.geneious.com) to edit chromatograms and perform sequence alignment with the MUSCLE algorithm under default parameters. Genetic diversity statistics were calculated for Beijing, Yantai, and South Korean specimens using DNAsp v6 (26), including the number of unique haplotypes (h), number of polymorphic sites (S), haplotype diversity (Hd), and nucleotide diversity (π) (Table 1). Uncorrected *p*-distances were calculated between haplotype groups, sampling years, and sampling locations in MEGA 11 (27). Mitochondrial genealogies were reconstructed using maximum likelihood (ML) analysis and Bayesian inference. *Anastatus fulloi* was chosen as the outgroup because it is the only species with both 192/720 and NJ/MD data available and also native to China (28). The best-fit nucleotide substitution model was selected by the corrected Akaike Information Criterion (AICc) in jModeltest 2.1.7 (29). The ML analysis was carried out by RAxML v8.2.11 (30) implemented in Geneious, using the best-fit substitution model with the algorithm that simultaneously searches for the best-scoring ML tree and does rapid bootstrapping. A parsimony tree was used as the starting seed for ML tree searching. Nodal support values were estimated through 500 non-parametric bootstrap replicates (BP). The Bayesian inference was carried out by MrBayes 3.2.6 (31) also implemented in Geneious using the best-fit substitution model with two independent runs, each of which had four heated chains running for ten million generations. Tree subsampling frequency was set to every 10,000 generations and the first 25% of trees were discarded as burn-in. Posterior probability (PP) was used to evaluate nodal support. To visually display connection between haplotypes, haplotype frequencies, and how haplotypes were shared between sampling years and locations, we further reconstructed a statistical parsimony network (TCS network) using PopART 1.7 (32).

Establishing iso-female lines

To build pure colonies of the three most common *A. orientalis* haplotypes that we detected, Haplotypes B, C, and D (see Results), we reared out and genotyped (see methods above) a subset of wasps from our live collections of *A. orientalis* from China described above and maintained the lines separately in colony. To start these lines, in April 2020, 100 *L. delicatula* egg masses were collected from the field in Beijing, China and 100 from Yantai, China and shipped to the USDA APHIS Forest Pest Methods Laboratory's Insect Containment Facility. Egg masses from Beijing were held in a growth chamber (Percival, Perry, Iowa) under conditions that simulated real-time temperature and light conditions in Beijing, China as conditions cycled from winter to spring, summer, fall, and winter conditions again. Hereafter, we refer to these conditions as "Beijing-match conditions". Similarly, the egg masses from Yantai were held under conditions that simulated temperature and light conditions in Longkou, Yantai, hereafter referred to as "Yantai-match conditions". Temperature data used were 11-year hourly averages (2007–2017) for each location from the NOAA National Centers for Environmental Information (Global Hourly – Integrated Surface Database). Chamber temperature conditions adjusted each hour to match the hourly averages obtained from the Integrated Surface Database. Light data (daily sunrise, sunset, and civil twilight times) were obtained from timeanddate.com. A relative humidity of 65% was maintained in both chambers. In the fall, on the date when maximum temperatures did not exceed 10°C (November 10 for Beijing and November 22 for Yantai), the chambers were set to constant 5°C with no lights for overwintering. In the spring, on March 7 for both Beijing and Yantai, the chambers resumed tracking the hourly temperature averages and daily light conditions. The full datasets used to program the chambers for "Beijing-match conditions" and "Yantai-match conditions" are available as a supplement (Supplementary Table 1). We collected emerging adult wasps three times per week (Monday, Wednesday, and Friday) for subsequent analysis, rearing, and experiments.

To develop a Haplotype B line, F1 progeny of wasps from April 2020 collections of parasitized egg masses from Yantai were collected three days per week (Monday, Wednesday, and Friday) in September 2020 so that the wasps were ≤ 72 hrs old when collected. Groups of up to five males and 15 females were placed in medium-sized rearing containers (473 ml plastic deli cup, SD16 GenPak, Charlotte, NC), which were modified to include a mesh lid. Wasps were provided with a streak of pure honey and held in Yantai-match conditions without access to egg masses for a mating and preoviposition period of five to nine days. Following preoviposition, 60 female *A. orientalis* were placed individually in small rearing containers (118 ml or 177 ml plastic deli cup, AD04 or AD06, GenPak, Charlotte, NC) modified to include a mesh lid. Each female wasp was provisioned with one *L. delicatula* egg mass and honey and remained in Yantai-match conditions for a one-week exposure. The *L. delicatula* egg masses had been collected from January to March of the same year in Lancaster County, Berks County, and Lebanon County, Pennsylvania. Prior to use, the egg masses were held in constant 5°C conditions with no lights to limit development. Parasitism of field collected eggs has been found to be extremely rare (unpublished data), and no or at least inconsequential numbers of *Anastatus* wasps native or resident to the U.S. would have been present in egg masses used for this study.

Following one week of exposure to the wasp, each egg mass was moved to its own cup. Each wasp was then provided with a second egg mass for a second exposure week and a third egg mass for a third exposure week. Following the third exposure week, each female wasp was preserved in 95% ethanol and genetically analyzed to determine haplotype. Egg masses that had been exposed to Haplotype C and D wasps were discarded. Egg masses exposed to Haplotype B wasps (16 out of the 60 total wasps) remained in Yantai-match conditions for approximately one month (33–35 d), after which they were moved to 25°C long-day conditions to promote emergence. No significant emergence

occurred after one month in 25°C conditions, and the egg masses were moved back into Yantai-match conditions for overwintering. The egg masses remained at Yantai-match conditions until their emergence in September 2021, at which point they were reared as described below.

To build pure colonies of Haplotypes C and D, 55 F2 *A. orientalis* female progeny of wasps from Beijing were held in Beijing-match conditions in May 2021, allowed one week to mate and develop eggs, and then provisioned with egg masses to produce progeny. The wasps were provided with new egg masses for a second exposure week, after which they were preserved in 95% ethanol and genetically analyzed. Egg masses exposed to Haplotype C (16 wasps) and Haplotype D (39 wasps) were labeled accordingly and held in Beijing-match conditions until their emergence in September 2021, at which point they were reared as described below.

Evaluation of rearing conditions

Adult *A. orientalis* from all three iso-female lines (Haplotypes B, C, and D) began emerging in September 2021. Adult wasps of each haplotype were collected three days per week (Monday, Wednesday, and Friday) and combined in groups separated by haplotype of up to five males and 15 females in medium-sized rearing containers (containers described above). Wasps of each haplotype (more than 40 of each) were then moved into environmental conditions that mimicked mid-September in Beijing, China (referred to as "Beijing-fall conditions") and another subset was moved to 25°C long-day conditions. Beijing-fall conditions cycled daily from a high of 25°C and a low of 14°C, lights on 5:55 AM to 6:23 PM, and 65% RH. The 25°C long-day condition maintained a constant 25°C temperature, 65% RH, and 17.5:6.5 (L:D) h (lights on 6:00 AM to 11:30 PM). Note however, that prior work with 25°C conditions set 16:8 (L:D) h with lights on from 6:00 AM to 10:00 PM. In this study, the lights were on 1.5 hrs longer due to a technical issue with the timer. The full datasets used to program the chambers for "Beijing-fall conditions" and "25°C long-day conditions" are available in Supplementary Table 1. A diagram depicting the full rearing protocol is also available in Supplementary Figure 1. These two temperature conditions were selected because Beijing-fall conditions had previously been identified as an effective rearing condition for *A. orientalis* Haplotype C (10) and 25°C long-day conditions had been identified as an effective rearing condition for *A. orientalis* (unknown haplotype) (6, 9).

The wasps were held in each rearing condition without access to egg masses for a one-week preoviposition period. Following preoviposition, one female *A. orientalis* was placed in a small rearing container (118 ml or 177 ml plastic deli cup, AD04 or AD06, GenPak, Charlotte, NC or 237 ml deli cup, 6011 NYHI, Canada) with one *L. delicatula* egg mass and a streak of honey for a one-week exposure. Egg masses of a comparable size (approximately 40-45 eggs per egg mass) were selected across replicates. Wasps remained in their respective rearing conditions (Beijing-fall or 25°C) for exposure. Following wasp exposure, each egg mass was moved to its own cup, and the wasp was provided with another *L. delicatula* egg mass for a second one-week exposure. Wasps were removed after the second exposure week and saved in 95% ethanol. The resulting parasitized egg masses were held for one month (28 d) in their respective rearing conditions to allow for wasp development. Replication of 80 egg masses for each haplotype in each exposure condition (an extra replicate was run for Haplotype B in Beijing-fall). Egg masses in the Beijing-fall condition were then moved to 25°C conditions to promote emergence as described by Broadley et al. (10). Egg masses assigned to 25°C conditions remained at 25°C conditions for emergence. The number of male wasps, female wasps, and *L. delicatula* nymphs that emerged were recorded three times per week (Monday, Wednesday, and Friday). All egg masses were allowed eight weeks (56 d) in 25°C conditions for emergence before they were discarded.

Statistical analysis

A two-way ANOVA with haplotype and rearing condition as factors and the interaction between these two factors on number of progeny produced as the response and another two-way ANOVA with the duration of emergence as the response were run. Replicates in which the parent wasp died during egg mass exposure were not included in the analysis; these accounted for a small number of replicates with only 1 to 9 replicates per each combination of haplotype and rearing condition. To test for an effect of haplotype or rearing condition on the resulting proportion of female progeny, we used a generalized linear model (GLM) with a binomial distribution and logit link. All statistical analyses were run using JMP 13.1.0 (SAS Institute Inc.).

Results

Molecular phylogenetic analysis

The ProtK DNA extraction method worked well for adult *A. orientalis* specimens, generating positive PCR amplifications for more than 80% of the samples that remained intact. Samples that failed the ProtK method had one leg removed, which was processed by the QIAGEN kit. In total, sequence data were generated for 160 A. *orientalis* collected in China (92 from Beijing and 68 from Yantai) and 134 collected in South Korea for the 192/720 and NJ/MD fragment. The two fragments overlapped by 38 bp and therefore were combined into a single 928 bp sequence within the span of the mitochondrial COI gene. No insertion/deletion nor premature stop codons were observed, as expected given its protein-coding function. After trimming both ends, the final sequence alignment included 900 bp. Population genetic statistics were provided in Table 1.

We reconstructed the gene tree based only on the Chinese specimens, as the South Korean population was secondarily introduced from China. The best-fit nucleotide substitution model was selected as the GTR+G model. The ML and Bayesian analysis produced identical haplotype groupings, albeit

relationships between groups remained unresolved since branch supports were low; only the ML tree is presented here (Figure 1). We identified six well-differentiated haplotype group (A–F), three of which (i.e., groups B, C, D) appeared in high frequencies and the other three (A, E, and F) were much rarer. All unique haplotypes were deposited in GenBank (accession numbers, OQ555811-OQ556104). The inferred phylogenetic relationships among those haplotype groups remained unclear as nodal support values were relatively low (ML BP < 80, Bayesian PP < 0.95). The maximum uncorrected *p*-distance between individual specimens in the Chinese *A. orientalis* was 1.56%, which can be found between groups C and E. After controlling for small within-group variations, net mean *p*-distance between haplotype groups ranged from 0.44% to 1.44% (Table 2).

In order to detect potential genetic differences between groups of specimens, which included multiple collecting years for Beijing, wasp emergence seasons for Yantai, and sampling locations for the South Korean population, those groups were labelled with separate colors in Figure 2. Among the Chinese samples, we observe no apparent association of haplotypes with sampling year or emergence season, although some year or emergence season categories recovered a greater number of haplotypes than others. Uncorrected *p*-distances within each sampling year or emergence season were close to distances between years or seasons (Supplementary Table 2). In Beijing, the majority of specimens belonged to Haplotype C or D and their derivatives, which differed from the respective main haplotypes by one or two substitutions. Collections made in different years had similar genetic composition.

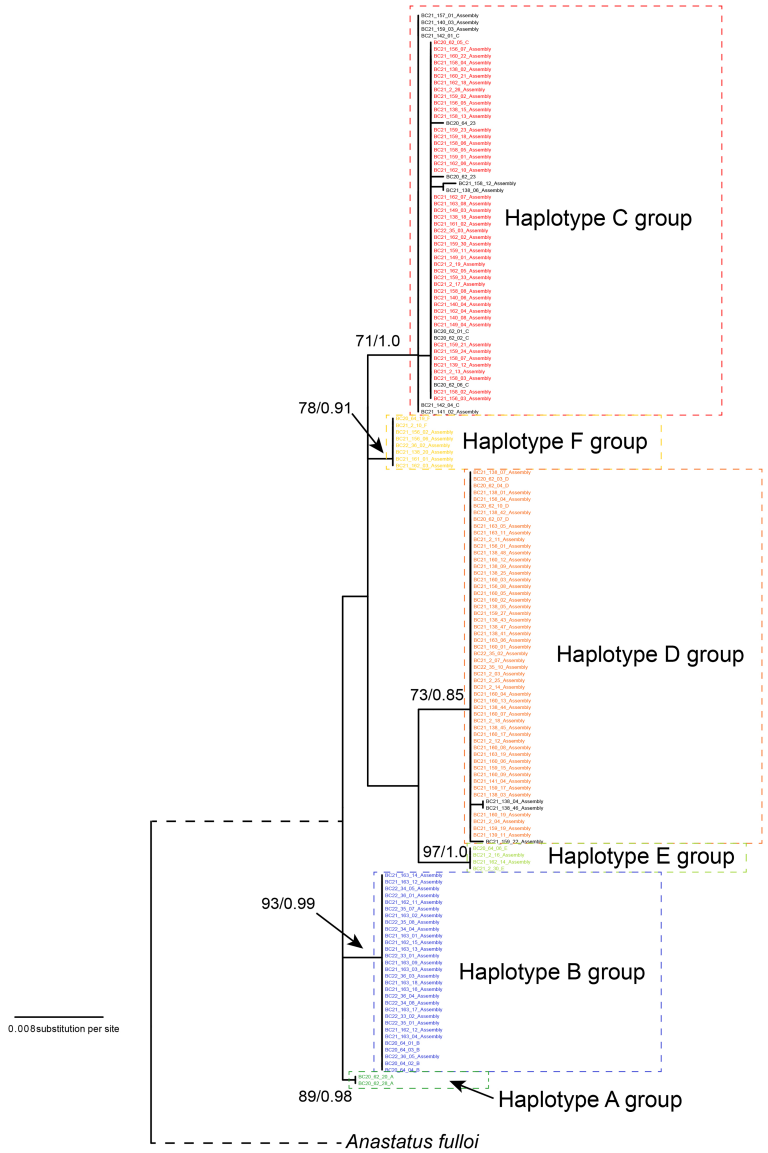

FIGURE 1
Maximum likelihood (ML) gene tree of *A. orientalis* collected from China. Tree is rooted by *A. ulloi*. The six haplotype **(A–F)** are color-coded and haplotype groups are designated by dashed lines. Samples in black color represent haplotypes that differ by a few substitutions from their respective main haplotypes. Numbers on branches are nodal support: ML bootstrap values/Bayesian posterior probabilities.

TABLE 2 Uncorrected p-distances between haplotype groups (lower diagonal) and within each group (diagonal).

	Group A	Group B	Group C	Group D	Group E	Group F
Group A	0.0000					
Group B	0.0044	0.0000				
Group C	0.0085	0.0108	0.0008			
Group D	0.0122	0.0144	0.0115	0.0001		
Group E	0.0122	0.0133	0.0139	0.0089	0.0000	
Group F	0.0056	0.0078	0.0055	0.0111	0.0111	0.0000

Group C, D and F are shared between Beijing and Yantai, while group A is restricted to Beijing and group B and E are endemic to Yantai. Group C and D are found in South Korea.

However, we did not find group B or E in Beijing, which seemed to be unique to the Yantai population. Indeed, Haplotype B was the most abundant haplotype among all Yantai specimens at a frequency of 44.12% (30/68), double the frequency of Haplotype C or D. However, Haplotype B was completely absent among all 15 wasps sampled from the fall 2020 emergence. In contrast to the high genetic diversity among Chinese specimens, the South Korean population had a much lower diversity and only possessed Haplotype C and D and a few derivatives, despite a sampling size close to China. Haplotype D is more prevalent than C in South Korea, occurring at a ratio of 1.5:1 in Buyeo, 5:1 in Nonsan, and up to 12.7:1 in Anseong. Together these two major haplotypes accounted for 91.8% (123/134) of sampled specimens. Interestingly, minor-frequency haplotypes found in the South Korean population were not recovered from the native populations in China.

Rearing of iso-female lines

The emergence rate of *Anastatus orientalis* Haplotypes B, C, and D varied significantly (Full model $F_{5,419} = 99.59$, $p < 0.0001$) in response to rearing in Beijing-fall and 25°C conditions (Figure 3A). Haplotype had the strongest effect ($F = 185.31$, $p < 0.0001$) followed by rearing condition ($F = 76.08$, $p < 0.0001$) and finally the interaction between these two factors ($F = 23.04$, $p < 0.0001$). Wasps presenting Haplotype B produced very few progeny in Beijing-fall conditions (0.3 ± 0.2 wasps per egg mass) and no progeny in 25°C conditions (i.e., they went into diapause). Wasps presenting Haplotype C produced a high number of progeny in Beijing-fall conditions (18.2 ± 1.1 wasps per egg mass) and a low number of progeny in 25°C conditions (3.6 ± 0.8 wasps per egg mass). Wasps presenting Haplotype D produced a high number of progeny in both Beijing-fall and 25°C conditions (21.9 ± 1.3 and 14.9 ± 1.5 wasps per egg mass, respectively). Overall, more female progeny emerged than male progeny, which is consistent with previous findings (10). There was no significant effect of haplotype, rearing condition, or their interaction on the resulting proportion of females ($p = 0.16$), though overall the proportion of females produced for Haplotype B was lower (49% female as compared to 81%–84% for Haplotype C and 73%–79% for Haplotype D). This is likely because neither rearing condition tested in this study was optimal for Haplotype B.

The timing of *A. orientalis* emergence also varied between the two rearing conditions (Figure 3B). Wasps began emerging later (first exposure date to first emergence date) in Beijing-fall conditions than in 25°C conditions (61.0 ± 0.5 and 43.0 ± 0.7 days to first wasp emergence, respectively). Wasps emerged over a

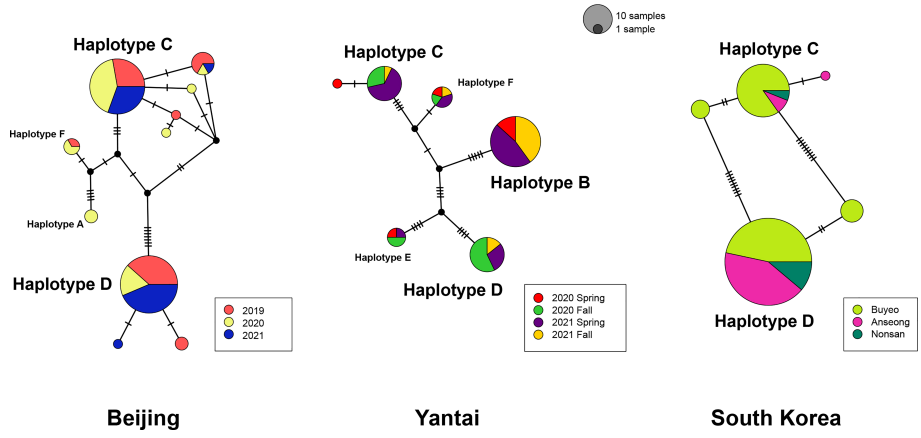

FIGURE 2
Statistical parsimony network of *A. orientalis* collected from Beijing, Yantai, and South Korea. Specimens are color-coded by collection year, emergence season, or location. Circle size is proportional to the number of samples, and all three networks are on the same scale. Each tick on branches represents a single mutational step.

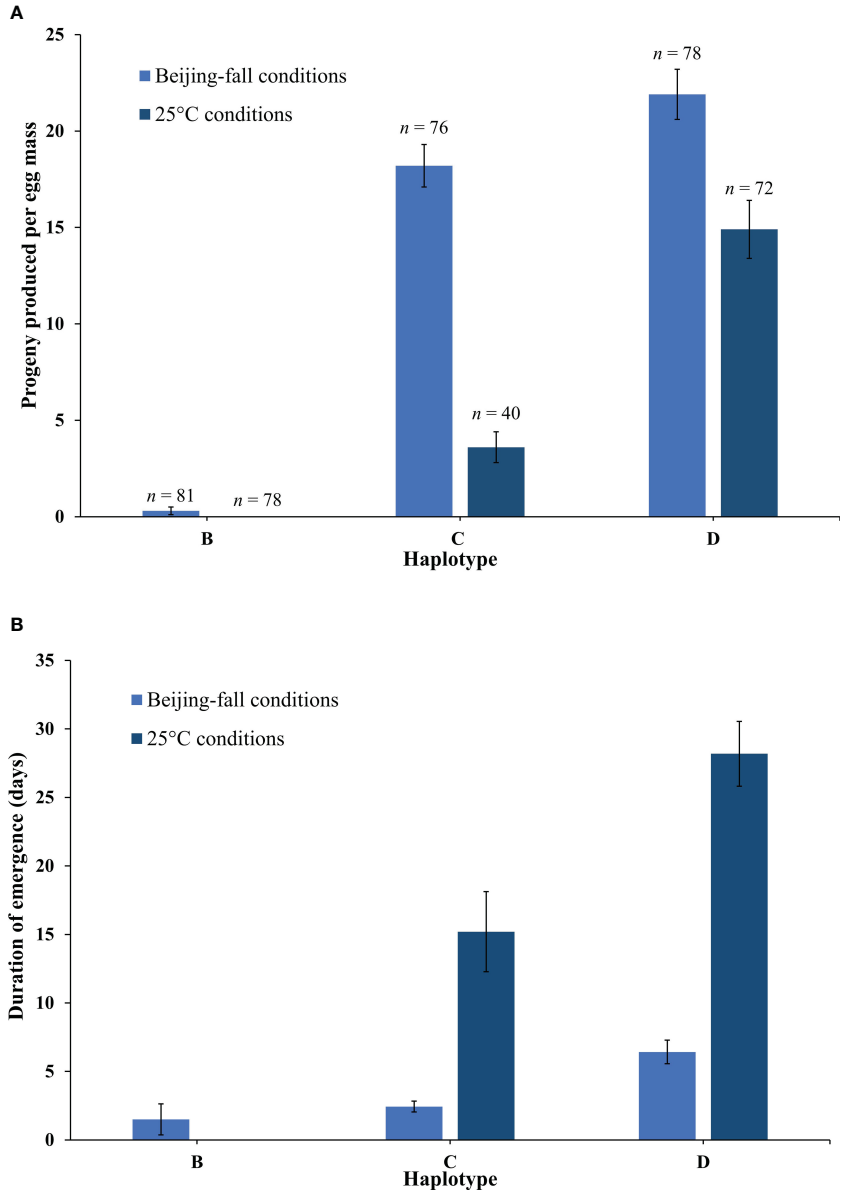

FIGURE 3
(A) The mean number of *Anastatus orientalis* progeny (female and males) produced per egg mass by Haplotypes (B, C, and D) in Beijing-fall conditions and 25°C long-day conditions, and **(B)** the number of days from the first wasp emergence date to the last wasp emergence date. The bars show means and standard errors. Because no Haplotype B wasps emerged in 25°C long-day conditions, no data on duration of emergence is available.

shorter duration (first emergence date to last emergence date) in Beijing-fall conditions than in 25°C conditions and this differed by haplotype ($F_{4,236}$ = 51.03, $p < 0.0001$). Haplotype had the strongest effect ($F = 23.26$, $p < 0.0001$) followed by the interaction between haplotype and rearing condition ($F = 6.54$, $p = 0.0112$).

Discussion

It has long been recognized that genetic variation has an impact on life history traits, even though the heritability of such traits may not be as strong as that of morphological traits (33). Intrigued by the contradictory observations of different diapause behaviors exhibited by *Anastatus orientalis* populations, we aimed to assess mitochondrial diversity within this parasitoid wasp—a potential biological control agent for the invasive spotted lanternfly, *Lycorma delicatula*—and the association between genetic variability and its life history traits. Our study contributes to the understanding of the biology of *A. orientalis*, which is critical for wasp rearing and subsequent tests of host specificity (34, 35).

Molecular analysis of mitochondrial data revealed considerable genetic variation by recovering six haplotype groups in *A. orientalis* collected from two locations in China (Figure 1). Two common Haplotypes C and D and a rare Haplotype F are shared between Beijing and Yantai, while the other three haplotypes (A, B, and E) seem to be restricted to either Beijing or Yantai (Figure 2). Sharing

only a portion of haplotypes between those two locations that are 500 km apart, which likely exceed the dispersal limit of the wasp, could potentially be attributed to inadvertent transportation of the host egg mass by humans. It must be noted that the designation of haplotype groups is not based on a specific cutoff value of genetic distance, which varied between group pairs, but rather based on the relative separation of those groups on the ML tree. Interestingly, a comparison to the prior study that included fewer specimens but more sampling locations (20) revealed a generally consistent pattern, namely five out of the six haplotype groups identified here had corresponding representatives from that study, suggesting a comparable level of genetic divergence recovered between the two studies despite different sample sizes and origins. Together these findings indicate high levels of local genetic diversity which contrasts with the lack of a broad-scale phylogeographic structure in *A. orientalis*.

Maximum intraspecific divergence observed among the 160 Chinese specimens reached 1.56%, and the largest divergence between haplotype groups after controlling for within-group variation was 1.44% between groups B and D, which can occur in sympatry in Yantai. This level of mitochondrial divergence exceeds the 1% threshold for a confident species identification set by BOLD (36) but is similar to that of other widespread parasitoid wasps such as *Aphidius ervi* (37) and *Diaeretiella rapae* (38). We have examined morphological characters of both male and female wasps from the three major Haplotypes B, C, and D using taxonomic keys offered in the original description of *A. orientalis* (6) and a review of the genus in China (39). We did not find noticeable morphological differences among those groups, indicating that the diversity is cryptic in this species.

Although Beijing and Yantai populations share some haplotype groups, the main distinction between their genetic composition is the occurrence of Haplotype B unique to Yantai, which accounts for nearly half of sampled Yantai specimens. This haplotype seems to be specially adapted to Yantai conditions, whereas the other two common haplotype groups (C and D) are more tolerant to varied environmental factors. However, even between Haplotypes C and D we observed different responses in diapause behaviors to Beijing-fall conditions vs. the 25°C conditions. Due to space and time constraints, we were unable to evaluate haplotype responses under Yantai-associated conditions, which would provide an avenue for future study. Reports in the literature indicate that *A. orientalis* can be continuously reared at 25°C long-day conditions for multiple generations in China (6) and South Korea (9), but a recent study using specimens from Beijing found contradictory results (10). Based on results from our genetic analysis and iso-female line rearing, it is highly likely that specimens from the two earlier studies were mostly composed of Haplotype D, which is predominant in South Korea, and the colony used by Broadley et al. (10) were mainly Haplotype C. Indeed, we subsequently genotyped this colony and determined it was a homogenous Haplotype C colony (30).

Anastatus orientalis has been introduced into South Korea to control the invasive population of *L. delicatula*. Reduced genetic diversity of the Korean *A. orientalis* compared to native Chinese populations is in line with its introductory nature. However, the actual introduction history is somewhat complicated. The formal introduction was initiated in 2011 *via* an international cooperative project between the National Institute of Agricultural Sciences of Rural Development Administration (RDA) of South Korea and the Chinese Academy of Forestry in Beijing (8). Those wasps were therefore presumably originated from Beijing. But prior to the release of introduced wasps, *A. orientalis* had already been reported in South Korea from overwintering egg masses of spotted lanternfly collected in April 2010 (40). At that time the parasitoid wasp could not be accurately identified and was referred to as *Anastatus* sp. similar to *A. japonicus* (40), because only in 2015 was *A. orientalis* described as a new species (6). Considering these details, *A. orientalis* have been introduced 1) inadvertently with the invasive *L. delicatula*, which were first documented as a pest in South Korea in 2005 (41), and 2) purposely in large quantity through the international biological control effort in 2011. Currently, *A. orientalis* appears to be distributed throughout South Korea. This scenario is supported by the genetic data, as over 90% of Korean *A. orientalis* possessed either Haplotype C or D, which are also the most abundant haplotypes in Beijing. Additionally, the presence of some minor rare haplotypes not recovered from Beijing may suggest additional sources of introduction.

From this study, we have gained a better understanding of the genetic differentiation within and across populations of *A. orientalis* and how these relate to rearing specifications. We designed new species-specific COI primers and detected six distinct haplotype groups, some of which were regionally specific and some of which coexisted in the same geographic area. The expanded sampling and sequence data provides new insights into the genetic diversity of *A. orientalis* both in its native and introduced range. Additionally, by developing iso-female lines of the three most common of these haplotypes, we determined that the lines responded to the same conditions differently demonstrating the direct impact of genetics on diapause behaviors. These findings help to explain the contradictions in rearing methods presented in prior studies. This work is essential for differentiating what haplotype groups were evaluated in prior studies and for optimizing the laboratory rearing, evaluating annual life cycle characteristics, and testing host specificity of each *A. orientalis* haplotype separately.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession numbers can be found below: NCBI; OQ555811 - OQ556104.

Author contributions

YW, HB, and JG designed the study. XW and LC provided the parasitoids for the study. YW, HB, KV, JM, HN, YK, CL, and AM ran the studies. YW, HB, JM, HN, YK analyzed the data. YW, HB, JM, and YK wrote the manuscript. All coauthors contributed feedback along the way and suggestions to manuscript drafts. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finsc.2023.1154651/full#supplementary-material

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Dendrochronology reveals different effects among host tree species from feeding by *Lycorma delicatula* (White)

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The spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), was first detected in the United States in Berks County, Pennsylvania, in 2014. Native to China, this phloem-feeding planthopper threatens agricultural, ornamental, nursery, and timber industries in its invaded range through quarantine restrictions on shipments, as well as impacts on plants themselves. The long-term impacts of *L. delicatula* feeding on tree species have not been well studied in North America. Using standard dendrochronological methods on cores taken from trees with differing levels of *L. delicatula* infestation and systemic insecticidal control, we quantified the impact of *L. delicatula* feeding on the annual growth of four tree species in Pennsylvania: *Ailanthus altissima, Juglans nigra, Liriodendron tulipifera*, and *Acer rubrum*. The results suggest that *L. delicatula* feeding is associated with the diminished growth of *A. altissima*, but no change was observed in any other tree species tested. The results also suggest that systemic insecticides mitigate the impact of *L. delicatula* feeding on *A. altissima* growth.

KEYWORDS

***Lycorma delicatula*, spotted lanternfly, *Ailanthus altissima*, tree of heaven, dendrochronology, tree core**

Introduction

The spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is native to China and was first detected in the United States, in 2014, in Berks County, Pennsylvania (1). This phytophagous phloem-feeder has over 100 identified host species worldwide and 56 host species confirmed in North America (2). As a phloem feeder, *L. delicatula* has the potential to cause serious economic and ecological impacts (3). In Pennsylvania, *L.*

delicatula has proven to be a major pest to grapevines. Some vineyards, with repeated seasons of high pest pressure from *L. delicatula*, have experienced yield losses of up to 90%, and have been subject to triple the number of insecticide applications (4). Studies have shown that some insecticides kill *L. delicatula*, but re-invasion by adult insects from surrounding forests and vegetation into vineyards continues through the late summer and fall (5, 6).

Although much of *L. delicatula* development can occur on cultivated plants, forest and ornamental/shade trees can be obligate hosts for some of the *L. delicatula* life cycle (4). The tree of heaven, *Ailanthus altissima* (Miller) (Sapindales: Simaroubaceae), is an invasive tree species in North America and is a preferred host of *L. delicatula* in its native range. Although *L. delicatula* may not require this tree to complete development, *A. altissima* certainly can constitute a significant proportion of the diet of *L. delicatula* and is a valuable host plant in the insect's development (7). The *L. delicatula* host range also comprises many economically important North American hardwoods, including black walnut [*Juglans nigra* L. (Fagales: Juglandaceae)], maple [*Acer* spp. L. (Sapindales: Sapindaceae)], oak [*Quercus* spp. L. (Fagales: Fagaceae)], and tulip poplar [*Liriodendron tulipifera* L. (Magnoliales: Magnoliaceae)] (1). The potential economic losses to the forest industry caused by *L. delicatula* have been projected at US$152.6 million per year in Pennsylvania alone (8). These estimates, however, do not fully account for the ramifications of *L. delicatula* invasion on tree health, as many of these effects have not been investigated.

Invasive phloem-feeding insects are a primary cause of disturbance in many forest ecosystems, altering community dynamics, biogeochemical processes, and carbon cycling (9, 10). Phloem sap is composed of carbohydrates and amino acids that are necessary for the production of proteins (11). Depending on phloem nutritional quality, phloem-feeding insects can feed continuously for many hours, ingesting high amounts of phloem sap and excreting excess glucose (11). Large aggregations of *L. delicatula* feeding on a tree effectively remove quantities of important nutrients from the tree manufactured during photosynthesis. In addition, the consumption of phloem sap results in *L. delicatula*'s excretion of honeydew, facilitating sooty mold growth that inhibits plant photosynthesis (12).

Although *L. delicatula* feeding can have detrimental effects on tree physiology in some forest species (13), our understanding of, and methodology for, assessing how sap-feeding insects alter tree growth are limited (14). Dendrochronology, the study of dating events using annual tree rings (15), has been used to identify historic defoliation events and beetle outbreaks in forests throughout the United States (16–18). To date, no study has looked into the effect of *L. delicatula* on the radial growth of host trees. In this study, we used dendrochronological methods to quantify the impact of *L. delicatula* feeding on host tree radial growth, and the ability of systemic insecticide treatments to mitigate this impact. The hypothesis we consider is that the presence of *L. delicatula* populations reduces the woody growth of host trees, as reflected by growth rings. This is important for two reasons. First, in the event that *L. delicatula* has a negative effect on the radial growth of economic hosts, there would be an argument for the value of preventative treatment. Second, regarding the effect of *L. delicatula* on the tree of heaven, field observations have revealed visible effects on tree vigor and health; knowledge of these effects could result in the extended lifespan of treated trap trees and our increased understanding of the ecological impacts of this insect on this host.

Materials and methods

Study area

To investigate the impact of *L. delicatula* feeding on the radial growth of known host trees, samples were collected from two sites in Pennsylvania where *L. delicatula* has been established in high densities. The Pennsburg site was first documented as containing *L. delicatula* in 2016, and the Blue Marsh Lake site was first documented as containing *L. delicatula* in 2017. The populations of both of these sites increased year over year [personal observations, BW and Brianna Treichler, the United States Army Corps of Engineers (USACE)] following initial infestation and continued to grow throughout 2020 at both sites. "High density" is a relative term and is often relative to the lifecycle stage and corresponding host species. The sites contained clear evidence of *L. delicatula* feeding on common host trees, particularly sooty mold growth on the trunks, cadavers from previous seasons abundant on the ground, and nearby understory stunted or killed by the sooty mold growth to the point of resembling the aftermath of a brush fire. It is not uncommon to document several hundred adult *L. delicatula* per tree in a 2-minute visual count on preferred hosts in the fall. Tree species composition at these sites was primarily mixed deciduous hardwood stands native to the area that have been invaded by the tree of heaven. The typical species at these locations include black walnut (*J. nigra*), red maple (*Acer rubrum* L.), silver maple (*Acer saccharinum* L.), tulip poplar (*L. tulipifera*), black cherry (*Prunus serotine* Ehrh.), sassafras [*Sassafras albidum* (*Nutt.0 Nees*)], mixed oak (red (*Quercus rubra* L.), chestnut (*Quercus montana* Willd.), white (*Quercus alba* L.), and hickories [shagbark—*Carya ovata* (Mill.) Koch; pignut—*Carya glabra* (Mill.) Sweet]. The habitat characteristics where trees were sampled generally consisted of fragmented edge habitats along farm fields or maintained parkland adjacent to roads and trails.

On 7 January 2020, tree cores were collected from Pennsburg, Upper Hanover Township of Montgomery County, Pennsylvania, USA (latitude, longitude: 40.36672, −75.54746). The cores of *A. altissima* (n = 10), *Ac. rubrum* L. (n = 8), *J. nigra* L. (n = 8), and *L. tulipifera* (n = 5), which had high densities of *L. delicatula* feeding on them, were collected between 2016 and 2019. In Pennsburg, the first trees selected were *A. altissima*, which were divided according to whether they were treated or untreated. Again, larger trees were selected with the expectation that they would provide a longer pre-infestation record. In Blue Marsh Lake, the trees that were selected were *A. altissima*, then treated or untreated (treated trees being previously selected by USACE personnel for treatment based on observed densities and proximity to areas with an increased risk of SLF hitchhiking to new locations on conveyances of park visitors), again with larger trees selected with the expectation of providing a longer pre-infestation record.

On 5 March 2020, *A. altissima* tree cores encompassing three insecticide treatment levels were collected from Blue Marsh Lake Recreation Area in northwest Berks County, Pennsylvania, USA (40.380709, −76.028454), where *L. delicatula* was initially discovered in 2016. The management of *L. delicatula* at Blue Marsh by the Philadelphia District USACE started in 2018 after high densities of adults were observed. The trees were selected for insecticide treatment based on the infestation level of *L. delicatula*. *A. altissima* trees that received 2 consecutive years of insecticide treatment were sprayed on 6 October 2018 and 26 July 2019. The *A. altissima* trees that received a single insecticide treatment were sprayed on 16 August 2019. Afterward, untreated trees still had large numbers of *L. delicatula*. Treated trees were sprayed until runoff with the systemic insecticide dinotefuran (Transtect 70 WSP insecticide; Rainbow Treecare Scientific Advancements, Minnetonka, MN, USA) as a basal bark application at 37.34 g AI/L from the ground to 30–38 cm on the trunk and 360° around the tree. Ten cores were collected from each treatment, for a total of 30 A. *altissima* cores.

Core collection and laboratory processing

All trees were cored at standard breast height (1.4 m aboveground) using a Jim-Gem® 35-cm increment borer (model 63084; Forestry Suppliers, Jackson, MS, USA) with a core diameter of 5.15 mm, and all trees cored had a diameter at breast height (DBH) longer than 25 cm. The extracted cores were immediately placed in labeled plastic straws lined with hole punches to allow the cores to remain straight while drying. The cores were air-dried on a baking sheet at room temperature for 2 weeks in accordance with standard practice (19).

Once dried, the cores were processed using standard dendrochronological methods (19). The cores were removed from the straws and individually mounted to 25 cm wood blocks with grooves cut down the center to accommodate the core. The groove was approximately 2 mm deep, allowing at least 50% of the core to remain exposed. The exposed surface of each core was then sanded with 220-grit sandpaper using a random orbital sander (DeWalt model DWE6420, Baltimore, MD, USA) for approximately 10–15 seconds to create a flat working surface. Each core was then sanded with progressively finer grit paper (320, 400, and 1,500 grit) for 2 minutes per grit. This was done to remove scratches from the previous grit and create a prepared surface with clearly defined rings and wood cells for dating and measurement under a microscope (20) Representative cores are illustrated in Figure 1.

Core measurement

The tree cores were cross-dated using the list method, a standard process by which narrow rings are matched between cores to ensure accurate dating (21). The ring widths in cores collected from Montgomery County, PA, USA, were measured to the nearest 0.01 mm using a dissecting microscope and Velmex measuring system. A sliding stage was incrementally moved via a small crank and a crosshair in the microscope was used to visually delimit the ring boundaries when taking measurements. The sliding-stage micrometer was connected to a computer and measurements were recorded in MeasureJ2X software (VoorTech Consulting, Holderness, NH, USA).

Due to the university building access restrictions as a result of COVID-19, *A. altissima* cores collected from Berks County, PA, USA, were measured digitally. Cores were placed under a dissecting scope equipped with a nine-megapixel digital camera (SKU: MU900; AmScope, Irvine, CA, USA) that was connected to a computer. Scope calibration and measurements were collected

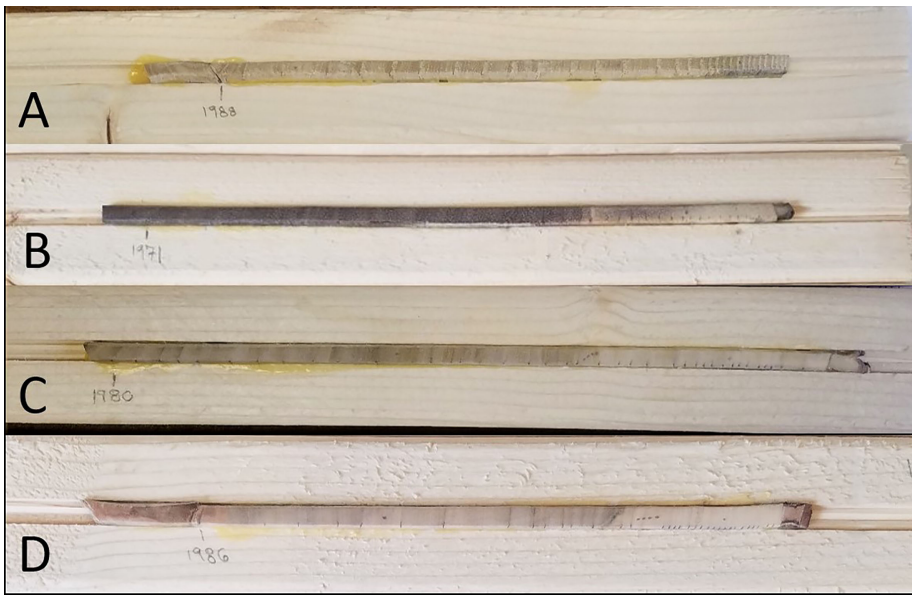

FIGURE 1
Representative core of each species. *Ailanthus altissima* **(A)**, *Juglans nigra* **(B)**, *Liriodendron tulipifera* **(C)**, and *Acer rubrum* **(D)**, with the year marker representing the first year's growth.

on-screen using AmScope software (version x64, 3.7.7303). The calibration was done at × 1 zoom using a 0.01-mm stage micrometer (SKU: MR096; AmScope). All the ring widths were measured to the nearest 0.01 mm.

After all the ring widths were measured, core dating accuracy was statistically evaluated using the computer program COFECHA (22, 23). COFECHA applies a 32-year cubic smoothing spline across all the data to create a master chronology for each site and species (24). Each tree core series is then compared with the master chronology by splitting it into 50-year segments and using 25 years of overlap to calculate the series intercorrelation for that site and species (24). Potential errors identified by COFECHA were investigated and corrected by re-cross dating. Any cores with unresolvable errors were excluded from further analysis.

Data standardization: tree size and age

The ring width tends to decrease over time as trees must allocate a greater proportion of resources to wood production to cover an increasing circumference (25). Dendrochronological studies often standardize ring width chronologies to control for varied growth rates among trees of differing sizes and ages (26). To standardize for age–size growth dependencies, raw ring width chronologies were standardized by fitting a negative exponential curve to the data using the computer program ARSTAN (27). ARSTAN was originally developed by Edward R. Cook of Columbia University and has been used since the late 1980s to conduct autoregressive time series standardization of tree ring data (27). If the negative exponential curve did not fit, a horizontal line through the mean was used for standardization (26, 28). The raw ring width value was then divided by the fitted curve value for each measurement, resulting in a dimensionless ring width index (RWI) with an average growth of approximately 1 (25). An RWI > 1 corresponds to greater than average annual growth, whereas a RWI < 1 corresponds to less than average annual growth.

Data standardization: climatic variables

Standardization has also proven to be useful in understanding the impacts of insects, climate, and other various environmental pressures on tree growth (25). Climatic variables have been shown to influence tree growth (17, 24, 29–31). In this study, we removed the most correlated climate variables from each data set to focus results on the effect of *L. delicatula* feeding. Climate data for both sampling locations were obtained from the National Oceanic and Atmospheric Administration (NOAA) database for Pennsylvania Climate Division 3, Southeastern Piedmont (32, 33). This data set consisted of monthly averages for minimum temperature, maximum temperature, average temperature, precipitation, and Palmer Drought Severity Index (PDSI) values from 1895 to 2019.

To identify the dominant climate variables that altered tree growth, each site and tree species standardized chronology was compared with each climatic parameter using a correlation matrix in Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) (34). Once the dominant climate variable [the climate variable that most affected tree growth (34)] was identified, all data for that variable were divided by their average to create a dimensionless climate index. To normalize by climate, and thus remove the dominant climate signal, the climate index was subtracted from the standardized chronology (RWI) for each site and species (34).

We attempted to standardize *A. altissima* chronologies obtained from Montgomery County by fitting a negative exponential curve to the raw ring-width data, but later year growth was close to zero and unrealistically skewed the RWIs. Therefore, to equalize the variance across series, we standardized *A. altissima* chronologies in ARSTAN by fitting a horizontal line through the mean, and the distribution of RWIs was then shown to be approximately normal. To maintain consistency all *J. nigra* and *L. tulipifera* series were standardized in ARSTAN by fitting a horizontal line through the mean to equalize variance across the series and the RWI distribution and were shown to be approximately normal (Table 1). The same method using ARSTAN was used for all cores.

Data analysis

The RWIs were combined for all cores to form a master chronology for each site and species. Pre- and post-*L. delicatula* infestation years were then compared to determine if there were detectable differences in radial tree growth. Since *L. delicatula* presence was confirmed in the region in 2016, initial populations were likely established in the area in 2015. Thus, tree growth prior to 2015 was considered pre-infestation growth, whereas that from 2015 to 2019 was considered post-infestation growth.

TABLE 1 Summary of COFECHA results characterizing radial growth of tree species from increment cores.

Site	Species	N_{cores}	Mean ring width (mm)	Series intercorrelation*	Mean sensitivity**
Upper Hanover	*Ailanthus altissima*	8	3.80	0.483	0.290
Upper Hanover	*Acer rubrum*	7	1.95	−0.103	0.347
Upper Hanover	*Juglans nigra*	5	2.46	0.307	0.361
Upper Hanover	*Liriodendron tulipifera*	5	5.74	0.592	0.298
Blue Marsh	*Ailanthus altissima*	22	4.79	0.485	0.327

*A measure of how well each tree core series correlates with the master chronology made by COFECHA; a larger number equals a higher correlation.
**A measure of year-to-year variation in tree ring width from 0 to 1. A mean sensitivity of around 0.2 is accepted for climate reconstruction (24).
Growth patterns are characterized among trees of the same species at the same site.

The RWI data were imported into R (The R Foundation for Statistical Computing, Vienna, Austria) (35), where the distribution was checked for normality using the Shapiro–Wilk test. If normality was met, paired t-tests were used to compare the RWI of infested years (2015–2019) to uninfested years (2010–2014) for each site and species. Similarly, a comparison of RWI from earlier uninfested years (2005–2009) to the uninfested years (2010–2014) for each site and species was also created to act as a control and to determine if environmental conditions may have had a significant impact on the mean growth of sampled trees. If normality was not met, RWIs would have been compared using the non-parametric paired Wilcoxon test (36). However, the residuals of all chronologies were shown to be approximately normal, so no Wilcoxon test was needed for analysis.

Results

Impact of *Lycorma delicatula* infestation on tree growth

Ailanthus altissima

The *A. altissima* chronologies, obtained from Montgomery County, ranged in length from 16 to 48 years, with a mean length of 30.7 years. For a two-tailed correlation of annual tree ring widths to climate data, with a sample size of 47 years at a confidence level of 0.05, the critical value for Pearson's correlation coefficient was 0.285 (31). All climatic variables were correlated with the standardized *A. altissima* chronology, and the September average temperature had the highest negative correlation of −0.576. A linear regression analysis was carried out for September's average temperature as compared with the standardized chronology (Figure 2A). The regression analysis showed that approximately 33% [R^2 = 0.331, degrees of freedom (df) = 47; $p < 0.001$] of the tree's reduced growth could be attributed to September's average temperature. After subtracting the normalized climate index from the standardized chronology, a Student's paired *t*-test showed significantly lower rates of growth from 2015 to 2019 than from 2010 to 2014 (t = 4.424, df = 4; p = 0.011). The growth from 2005 to 2009 and 2010 to 2014, periods when *A. altissima* was presumed to be uninfested, was not significantly different (t = 2.366, df = 4; p = 0.077; Figure 2B).

Juglans nigra

The *J. nigra* chronologies, collected from Montgomery County, ranged in length from 26 to 81 years, with a mean length of 48.8 years. For a two-tailed correlation of annual tree ring widths to

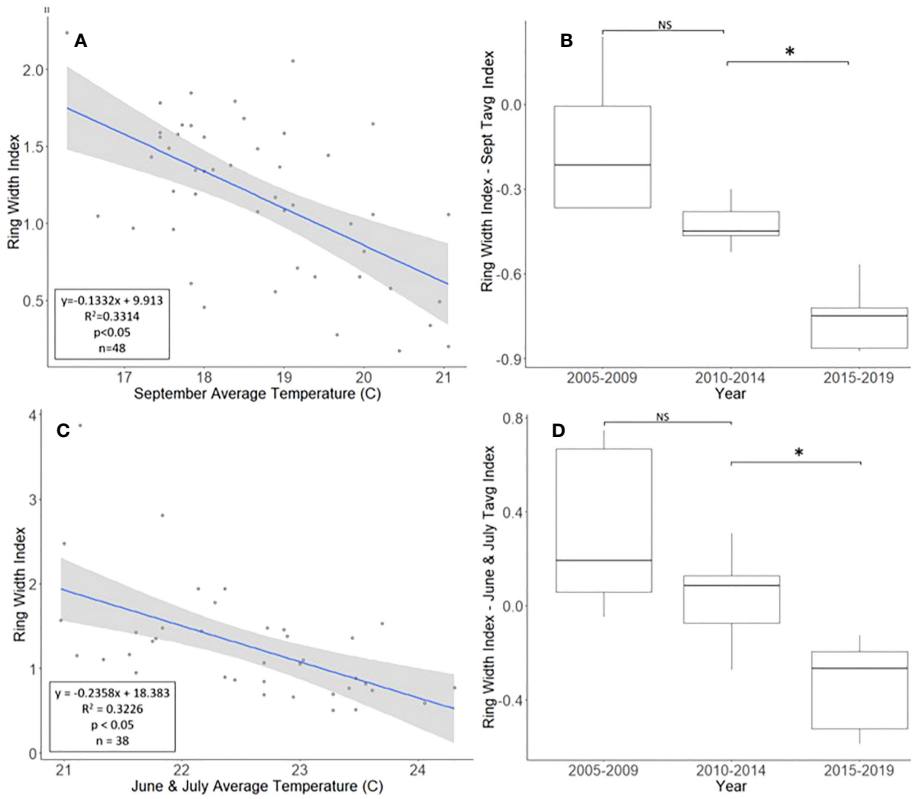

FIGURE 2
Impact of climate conditions and *Lycorma delicatula* on the ring width index for *Ailanthus altissima* in Upper Hannover Township, PA, USA **(A, B)** and Blue Marsh Recreation Area, Berks County, PA, USA **(C, D)**. Regression analysis of *A altissima* ring width index values and September's average temperature **(A)**; a comparison of *A altissima* ring width index values with the dominant climate variable removed in years before (i.e., 2005 to 2009 and 2010 to 2014) and after (2015 to 2019) the likely start of *L. delicatula* infestation **(B)**; a regression analysis of *A altissima* ring width index values and the average temperatures for June and July **(C)**; and a comparison of *A altissima* ring width index values with the dominant climate variable removed in trees without insecticide treatment. **(D)**. NS, the difference between means not significantly different from zero; *, the difference between means significantly different from zero ($p < 0.05$).

climate data, with a sample size of 81 years at a confidence level of 0.05, the critical value for Pearson's correlation coefficient was 0.216 (36). All climate variables were correlated with the standardized chronology for *J. nigra*, and September's minimum temperature had the largest negative correlation of −0.262. A linear regression analysis was carried out for September's minimum temperature as compared with the standardized chronology (Figure 3A). The regression analysis showed that approximately 7% (R^2 = 0.069, df = 81; p = 0.018), of the tree's reduced growth could be attributed to September's minimum temperature. After subtracting the normalized climate index from the standardized chronology, a Student's paired t-test showed no significant reduction in growth after *L. delicatula* infestation (t = 2.056, df = 4; p = 0.109). However, the climate-adjusted RWI from 2010 to 2014 was significantly less than from 2005 to 2009 (t = 3.559, df = 4; p = 0.024; Figure 3). The fact that there are differences in the RWI between the two-time intervals in the absence of *L. delicatula* shows that factors other than *L. delicatula* can influence tree regrowth.

Liriodendron tulipifera

The *L. tulipifera* chronologies, collected from Montgomery County, ranged in length from 17 to 40 years, with a mean length of 25.8 years. For a two-tailed correlation of annual tree ring widths to climate data with a sample size of 40 years at a confidence level of 0.05, the critical value for Pearson's correlation coefficient was 0.301 (36). All climate variables were correlated with the standardized chronology for *L. tulipifera*, and July's maximum temperature had the largest negative correlation of −0.474. A linear regression analysis was carried out for July's maximum temperature as compared with the standardized chronology (Figure 4A). The regression analysis showed that approximately 23% (R^2 = 0.2251, df = 40; p < 0.001) of the variation in the RWI could be attributed to July's maximum temperature. After subtracting the normalized climate index from the standardized chronology, a Student's paired t-test showed a significant reduction in the growth of *L. tulipifera* after *L. delicatula* infestation (t = −2.961, df = 4; p = 0.042). There was no significant difference in the two uninfested time periods when the dominant climate variable was removed (t = 2.288, df = 4; p = 0.084; Figure 4B).

Acer rubrum

The *A. rubrum* chronologies, obtained from Montgomery County, had a high degree of variation in ring width and ranged in length from 19 to 151 years, with a mean length of 61.4 years. None of the seven *Ac. Rubrum* trees sampled correlated well with the master chronology created in COFECHA (Table 1) and were excluded from further analysis.

Impact of chemical treatment on *Ailanthus altissima* growth

The *A. altissima* chronologies, collected from Blue Marsh, ranged in length from 5 to 37 years, with a mean length of 19.4 years. The eight cores did not date well with the master chronology. Discrepancies in the wood could not be identified and the cores were removed from further analysis. All other series dated well in COFECHA, with an interseries correlation of 0.485 (Table 1). To remain consistent, we standardized Blue Marsh *A. altissima* chronologies by fitting a horizontal line through the mean, and the distribution of RWIs was found to be approximately normal.

For a two-tailed correlation of annual tree ring widths to climate data, with a sample size of 38 years at a confidence interval of 0.05, the critical value for Pearson's correlation coefficient was 0.312 (37). All climate variables were correlated against the standardized chronology for *A. altissima* and it was found that June's and July's average temperatures had the largest negative correlation, at −0.520 and −0.447, respectively. A linear regression was calculated for June's and July's average temperatures as compared with the standardized chronology (Figure 2C). The regression analysis showed that approximately 32% (R^2 = 0.323, df = 38; p < 0.001) of reduced tree growth could be attributed to June's and July's average temperatures. After subtracting the normalized climate index from the standardized master chronology, the data

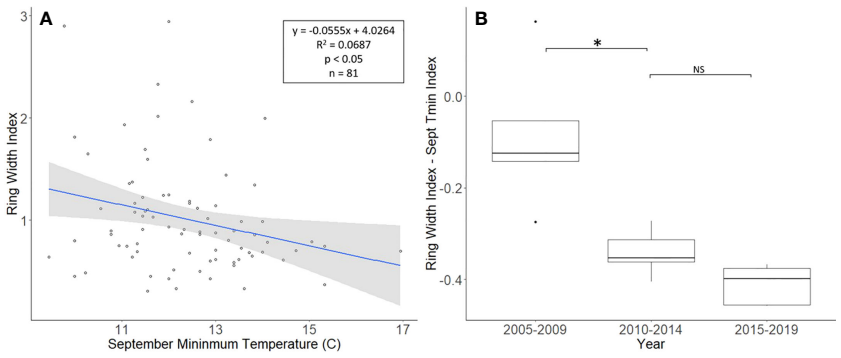

FIGURE 3
Impact of climate conditions and *Lycorma delicatula* on the ring width index for *Juglans nigra* in Upper Hanover Township, PA, USA. Regression analysis of *J nigra* ring width index values and September's minimum temperature. **(A)** and a comparison of *J nigra* ring width index values with the dominant climate variable removed in years before (i.e., 2005 to 2009 and 2010 to 2014) and after (2015 to 2019) the likely start of *L. delicatula* infestation. **(B)**. NS, the difference between means not significantly different from zero; *, the difference between means significantly different from zero (p < 0.05).

were broken up into treatments for analysis using a Student's paired *t*-test (0, 1, and 2 years of insecticide treatment, respectively).

No insecticide treatment

After accounting for the dominant climate variables, *A. altissima* without insecticide treatment showed a significant reduction in RWI from 2015 to 2019 than from 2010 to 2014 (t = 3.513, df = 4; p = 0.025). However, no significant difference in climate-adjusted RWIs was found when we compared the two periods presumed to be before the *L. delicatula* invasion period, that is, the period from 2005 to 2009 to that from 2010 to 2014, (t = 1.308, df = 4; p = 0.261; Figures 2D, 5A).

One year of insecticide treatment

After accounting for the dominant climate variables, *A. altissima* that received 1 year of insecticide treatment did not show a significant reduction in climate-adjusted RWI after the presumed introduction of *L. delicatula* (t = −0.264, df = 4; p = 0.805). Similarly, no significant difference in RWI was found when we compared the two periods before the *L. delicatula* invasion (t = 1.818, df = 4; p = 0.143; Figure 5B).

Two years of insecticide treatment

After accounting for the dominant climate variables, *A. altissima* that received 2 years of insecticide treatment did not show a significant reduction in RWI post-*L. delicatula* invasion (t = −2.612, df = 4; p = 0.059). Similarly, no significant difference in RWI was found when we compared the two preceding periods of uninfested years prior to *L. delicatula* invasion (t = 2.153, df = 4; p = 0.098; Figure 5C).

Master chronologies

Master chronologies indicate differences in growth patterns among the *L. delicatula* hosts examined. *A. altissima* had suppressed growth in 2007 (likely from a severe drought that year) and 2015 (potentially from *L. delicatula* feeding) (Figure 6A). Interestingly, *J. nigra* had suppressed growth in 2010, perhaps due to a late-season drought, but no negative impacts on growth that could be associated with *L. delicatula* feeding from 2015 onward were found. *L. tulipifera* had a substantial increase in growth rate in 2016, the year *L. delicatula* was confirmed in the area, but no other notable growth observations were made.

Discussion

Lycorma delicatula impact on *Ailanthus altissima*

We found evidence of *L. delicatula* reducing the annual growth of *A. altissima* at two field sites. Similar impacts on trees have been reported in related systems. Research in Mexico used dendrochronological methods and found that a phloem-feeding scale insect, *Stigmacoccus garmilleri* Foldi (Hemiptera: Stigmacoccidae), negatively affected the growth of oak trees as scale densities increased (37). Similarly, dendrochronological research has shown that *Tsuga canadensis* (L.) (Pinales: Pinaceae) exhibits a sharp reduction in growth immediately following infestation from the xylem feeder *Adelges tsugae* (Annand) (Hemiptera: Adelgidae) (38). Tree ring analysis has also shown that increasing densities of xylem-feeding periodical cicadas, *Magicicada* spp. Davis, can negatively affect the growth of many tree species (14, 31).

Not all observed variations for *A. altissima* RWI seen in the master chronology from the Upper Hanover Site (Figure 6A) can be attributed to *L. delicatula* feeding. This result is not surprising because many variables affect tree growth (30). For example, a suppression in *A. altissima* growth occurred prior to *L. delicatula* introduction, beginning in 2007 (Figure 6A). This reduction can likely be attributed to a severe drought that occurred during the summer and fall of 2007 in the mid-Atlantic region (39).

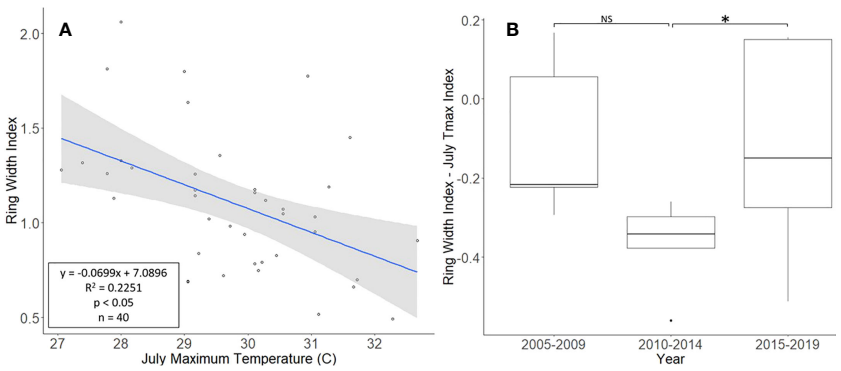

FIGURE 4
Impact of climate conditions and *Lycorma delicatula* on the ring width index for *Liriodendron tulipifera* in Upper Hanover Township, PA, USA. Regression analysis of *L. tulipifera* ring width index values and July's maximum temperature. **(A)**; and a comparison of *L tulipifera* ring width index values with the dominant climate variable removed in years before (i.e., 2005 to 2009 and 2010 to 2014) and after (2015 to 2019) the likely start of *L. delicatula* infestation **(B)**. NS, the difference between means not significantly different from zero; *, the difference between means significantly different from zero (p < 0.05).

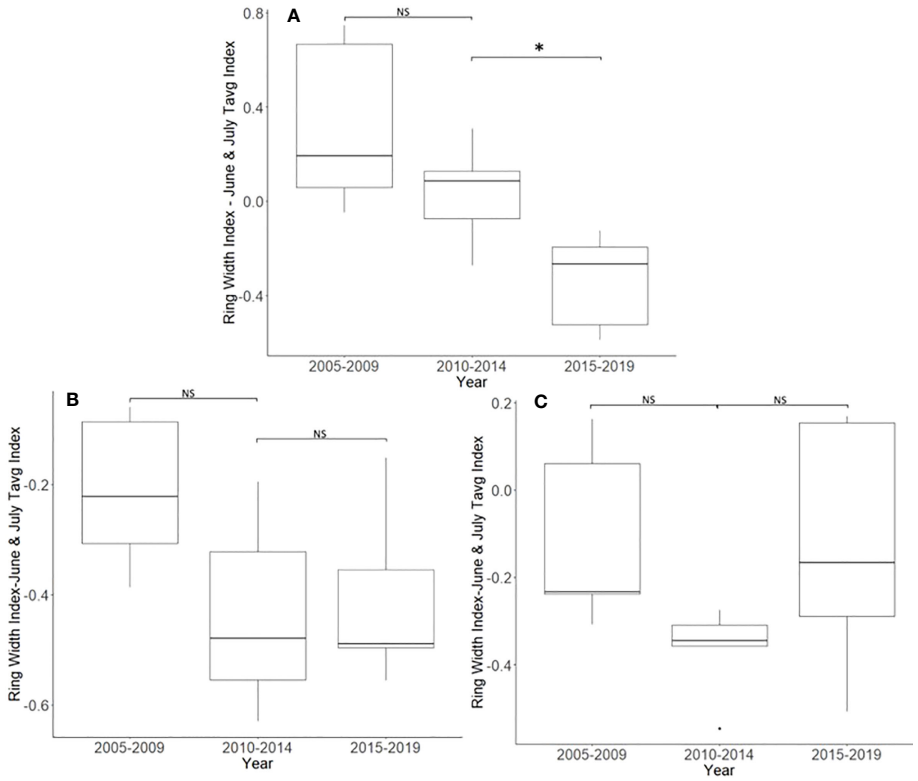

FIGURE 5
Impact of insecticide treatment and *Lycorma delicatula* on the ring width index with the dominant climate variable removed for *Ailanthus altissima* in Blue Marsh Recreation Area, Berks County, PA, USA. Trees had no insecticide treatment **(A)**, 1 year of treatment **(B)**, or 2 years of treatment **(C)**. Comparisons apply to years before (i.e., 2005 to 2009 and 2010 to 2014) and after (2015 to 2019) the likely start of *L. delicatula* infestations. NS, the difference between means not significantly different from zero; *, the difference between means significantly different from zero ($p < 0.05$).

This drought potentially caused a reduction in growth for the following several years as the trees recovered.

Treating *A. altissima* with the insecticide dinotefuran reduces the impact of *L. delicatula* on tree growth. We were therefore able to compare the radial growth of different *A. altissima* trees over the same time period and location with the only difference being heavy *L. delicatula* feeding influenced by insecticide treatment. In addition, in North America, there are very few arthropod enemies associated with *A. altissima* (40). *Atteva aurea* (Cramer) (Lepidoptera: Attevidae), the *Ailanthus* webworm, has been reported as a non-native herbivore to *A. altissima*, but severe damage has been documented only rarely on seedlings and young saplings (40). All trees sampled in this study measured greater than 25 cm DBH. Therefore, it is unlikely that an additional herbivore of *A. altissima* was responsible for the decreased growth observed in the untreated trees at Blue Marsh. This may indicate, where warranted, that treating high-value trees, such as timber, ornamental, or other economically valuable species, may help to reduce the impacts of *L. delicatula*. In some areas, *A. altissima* is a valuable tree and may benefit from protection. Other tree species, not studied here, may in the future be shown to also be sensitive to feeding by *L. delicatula* (41, 42).

In our experimental design, no trees uninfested with *L. delicatula* were treated with dinotefuran; in theory, the larger tree rings could have been due to the application itself. There have been cases where insecticides have elevated plant functions, including photosynthesis (43). In that study, which featured an evaluation of apple tree response to 33 insecticides, most had no effect on photosynthesis; 12 had an effect, but only two increased photosynthesis. No neonicotinoids were included in that study. However, there has been no evidence reported for elevated plant function by dinotefuran. In fact, this insecticide has been shown to have a negative effect on plant roots (44) and increases oxidative stress in plants (45). It is unlikely, then, that the dinotefuran application itself was responsible for the larger tree ring growth noted in dinotefuran-treated trees.

Our dendrochronological methods did not provide evidence of *L. delicatula* significantly reducing the growth of *J. nigra*. *J. nigra* had sample chronologies that correlated well with their master chronology in COFECHA, indicating that they were accurately dated (Table 1). Additional sampling may have discerned a significant difference; a downward trend was apparent. We did detect a significant reduction in the growth of *J. nigra* between the two preceding time periods before we presume *L. delicatula* was introduced. This growth suppression appears to have begun in 2010 (Figures 3B, 6B) and may be the result of a late-season drought affecting the sampling area. A similar decrease in growth can be seen in the master chronology of *A. altissima* from Upper Hanover (Figure 6A), but this did not appear to affect significance in the analysis of *A. altissima* cores. The reason for this phenomenon is

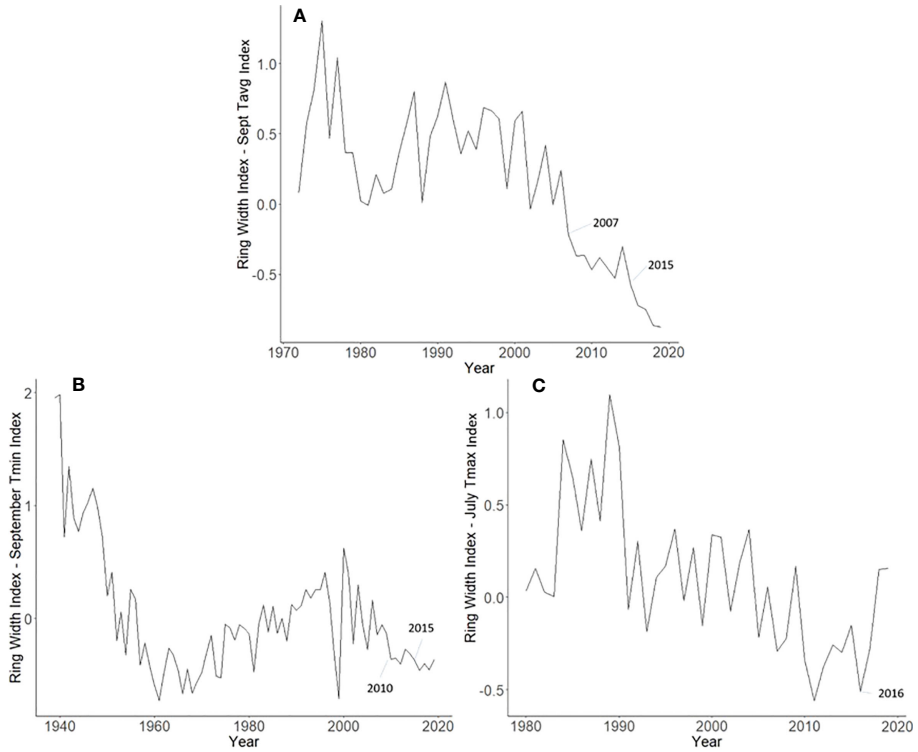

FIGURE 6
Master chronologies for *Ailanthus altissima* **(A)**, *Juglans nigra* **(B)**, and *Liriodendron tulipifera* **(C)**, showing the annual ring width index with the dominant climate variable removed over the life course of the sampled trees in Upper Hanover Township, PA, USA. *Ailanthus altissima* had suppressed growth in 2007 (likely because of a severe drought that year) and 2015 (potentially from *L. delicatula* feeding). *Juglans nigra* had suppressed growth in 2010, perhaps due to late-season drought. *Liriodendron tulipifera* had a substantial increase in growth rate in 2016, the year *L. delicatula* was confirmed in the area.

unclear and beyond the scope of this article; further research is needed.

Liriodendron tulipifera also had cores that correlated well with their master chronology in COFECHA (Table 1). Once the dominant climate factor of July's maximum temperature for *L. tulipifera* was removed, there was evidence suggesting a significant increase in growth occurred after *L. delicatula* invasion (Figure 4B). This phenomenon could be evidence that some tree species benefit from *L. delicatula* invasion. Yang (2004) tested a hypothesis where he looked at the effect of periodical cicada density on the growth of the American bellflower, *Campanulastru americanum* L. (Asterales: Campanulaceae) (46). He enriched the soil of American bellflowers with different densities of periodical cicada carcasses that resulted in bellflowers in the experimental group having larger seeds and leaves, and higher nitrogen concentrations in leaves than the control group (46).

The impact of *L. delicatula* on *J. nigra* and *L. tulipifera* may still be occurring, despite no impact being detected using our methods. For example, *L. tulipifera* is often less infested than *A. altissima*, and not considered a consistent primary host, whereas *J. nigra* is often seen as a primary host during the fourth instar and early adult life stages of *L. delicatula* (authors' observation). By contrast, *A. altissima* is frequently documented to host all *L. delicatula* life stages and fed on throughout the entire growing season. Reduced feeding durations on *L. tulipifera* and *J. nigra* may result in growth impacts not being detectable within just 5 years. A larger sample size that includes a diversity of different sites could help clarify if *L. delicatula* does impact growth in non-*A. altissima* tree hosts and ensure that we were not just looking at trees that had escaped herbivory. Furthermore, as *L. delicatula* is often found feeding in the canopies of trees, stem analysis of canopy branches may provide useful information in future studies (40).

Lastly, this difference in impact level between *A. altissima* and *L. tulipifera* and *J. nigra* could be explained by the large number of *A. altissima* at this Upper Hanover Site. *L. delicatula* feeding may have been focused on its preferred host *A. altissima*, to the exclusion of *L. tulipifera* and *J. nigra*, and the results of sampling a site without *A. altissima* may have indicated a significant feeding impact on *J. nigra* and *L. tulipifera*.

Conclusions

Dendrochronology can be used to identify and quantify long-term *L. delicatula* feeding injury to certain trees, such as *A. altissima*, as it has been used with other phloem feeders or invasive tree-feeding herbivores. We were unable to quantify any negative impact of feeding by *L. delicatula* on *J. nigra* or *L. tulipifera*. Either the radial growth of those species is not affected by *L. delicatula* feeding, or it may be that standard dendrochronology

methods may not be the most effective way of identifying a feeding signal and studying the long-term impacts for these tree species. It is possible that the use of quantitative wood anatomy and the hydrologic conductance measured by pore size could be used as a better measure of insect injury. It is also possible that these tree species are simply not as affected by *L. delicatula* feeding. Basal insecticide applications of dinotefuran appear to reduce and prevent damage to *A. altissima* trees that experience heavy feeding by *L. delicatula*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

AD performed, analyzed, and wrote the original version. DP and TK supervised research and advised student development. SS and TL participated in the design and analysis. KM assisted in analysis and writing. BW assisted in the field research carried out in Pennsylvania. JS assisted in core analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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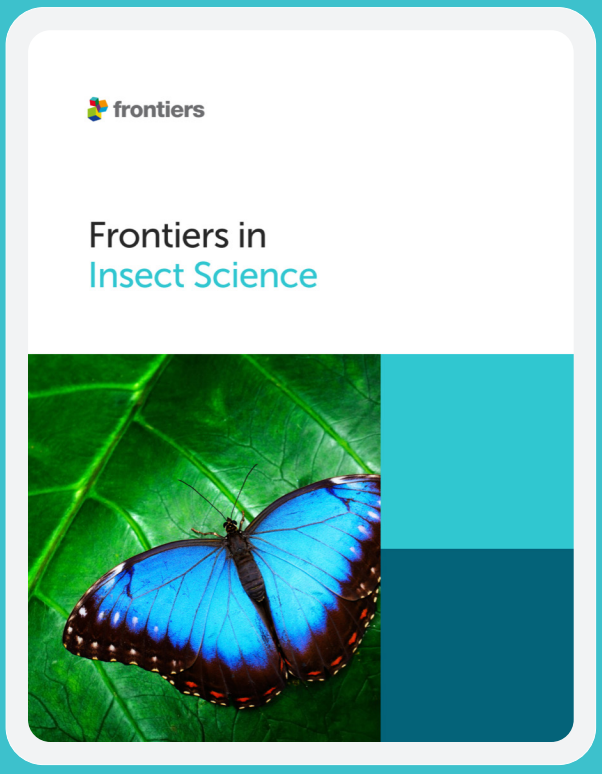

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