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Research

Sampling

A Method for a Long-Term Marking of Spotted Lanternfly (Hemiptera: Fulgoridae) Using a Stable Isotope of Nitrogen

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Abstract

Developing a lifelong marking method for *Lycorma delicatula* (White, 1845) is crucial to investigate ecological processes. Here we validate a marking method using stable isotope enrichment (^{15}N) of host plants to track spotted lanternfly (SLF), an invasive species causing economic damage on grapes, hardwood forest and landscape tree species. To validate this method, we first determined the isotope dosage to be sprayed on the host plants and subsequently detected in SLF. Second, we examined whether ^{15}N mark remains detectable from the nymphal to adult stage. We demonstrated that two stable isotope dosages applied to the host plants were assimilated by the insect and equally detectable in the exoskeleton, wings, and mature eggs ready to be oviposited. This safe and reliable method can be used to examine fundamental processes of the biology and ecology of SLF that range from dietary resources and resource allocation to food-web structure and dispersal patterns.

Key words: invasive species, ^{15}N, stable isotope, feeding, grape

Since 2014, spotted lanternfly *Lycorma delicatula* (Hemiptera: Fulgoridae) (White, 1845) has been a major invasive species in Pennsylvania. The expansion of the range of this invasive species into the mid-Atlantic states and beyond is an increasing concern (Barringer et al. 2015). SLF is native to China, India, and Vietnam, and was first discovered in the United States (Berks Co., PA) in September 2014. SLF is polyphagous, but to date, economic damage has only been documented on grapes and some hardwood forest and landscape tree species (Lee et al. 2019, Harper et al. 2019). The mid-Atlantic states of the United States have mounted an extensive management effort to limit SLF population expansion and to protect agricultural crops and ornamental plantings. However, being an invasive species, little is known about the movement, host preferences, and general ecological relationships in the United States; thus, designing management strategies has been a challenge (Lee et al. 2019).

There are numerous methods for marking insects, which include fluorescent powders and radioisotopes. The fluorescent powders only provide short-term marking, disappearing after the insect molts. Other methods like radioisotopes pose biosafety and environmental issues (Knight 1989, Meserve 2001). An optimal marking method should be long term, lasting throughout a lifecycle or more, not alter the physiology or behavior of the insects, or pose a threat to the environment (Hood-Nowotny and Knols 2007, Hyodo 2015). Some of these long-term marking tools compete with elements that naturally occur in the target and act as labels that can mark individual insects. For example, rubidium, which competes with calcium and is incorporated into body tissues, has been used in insects (Knight et al. 1989, 1990). Stable isotopes is another long-term marking tool that are incorporated into body tissues as their light forms, but are detectable using special laboratory analyses. Stable isotopes of carbon, nitrogen, and hydrogen have been used as tools to track numerous insect such as aphids (Nienstedt and Poehling 2000), coccinellids (Steffan et al. 2013), mayflies (Caudill 2003), dipterans (Hershey et al. 1993; Hood-Nowotny et al. 2006; Hamer et al. 2012, 2014; Faiman et al. 2019), and butterflies (Hobson et al. 2017). The use of stable isotopes to examine the physiology, behavior, and ecology of SLF has not been tested.

The stable isotope of nitrogen, ^{15}N, is a heavy form of the element, which is naturally present at low concentrations (0.36%). The natural abundance of ^{15}N may be used as a natural signature of populations (Hood-Nowotny and Knols 2007, Hyodo 2015), but another approach, using ^{15}N to enrich host plants generates a strong isotopic signature easily traceable in laboratory and field

studies (Hood-Nowotny and Knols 2007). Nitrogen isotopes can be delivered to plants in numerous ways such as foliar-spraying, soil-watering, or trunk injections. In the case of foliar-spraying isotope solutions, the nitrogen enters the plant through the epidermis, and the ^{15}N is used in metabolic functions and is found in tissues throughout the plant including the vascular system. Stable isotope spraying results in labeling all plant tissues with ^{15}N (Coradier 1990, Zapata et al. 2004). The stable isotope can be identified using isotope ratio mass spectrometry (IRMS), and the stable isotope ratios are expressed in delta (δ) notation [$\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ (‰ parts per thousands), where R_{sample} and $R_{standard}$ represent the ratios of ^{15}N to ^{14}N (Fry 2006)], or Atom percent (absolute number of atoms of an isotope in 100 atoms). Several enrichment studies of insects have used delta notation, for example Unsicker et al. (2005); Hood-Nowotny et al. (2006, 2016); Peters et al. (2012); Medeiros et al (2017); and Healy (2018).

This study describes a method for marking SLF using a stable isotope of nitrogen under shadehouse conditions. The goal was to validate the suitability of ^{15}N-enriched host plants for labeling SLF. This goal was addressed by determining the isotope dosage for labeling a host plant (grape, *Vitis vinifera*) in the field, and then feeding SLF nymphs and adults on labeled and unlabeled grapes to evaluate the persistence of the isotopic signature throughout the SLF life cycle.

Materials and Methods

Insects and Study Area

All experiments were carried out at the Pennsylvania State University, Berks Campus near Reading, PA in the United States (40.36 lat. -75.97 long.), a site that is within the SLF quarantine area. SLF has been present in this area since 2014. Spotted lanternfly nymphs were collected in Wyomissing, Berks County. Specimens were transported to the Berks Campus area using cages (32 × 32 × 60 cm white fabric, w/vinyl window; Bioquip, CA) and were fed on *Ailanthus altissima* (Mill.) Swingle (Sapindales: Simaroubaceae) leaves (a preferred host) for 2–24 h.

Experiment 1: Plant Culture and Labeling Procedure

The isotope ratios of grape plants enriched at three different doses of ^{15}N were measured under field conditions in July 2019, at the Pennsylvania State University, Berks Campus, PA. Grape plants (*Vitis vinifera* L. [Vitales: Vitaceae] var. Chardonnay 95/3309, approx. 90 cm height) were transplanted into 2-gallon pots with the soil substrate Lambert LM-16 Bark mix, watered every morning using a gravity system, and arranged in a randomized block design in an open field. Three ^{15}N doses (1.0, 0.5, 0.1 g/liter) were evaluated using three different plants per dose, and an unsprayed plant was used as a control. The isotope solutions (Ammonium-nitrate 98.9 atom%; Cambridge Isotope Laboratories Inc. MA) were prepared using distilled water to ensure the consistence of the treatment effects, and 0.0005 liter of Tween-20 (Sigma Aldrich. St. Louis, MO) to improve adhesion and contact to plant surface (Carlo et al. 2009). To apply the ^{15}N solutions, plants of each treatment group were isolated using three waterproof boards (2 × 2 m) placed on each side of the group of plants to avoid drift among the treatments. A hand-operated mister was used to spray 30 ml of solution per plant, and all leaves and trunk surfaces were moistened. In total, 12 grape plants were used in our experiment, three plants per isotope dose. Plants were sprayed every 3 d, eight times in total. Next, leaf and trunk tissue (one leaf and 15 mm length × 5 mm width × 3 mm thick stem per plant) were collected weekly during the spraying period to evaluate the ^{15} N uptake over time.

The samples were stored in paper envelopes, dried at 60°C for 72 h and ground into powder form. Ground samples (0.5–1.0 mg of each sample) were loaded into tin capsules (8 × 5 mm) for mass spectrometry to determine the stable isotope ratio. Analyses were performed at the Isotope Ratio Mass Spectrometry facility at Penn State University (University Park, PA) following the Werner et al. (1999) protocol using a Costech 4010 analyzer with helium as acarrier (100°C/min). N_2 was chromatographically separated. The N_2 peaks were routed to a Con Flo III which feeds the MAT 252 mass spectrometer along with reference peaks of the standard [atmospheric N_2 provided by the International Atomic Energy Agency, δN^{15} (‰) = 0.40, error was less than 0.15‰]. The peaks were integrated and δN^{15} values were calculated relative to the reference peak.

Experiment 2: ^{15}N Uptake Throughout SLF Life Cycle

Fifty second instar nymphs were released on a ^{15}N-enriched grape plant (1 g/liter isotope solution, following the same spraying procedure as in experiment 1). Once the individuals reached the fourth instar, half of the population was transferred to a new ^{15}N-enriched grape, and the other half was moved to a nonenriched plant. The individuals fed on the plants until they reached the adult stage and mated (about 40 d). Third and fourth instar nymphs, exuviae of each nymphal instar, and mated females were collected and frozen for a month. As in experiment 2, structural (whole exoskeleton, wings, and fatbody) and reproductive tissues (ovarioles and eggs) were isolated, dried, ground, weighed, and placed in tin capsules.

Experiment 3: Isotopically Labeling SLF Adults

In a shade-house experiment, the ^{15}N isotope uptake of SLF adults was determined through feeding trials using ^{15}N-enriched grape plants at different isotope doses (1.0, 0.5, 0.1, g/liter, three different plants per dose from experiment 1). Each plant was placed inside a mesh bag (tule fabric, 60 × 120 cm), then we released 30 SLF nymphs (fourth instar) and allowed them to feed and reach the adult stage (approximately 20 d). This experiment was replicated three times per isotope dose. Three SLF females from different plants were stored frozen at −20°C for a month. Then structural (wings and exoskeleton) and reproductive tissues (ovarioles of mated females) were isolated, dried, ground, weighed, and placed in tin capsules for spectrometry analysis.

Data Analysis

For all the experiments, normality (Shapiro–Wilks test) and homogeneity of variance (Levene's test) were tested. Because data sets were not suitable for parametric analysis and transformation did not normalize the residuals, nonparametric ANOVAs (Kruskal–Wallis) were used and followed by posthoc nonparametric pairwise comparisons with Wilcoxon tests. All statistical analyses were performed in JMP Pro 14 (SAS Institute Inc., Cary, NC) and R (v. 3.4.3., CRAN project).

Results

Experiment 1: Isotopically Labeling Host Plants

The $\delta^{15}N$ of leaves and stems of grape sprayed with the isotope solutions were significantly higher than the natural ^{15}N-content level (leaf: KW = 85.095, df = 3, $P < 0.0001$; trunk KW = 42.50, df = 3, $P < 0.0001$). The isotope values of the grape plants substantially increased over time in response to repeated applications. The $\delta^{15}N$ of plants enriched with 1.0 g/liter solutions was significantly

higher after two isotope applications (trunk KW = 10.45, df = 3, $P = 0.0149$; leaf KW = 10.38, df = 3, $P = 0.0156$). By the last application, trunk and leaves of ^{15}N- enriched plants at 1.0 g/liter solution were 478 and 281 times higher, respectively, than ^{15}N content of control plants (leaf $P = 0.0194$) (Fig. 1; Supp Fig. 1 [online only]). The δ^{15}N of plants sprayed with isotope solution at 0.5 g/liter were also significantly higher relative to control plants. However, plants sprayed with isotope solutions at 0.1g/liter were not significantly different from control plants.

Experiment 2: ^{15}N Uptake Throughout SLF Life Cycle

All SLF individuals that fed on ^{15}N-enriched plants assimilated the stable isotope, and the isotope's detectability persisted throughout all developmental stages (Fig. 2A and B; Supp Fig. 2 [online only]). The δ^{15}N of SLF kept on enriched plants were significantly higher than the controls, and these differences were observed even in exuviae where the δ^{15}N was seven times higher (Fig. 2A). The isotope signature in adults was significantly higher in individuals kept on ^{15}N-enriched plants compared to controls (KW = 6.48, df = 2, $P = 0.0390$). Switching SLF fourth instars individuals from ^{15}N-enriched plants to unsprayed plants did not affect the isotope signature in the structural and reproductive tissues (Fig. 2B).

Experiment 3: Isotopically Labeling SLF Adults

The δ^{15}N of structural and reproductive tissues of females increased with increasing isotope solution concentration (Fig. 3; Supp Fig. 3 [online only]). The ^{15}N was found in all tissues of adults kept on enriched plants. The δ^{15}N of females kept on plants sprayed with isotope solutions at 0.5 and 1.0 g/liter were significantly higher than controls. For example, the δ^{15}N was, on average, 105 times higher than control females (Fig. 3) in insects kept on enriched plants at 1.0 g/liter isotope solution, but differences were not significant among all tissues of adults kept on control and enriched plant with solutions at 0.1 g/liter.

Discussion

This study demonstrated that ^{15}N-host plant enrichment enables SLF lifelong marking. All SLF fed on ^{15}N-enriched plants assimilated the stable isotope, and it persisted through the insect development. The δ^{15}N of SLF kept on enriched plants were significantly higher from that of the controls; these differences were observed even in exuviae. Switching from ^{15}N-enriched plants to unsprayed plants did not affect the persistence of isotope marking in the SLF structural and reproductive tissues. Interestingly, δ^{15}N did not significantly vary between SLF tissues of individuals switched to unlabeled plants and those kept on enriched plants, but both significantly higher from the control. The stable isotope did not affect SLF metamorphosis and was incorporated into the wings, exoskeleton, fat body, ovarioles, and eggs.

Enriched host plants successfully delivered a persistent label to nymphs and adults of SLF. The host plant rapidly assimilated the stable isotope, the δ^{15}N of leaves and trunk were significantly higher than the control in every plant sprayed with isotope solutions at 0.5 and 1.0 g/liter. Early^{15}N uptake may be due to young leaves rapidly assimilating nitrogen (Bowman and Paul 1992). Grape trunks showed higher δ^{15}N than leaves, which might be explained by nitrogen remobilization and storage after blooming (Below et al. 1985, Deléens et al. 1994). The last aspect may become advantageous for SLF marking since adults predominantly feed on host plant trunks.

Nymphs and adults of SLF were labeled by the stable isotope even over short periods of exposure to enriched plants (15–20 d). Furthermore, the high δ^{15}N of structural body parts such as wings of marked-SLF that were transferred to unlabeled host plants (Fig. 2) indicate low isotope dilution. A previous study showed that isotope turnover of structural tissue was lower than in metabolically active thoracic or abdominal tissues (Tibbets et al. 2008). Therefore, wings may be of particular interest for SLF mark-recapture studies.

The advantages of labeling SLF through host plant-enrichment are: 1) the method is metabolically benign allowing insects to have the necessary nutritional intake to grow and complete their life cycle; 2) the spray application system is easy to perform; and 3) the isotope creates a robust label in both plants and SLF, making ^{15}N an ideal tool for field studies, since^{15}N has a slow turnover in grape plants and did not affect the development or behavior of the insects. Previous studies showed that grapes still presented 46% of the ^{15}N after 10 mo (Coradier 1990). This affordable method required

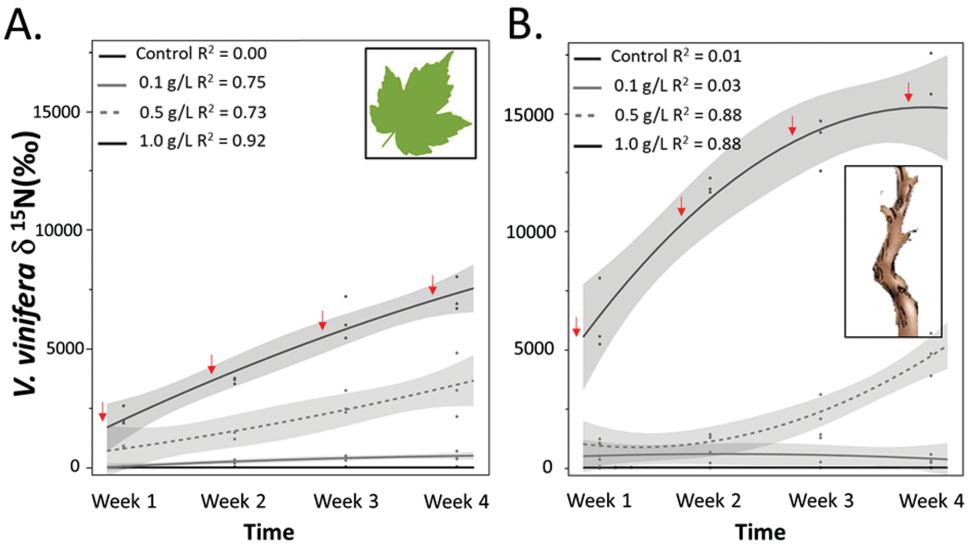

Fig. 1. Changes of *V. vinifera* δ^{15}N (‰) over time (weeks) since the start of isotope solution spray applications at 0.1, 0.5, and 1.0 g/liter. (A) Leaf (entire leaves). (B) Trunk (sample included phloem tissue). Lines are the best-fit quadratic regressions (bands are the 95% CI, $n = 3$). Arrows in (A) and (B) indicate the time of the isotope solution application.

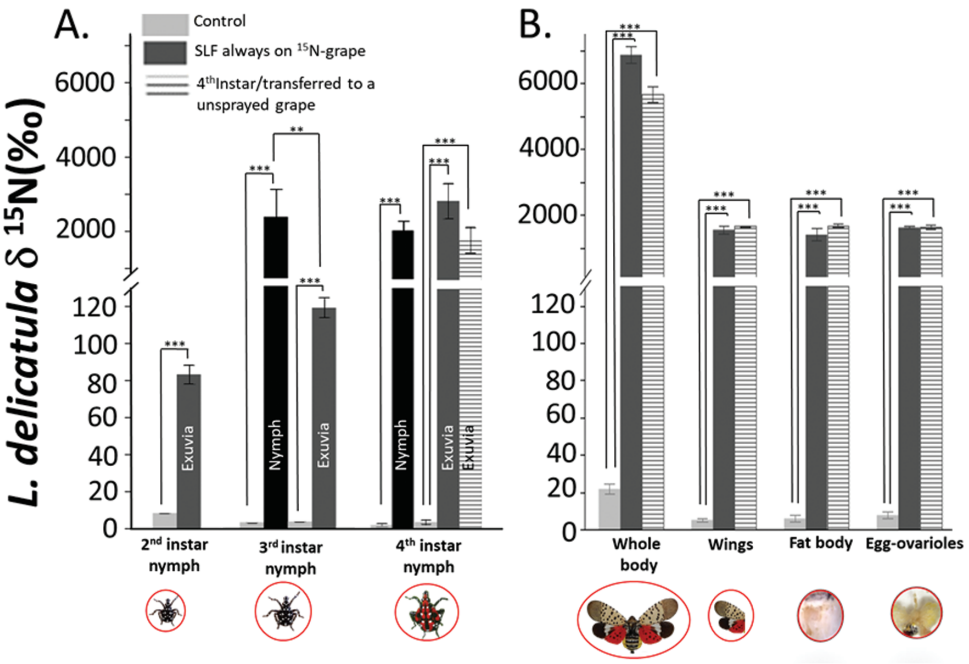

Fig. 2. δ^{15}N (‰) in different developmental stages of *L. delicatula* fed on ^{15}N-enriched grape plants (1.0 g/liter). (A) δ^{15}N (‰) of second, third, and fourth instar nymphs and exuviae of *L. delicatula*. (B) δ^{15}N (‰) of structural and reproductive tissue of females. Individuals were collected a week after they reached a different developmental stage (multiple comparisons, $P = 0.0156$, mean ± SE, $n = 3$).

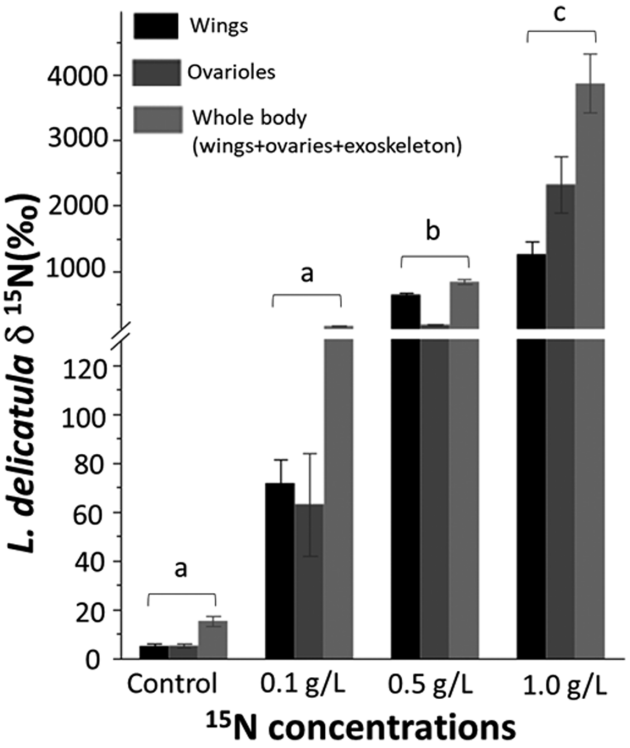

Fig. 3. δ^{15}N (‰) in different tissues of *L. delicatula* feeding on ^{15}N-enriched grape with three stable isotope solutions at 0.1, 0.5, and 1.0 g/liter. Females were collected after 15 d of continuous feeding on grapes (multiple comparisons, $P = 0.0156$, mean ± SE, $n = 3$).

minimal infrastructure for sample preparation, and samples analysis is inexpensive ($6–10 each sample) compared to molecular methods (Hood-Nowotny and Knols 2007). The stable isotope of nitrogen can be used to examine different aspects of the biology and behavioral ecology of SLF, such as host plant preference, nutrient assimilation, metabolic rates, interspecific interactions, and long-distance dispersal patterns.

To produce an effective ^{15}N-mark on SLF, we suggest spraying solutions at concentrations between 0.5 and 1.0 g/liter with a drop of Tween-20 to increase the adhesion of the isotope to the plant epidermis. The isotope solution may be sprayed every other day, at least three times for solutions at 1.0g/liter or six times for low isotope concentration (<0.5g/liter). Finally, plant and SLF samples can be preserved in glass containers stored at –20 or –80°C until isotope analysis. Preserving samples in plastic bags or containers should be avoided because plastic can negatively affect the isotopic signature of the samples (Fraser 2008).

Although, we were not able to test eggs after oviposition, we found high levels of ^{15}N in preoviposition eggs in mated females, which suggests that ^{15}N may be found in oviposited egg masses or even F1 individuals that may potentially be used as SLF tracers for field studies. Further research on ^{15}N-enrichment for SLF mark-recapture studies in the field such as studying differences of feeding preference, feeding behavior, food assimilation, and allocation. To study dispersal patterns, stable isotope enrichment should combine the labeling method (host plant injection and/or spraying) and the development of a two-source mixing model to infer the number of marked SLF in a mixture of marked and unmarked individuals. This method, together with the sampling method (i.e., concentric bands around the enriched SLF host plants or SLF hot spot) will help to track the marked population over long distances such as hundreds or thousands of meters.

In conclusion, to our knowledge, this study is the first to develop a long-term marking method using a stable isotope for SLF. These results revealed that structural and reproductive tissues of SLF incorporate ^{15}N, and especially wings have a strong isotopic signature that indicates its potential as a tracer for field studies. We also demonstrated ^{15}N assimilation by the host plant at every time

point of foliar spraying. Finally, this study provides evidence that the ^{15}N-enrichment of host plants is an efficient, affordable, and reliable system for labeling SLF.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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