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METHOD

Evaluation of sample collection and storage protocols for surface eDNA surveys of an invasive terrestrial insect

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Abstract

Environmental DNA surveys have revolutionized monitoring of rare or cryptic species and species inhabiting areas where conventional sampling is difficult or dangerous. Recent advancements within terrestrial environments include the capture of eDNA deposited by animals on surfaces such as tree bark and foliage, hereafter "surface eDNA." Notably, a technique which uses commercial paint rollers to aggregate surface eDNA has been deployed with success to detect the presence of forest insect pests providing a potentially powerful new management tool. However, before widespread adoption is feasible, the efficiency and logistics of roller sample collection and study design, especially relative to realistic survey conditions, must be evaluated. We compared the performance of two DNA preservation treatments—cold and ethanol—on their ability to reduce the loss of captured eDNA on rollers over time. Additionally, we evaluated how the detection probability of our target species, the spotted lanternfly (*Lycorma delicatula*), varied with sampling effort (time spent rolling per sample) and the initial quantity of eDNA present. Finally, we evaluated how the number of trees sampled per roller influenced the final concentrations of lanternfly eDNA remaining on the roller. We found storing rollers with ethanol or cold temperatures resulted in 3–10-fold greater concentrations of experimentally controlled eDNA relative to no treatment after 24 h. Detection probability declined as the amount of lanternfly eDNA decreased, but did not change in response to sampling effort over sample time (10–80 s/tree). Finally, recovered lanternfly eDNA decreased as more trees were sampled by a single roller—a 91% reduction after 7 trees—potentially due to captured DNA being transferred back from the roller onto the bark. Our results provide improved guidance for deploying roller surface eDNA methods for spotted lanternfly surveys, and for invasive insect pest surveillance and monitoring programs generally.

KEYWORDS
biosecurity, detection probability, early detection, invasive species, *Lycorma delicatula*, terrestrial sampling

1 | INTRODUCTION

Non-native damaging pests, like invasive insects, cost billions of dollars annually in lost annual yields, threaten ecologically important tree species, and disrupt the delivery of key ecosystem services (Bradshaw et al., 2016). These pests reach new geographic regions due to the high volume of international trade and become established from failed intercept or detection efforts (Tobin et al., 2014). Once established, these pests can quickly increase in number reaching levels that are damaging and become difficult eradicate (Tobin et al., 2014). Detecting initial incursions of harmful pest insects, establishing the spatial extent of their initial populations, and tracking their subsequent geographical spread are all critical to reducing these the ecological and economic impacts (Martinez et al., 2020; NISC, 2016; Tobin et al., 2014). Due to these high environmental and economic costs, any survey tool that can significantly increases the detection rates of invasive pests is critical (Epanchin-Niell, Haight, Berec, Kean, & Liebhold, 2012; Tobin et al., 2014). The principle hurdle to detecting pest insect incursions is the low power of conventional surveys to confirm the presence of a target species when spatially rare and at low abundance (Epanchin-Niell & Hastings, 2010; Epanchin-Niell et al., 2012; Tobin et al., 2014). Sampling efforts based on environmental DNA (eDNA) may offer substantial benefits in this context as they have been repeatedly shown to increase detection probability for rare populations (Darling & Blum, 2007; Jerde, Mahon, Chadderton, & Lodge, 2011), including for detecting low-abundance terrestrial insect pest populations (Allen et al., 2021; Valentin et al., 2018; Valentin et al., 2020). Valentin et al. (2020) introduced novel eDNA survey approaches for terrestrial pest insects, including the "roller" method—the use of commercial paint rollers to sample eDNA from tree surfaces—representing a potential breakthrough for use in forest health monitoring (Valentin, Kyle, Allen, Welbourne, & Lockwood, 2021). However, using the roller method in forests requires exploration in regard to sampling design, eDNA capture rates, and detection probability. In particular, adoption of this method will depend on whether sampling designs can address logistical hurdles associated with surveying in remote forested ecosystems. Here, we conduct a series of experiments that inform the design and interpretation of roller surface eDNA surveys in the context of forest insect pest surveillance and monitoring.

Based on forensic DNA techniques (Verdon, Mitchell, & van Oorschot, 2014), the roller method of Valentin et al. (2020) targets DNA that is deposited by insects such as exuvia, salvia, excrement, or other material onto the trunk or branches of trees. The roller, a cylinder of woven synthetic fabric commercially available as a paint applicator, is attached to a pole, dampened with water, and rolled over the surface of a tree, acting in a similar fashion as a forensic DNA swab (Valentin et al., 2020). The roller is then bathed in water, moving the eDNA into solution, after which standard filtering and preservation methods are applied. Valentin et al. (2021) showed that terrestrial insect DNA can be recovered from dry surfaces and amplified using qPCR for up to a week after deposition, but rarely beyond this time period. Thus, any eDNA collected by the roller likely indicates recent activity of the pest insect on, or very near, the surveyed trees. Preliminary field trials using the roller method revealed that, coupled with a sensitive qPCR assay, it readily detected target insect eDNA where conventional visual detection methods failed (Valentin et al., 2020). Yet, rigorous, quantitative field trials are needed and remain lacking.

Successful eDNA-based sampling designs should maximize detection probability, via maximizing eDNA capture and persistence, while also minimizing survey costs and simplifying logistics (Hinlo, Gleeson, Lintermans, & Furlan, 2017). The adoption of emerging techniques such as roller eDNA sampling, therefore, not only requires an understanding of per-sample detection rates, but also how the range of possible survey design options will affect these rates as well as any tradeoffs in terms of cost and logistical complexity. For example, using the roller method to conduct large-scale monitoring efforts of forest pests may require sampling in remote locations where transporting heavy or bulky equipment and supplies used to process samples (e.g., water, peristaltic pumps, and rollers; Valentin et al., 2020) could be difficult or prohibitively expensive. An alternative solution is to collect the samples and then transport the rollers to a laboratory for filtering and other processing, thereby reducing the amount of field equipment and the time required to collect samples. However, this solution prolongs the time between initial eDNA transfer onto the rollers and the eventual filtering and preservation of the eDNA for later PCR-based detection in the laboratory. In aquatic systems, increased time between sample collection and DNA extraction negatively influences DNA detection probability (Barnes et al., 2014; Strickler Fremier, & Goldberg, 2015) and is commonly addressed in eDNA studies using cold storage or chemical preservation methods (Minamoto, Naka, Moji, & Maruyama, 2016). In a terrestrial system, it is presently unknown how long eDNA may persist on rollers and how best to slow degradation rates. Identifying a storage and transport protocol for rollers that minimizes survey false-negative errors would increase the efficiency and feasibility of the method by making it possible to forego filtering samples in the field.

Sampling effort is also key to optimizing eDNA survey design. For example, in aquatic surveys, the sample volume and the number of samples per site are common considerations (Goldberg et al., 2018; Rees et al., 2014). As a newer method, roller sampling needs to address unique questions including survey effort, specifically how much time is needed to be spent rolling per sample (e.g., time spent per tree), and how much total surface area (e.g., number of trees) is optimal to sample per roller. Time spent rolling could influence detection probability by affecting the probability of a roller contacting DNA on a substrate, the DNA transfer efficiency, or the fraction of DNA that transfers from the substrate onto the roller. The total surface area sampled could affect detection probability in two ways. First, oversampling, or including too much surface area with a single roller, may reduce the total amount of captured eDNA on the roller via back-transfer from the

roller onto the surface of substrates sampled after the initial encounter. Second, the likelihood of a DNA detection could be reduced from the build-up of PCR inhibiting materials on the roller. Both of these factors would have the effect of reducing detection probability.

We explore these issues using laboratory and field experiments centered around the use of rolling tree surfaces for detecting the DNA of the spotted lanternfly (*Lycorma delicatula* White; Hemiptera: Fulgoridae; SLF), a rapidly spreading invasive pest in the USA and eastern Asia (Urban, 2020). The roller eDNA survey method was initially developed for this species, and related management efforts and logistical survey constraints, in mind (Valentin et al., 2020). To better inform integration of roller eDNA surveys into early detection efforts for this species, we investigate: (1) how best to preserve SLF eDNA transferred onto rollers until the DNA can be suspended in water and the water filtered (Experiment 1); (2) how the amount of time spent sampling per tree and the amount of SLF eDNA present on tree surfaces affects per-sample detection probability (Experiment 2); and (3) the optimal number of trees to sample with one roller to maximize the concentration of SLF eDNA found in each sample (Experiments 3 and 4).

2 | METHODS

2.1 | Study species and honeydew collection

The spotted lanternfly is a phloem-sucking insect that feeds on woody plants and excretes copious amounts of a sugary solution termed "honeydew." Originally from China, India, and Vietnam, this pest invaded forests and vineyards in South Korea and Japan where its status as a global threat to agriculture and forestry was recognized (Han et al., 2008; Kim et al., 2013). In 2014, it established as an invasive pest in the United States and now threatens US$915 million of grapes and tree fruits and over US$5 billion worth of timber in its current US distribution, including Pennsylvania and neighboring states (PCNR, 2020; Urban, 2020; USDA-NASS, 2020). This distribution in the United States is rapidly expanding and is likely to increase substantially in extent in the future (Cook et al., 2021). Early detection efforts are seen as key to preventing its spread into especially vulnerable areas such as the wine-growing regions of New York, California, Australia, and South America (Urban, 2020).

We collected several ml of SLF honeydew in 1.5 ml tubes from wild individuals at Rutgers Snyder Research Farm (New Jersey, USA) in late September to early October 2020 that were feeding on red maple (*Acer rubrum*) and grape vines (*Vitus* spp.). This time of year was chosen because adult SLF females are abundant and are producing more honeydew due to increased feeding in preparation for egg laying. Our methods followed Valentin et al. (2020) and involved gently squeezing the abdomens of adult SLF and collecting the resulting excreted honeydew directly into 1.5 ml centrifuge tubes. Tubes were stored in a −20°C freezer until thawed and used within experiments.

2.2 | Roller method protocols

Two to four days before each field experiment, all rollers (15 cm long × ~3 cm diameter; synthetic woven fabric with 0.6 cm nap) were sterilized using a 10% bleach solution, quadruple-rinsed in deionized (DI) water, and wrung of excess water. Until the rollers were used in the field, we stored them in 1.75 L high-density polyethylene buckets with lids attached, which were sterilized in the same manner as rollers. Once in the field, we took a sterilized roller from a bucket with a gloved hand and attached it to a flame-sterilized (Valentin et al., 2020), conducted to reduce contamination, commercially available metal paint roller handle mounted to a pole. We then placed the roller onto the surface of the tree and moved it across these surfaces using consistent, gentle pressure (Valentin et al., 2020). A single roller can be used to sample one tree, or it can be used across many trees via the surveyor walking to another tree and repeating the surface rolling technique. After one or more trees were sampled (depending on experimental protocol, see below), we removed the roller by placing a 304 × 114 mm sterile plastic bag (Wards, Rochester, New York, USA) over the roller and pulling it off the roller handle. These bagged rollers were then be stored and transported to the laboratory for eDNA transfer and filtering, or they were field-processed leaving the resultant filter(s) to be stored and transported to the laboratory (see below).

Whether executed in the field or laboratory, we removed eDNA captured by the roller and placed it into solution by adding 100 ml of DI water to the sterile bag, partially submerging the roller (approximately half submerged). We shook the sealed bag with DI water and roller for approximately 30 s to mix the gathered material (i.e., eDNA and other plant matter) into the water solution. Next, we massaged the roller in the bag to further dislodge any captured eDNA and suspect it in the water. We then removed the roller from the bag and filtered this water using 10-micron × 47 mm (polycarbonate track-etched) filters housed in reusable plastic filter holders (Whatman Swin-Lok, Cytiva, Marlborough, Massachusetts, USA) using silicone tubing and a Pegasus Alexis peristaltic pump (Proactive Environmental Products, Bradenton, Florida, USA; Valentin et al., 2020, 2021). Prior to filtering, the filter assemblies were sterilized and quadrupled rinsed with DI water as described for roller sterilization (above) in the laboratory and stored in sterile buckets with lids to prevent contamination. We filtered as much of the 100 ml water from each sterile bag as possible until clogging of the filter (no less than 40–50 ml/sample). We used flame-sterilized forceps to transfer filters into sterile 1.5 ml centrifuge tubes. If we did not immediately extract DNA from a filter, it was preserved within the tube at −20°C or, in the case of Experiment 2, using 100% non-denatured ethanol for one day.

Following Valentin et al. (2020, 2021), we used the HotSHOT method (Truett et al., 2000) to extract eDNA from filters. If filters were frozen, we thawed them at room temperature (~23°C) no more than 2 weeks after initial sampling. For ethanol preserved samples, we evaporated off the ethanol in a vacuum centrifuge for two to three hours. For all filters, we pipetted 75 µl of Lysis buffer into

1.5 ml tubes, and the pipette tip was used to fully submerge the filter in solution. The samples were then placed in a dry bath for 30 min at 95°C. We then pipetted 75 µl of neutralization buffer into the sample tube. We included one extraction negative control in a tube with each extraction batch to test for in-lab contamination with the same, previously described extraction process minus the filter.

2.3 | Real-time PCR analysis

Samples were tested for the presence of SLF eDNA using a TaqMan genetic assay on a quantitative real-time PCR system (StepOnePlus™, Applied Biosystems, Foster City, California, USA). This assay uses a 63 base-pair segment within the ribosomal gene ITS1 and is highly specific and sensitive to SLF DNA (Valentin et al., 2020).

We performed all qPCR laboratory preparations in a dedicated "low-copy" room and within a UV-sterilized positive-flow hood (AirClean 600 PCR Workstation, AirClean Systems). Each 96-well plate included a five-step 1:10 serial dilution of genomic DNA extracted from SLF leg tissue (DNeasy Blood and Tissue Kit, Qiagen) to create a standard curve. The standard curve samples, a negative (no template DNA) control, and all field samples were run with three technical replicates per sample. Reactions consisted of 500 nM of each primer, 250 nM of probe, 1x TaqMan® Environmental Mastermix II with no UNG, 2 µl of extracted template, and 5.5 µl nuclease-free water to achieve a total reaction volume of 20 µl/well. The optimized PCR reaction included an initial denaturing step of 96°C for 10 min, then 45 cycles of denaturing at 96°C for 15 s and annealing and extension at 60°C for 1 min (Valentin et al., 2020). A Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Carlsbad, California, USA) was used to quantify the amount of DNA in the most concentrated dilution (1/10; range: 0.669–0.896 ng/µl among experiments). In all of our experiments, we estimated eDNA sample concentrations per reaction based on the standard curves derived from the serial dilutions described above (mean efficiencies = 89.7%, range: 82.7–100%; mean R^2 = 0.983, range: 0.968–0.993).

For our experiments, we "spiked" surfaces (rollers or tree surfaces) with diluted SLF honeydew. We estimated that a single adult SLF is likely to excrete 5 µl of honeydew per hour by assuming an average adult mass of 2.47 mg (Johnson & Strong, 2000), and then, estimating that an adult of this size will excrete 500 µg of honeydew per hour. The latter calculation was based on an allometric relationship from the closely related family Delphacidae (Moir, Renton, Hoffmann, Leng, & Lach, 2018; their Figure 1). We used this SLF honeydew excretion rate to benchmark how much DNA very low densities of SLF will deposit in a single location. Thus, our very low concentration of SLF DNA roughly represents a single adult excreting honeydew for an hour within a single tree. We consider the ability of an any eDNA survey to consistently detect the short (1 h) presence of a single feeding SLF adult in a tree to be consistent with its uses within pest insect early detection and rapid response programs.

2.3.1 | Experiment 1: Degradation and preservation

Within realistic field deployment contexts (e.g., remote settings), it may not be logistically feasible to take rollers that have been used to collect surface eDNA, suspend them in DI water, filter this water using

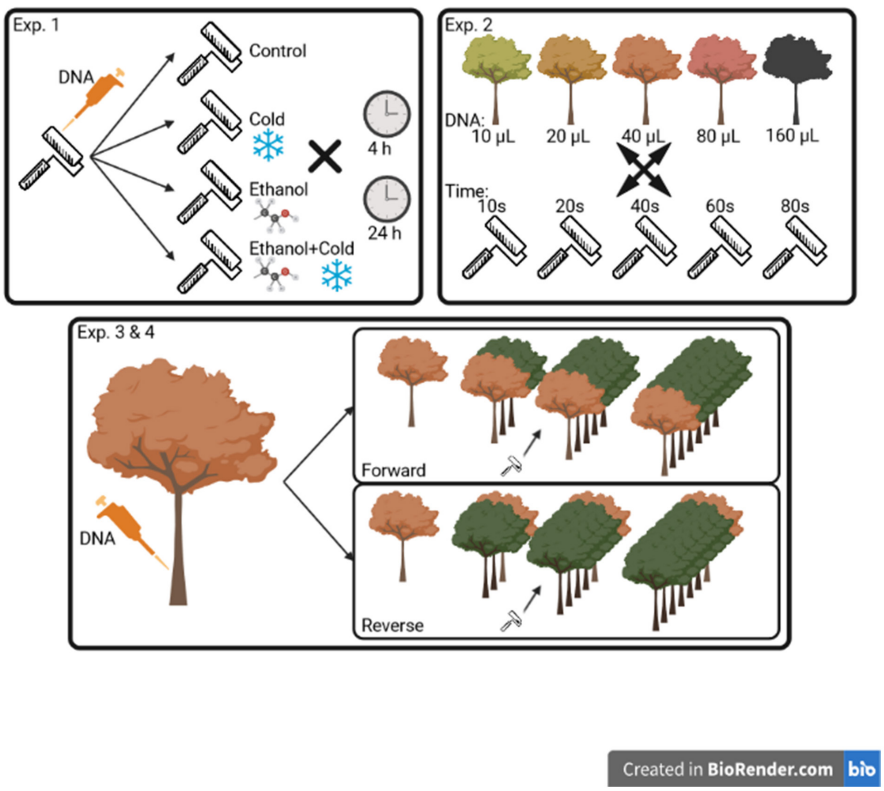

FIGURE 1 General schematic of studies conducted: Experiment 1, degradation of insect DNA on paint rollers with no treatment, cold (ice), ethanol spray, and ethanol spray and cold over four and 24 h; Experiment 2, detection, and non-detection of insect DNA placed onto trees with combinations of five volumes (DNA) and five time limits (s) to rolling; Experiment 3 and 4, recovery of insect DNA by rolling one, three, five, or seven trees with either the initial (forward) or final (reverse) tree spiked with DNA

a pump, and preserve resultant filters for transfer to a laboratory in ethanol. Thus, our first experiment explored the efficacy of storing rollers within sterile bags in conditions that will slow the rate of DNA degradation (Figure 1). We selected two methods of preservation, ethanol, and cold storage, as they are practical to use in the field and are used in other eDNA survey protocols (Goldberg, Sepulveda, Ray, Baumgardt, & Waits, 2013; Minamoto, Naka, Moji, & Maruyama, 2016; Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Renshaw et al., 2015; Spens et al., 2017; Strickler et al., 2015). Cold temperature storage was achieved using a commercially available cooler filled with a bottom layer of ice. For ethanol preservation, we pump sprayed 100% non-denatured ethanol onto each roller using a hand spray nozzle (12 sprays per roller). We selected time points between initial sample collection and rinsing-filtration to reflect likely scenarios in field applications. These time points include (1) time zero, where processing of rollers can happen immediately after sampling in the field; (2) 4 h, where roller processing cannot happen until after they are transported from a field site to a laboratory or a site with access to DI water/electricity; and (3) 24 h, where overnight travel between the field site and a laboratory, or overnight shipping of rollers, are required.

We applied 10 μl of 1/5 dilution of SLF honeydew on 90 rollers total. Ten rollers were immediately rinsed in water within the bag (time zero). The rest of the rollers (n = 80; 10 per time period per treatment) were stored for either four or 24 hours with no treatment (room temperature ~23°C), placed in cold storage (with ice in a cooler), misted with ethanol, or cold storage and an ethanol misting. In this way, we evaluated the influence of cold storage of rollers and application of ethanol to rollers, independently and combined, across time. We used the DNA concentration recovered from each roller as the dependent variable and a two-way ANOVA to test for significant effects of treatment, time, and their interaction. DNA concentration data were log transformed to improve normality.

2.3.2 | Experiment 2: DNA density and per-tree sampling effort

Experiment 2 was designed to inform the per-tree sampling protocol, where the time spent rolling any single tree should be set to maximize detection probability while minimizing effort (Figure 1). We expected maximum detection probability per unit of time sampling to be influenced by both the time spent rolling each tree and the amount of DNA available on the tree surface (honeydew density), which would correspond to the density of SLF individuals. This experiment (10 March 2021), along with Experiments 3 and 4, was conducted at Rutgers Fruit and Ornamental Research Extension Center in Cream Ridge (Monmouth County), New Jersey, USA. These three field experiments were conducted over winter months when SLF were not present, and in years when SLF was yet to be detected at the site. Any potential background DNA from SLF that may have been present from 2020 would have degraded and been washed away by the time of our study (Valentin et al., 2021). We selected peach trees (*Prunus persica* L.) for this experiment due to their smooth, relatively consistent bark texture and even size distribution (~25–40 cm in diameter at soil line, ~4–5 m in height). Standardizing bark texture and tree size removed two variables that likely influence the probability with which a roller encounters and transfers SLF DNA and, thus, detection probability.

For this experiment, we applied 10, 20, 40, 80, or 160 μl of 1/10 dilution of SLF honeydew to the bark surface of individual trees (replicates) and a surveyor actively rolled the upper and side areas of branches between 0.5–2 m high on the trees for 10, 20, 40, 60, or 80 seconds each. We used a fully crossed regression design, with 3 replicates of each DNA density and effort treatment combination for a total of 75 replicates. To prevent sampling bias and contamination, one surveyor spiked trees with SLF honeydew by aliquoting the 10–160 μl in fourths (2.5–40 μl) to four random locations on the bark of a peach tree. The specific locations were unknown to the surveyor that rolled the trees. Honeydew was only applied to the upper surface of branches, where honeydew excreted by SLF would naturally accumulate. After the honeydew was applied, a second surveyor would roll as much of the bark surface area as possible for between 10 and 80 s depending on treatment, with duration of sampling initiated and halted by the initial surveyor who served as timekeeper. The second surveyor removed the roller from the pole as previously described. A third and fourth surveyor then added water to the sterile bag, filtered the water, and using flame-sterilized forceps, placed the filter into a sterile 1.5 ml microcentrifuge tube containing 1 ml of ethanol (100%, non-denatured) for preservation and transport to the eDNA processing laboratory. We collected equipment negatives that were rollers that we processed as described above, except for being exposed to tree treatments/honeydew. Three field negatives were collected by haphazardly rolling peach trees throughout the orchard to test for background honeydew (contamination), and before the honeydew was deposited onto experiment trees. We spiked clean rollers in the field with 10 μl of SLF diluted honeydew as a field positive. At the laboratory, we analyzed each sample using qPCR as described above, recording SLF as "detected" in a sample if one or more of the three technical replicates showed amplification, and "not detected" otherwise.

We evaluated the effects of SLF honeydew density and per-tree sampling effort on detection probability using a generalized linear model with a logistic link function. The dependent variable was the binary detection or non-detection data (see above), treated as outcomes of a Bernoulli distribution with the parameter p, or the probability of detecting the presence of the SLF DNA that we placed on the trees. We modeled p as a function of both density and per-tree sampling effort as follows, with parameters estimated using maximum likelihood (function "glm"; R Core Team, 2020):

$$logit(p) = \text{intercept} + \beta_1(\text{density}) + \beta_2(\text{effort})$$

2.3.3 | Experiments 3 and 4: Number of trees to sample

To test the influence of the number of trees sampled per roller on eDNA concentrations captured, we conducted two field experiments

at the Rutgers Fruit and Ornamental Research Extension Center. For each experiment, we set up 24 plots of either one, three, five, or seven peach trees. On an individual tree in each plot, we pipetted two 10 μl aliquots of a 1/5 dilution of SLF honeydew on the upper surface of the lower branches.

In the first experiment (18 November 2020, 2–3°C ambient temperature), the "forward" experiment, the tree with honeydew applied to the bark was sampled first and then a subset of the remaining (up to six) trees, without DNA, in that set were sampled using the same roller (Figure 1). There were four treatments in this experiment; (1) only the initial tree with SLF honeydew was sampled, (2) the initial tree with SLF honeydew was sampled followed by two more trees using the same roller (3) the initial tree plus four more trees with the same roller, and (4) the initial tree plus six more trees with the same roller. For each tree, we sampled the same 0.5–2.0 m of tree trunk as previously described for 25–30 s. This design allowed us to test whether, after the roller initially encountered SLF eDNA, increasing the number of trees affected final DNA concentrations.

To determine whether the build-up of material on rollers influenced eDNA concentrations (e.g., via PCR inhibition or a physical reduction in the ability to pick up SLF honeydew), we conducted a "reverse" field experiment (15 December 2020, >0°C ambient temperature). Here, we executed the same treatment design as above but placed the SLF honeydew on the last tree in a set instead of the first (Figure 1). All other aspects of the protocol were identical to the forward experiment.

For these experiments, all rollers were placed into sterile bags, placed into a cooler with ice (0–4°C), and transported to our laboratory at Rutgers University. Immediately upon arrival to the laboratory (<2 h from initial collection), DI water was added and all remaining processing steps completed (see above). Filters were stored frozen at −20°C, and DNA extracted and qPCR analyses completed within 2 weeks of sample collection. We collected equipment negatives, two field negatives from peach trees before the deposition of honeydew, and a field positive for each experiment to test for contamination within any portion of these field experiments as previously described.

For both experiments (forward and reverse) DNA concentration for each roller derived from qPCR runs were considered the dependent variable, with treatment category the independent variable within one-way ANOVAs and post hoc Tukey's tests. Four samples for which DNA failed to amplify during qPCR were excluded from this analysis: two from the 3-tree treatment in Experiment 3, one from the 1-tree treatment in Experiment 3, and 1 from the 7-tree treatment in Experiment 4. For the forward experiment, exponential decay regression (y ~ a*exp[b*x] in R) line was used to describe potential reductions in DNA quantity recovered, as observed in aquatic systems (i.e., Barnes et al., 2014) with increasing numbers of trees sampled. DNA quantity data were log transformed prior to analysis to improve normality. We used the limit of detection (LOD) to benchmark DNA concentrations with which to compare those estimated in experiments. LOD is defined as the lowest DNA concentration that can be detected by a qPCR assay with a 95% detection rate (Klymus et al., 2020). The LOD for the SLF assay we used is 0.000006635 ng/μl (95% CI = [0.000005485, 0.00000803]), when three technical replicates are used (Allen, Nielsen, Peterson, & Lockwood, 2021).

3 | RESULTS

In Experiment 1, we tested the effect of preservation methods on the rate at which eDNA concentration on rollers declined through time. We found that, over 24 h, DNA concentrations on rollers decreased significantly ($F = 49.97$; $df = 2, 103$; $p < 0.001$) from a high of 0.0276 ng/μl (0.0081 SE) in the immediate filtration group, declining by 77% after four hours (mean across treatments: 0.0063 ng/μl; 0.0026 SE); an 84% decline was observed after 24 hours (mean: 0.0042 ± 0.0027 ng/μl; Figure 2; Table 1). We found strong evidence for both a treatment effect ($F = 4.02$; $df = 3, 103$; $p = 0.009$; Figure 2) and a treatment and time interaction ($F = 3.13$; $df = 6, 103$; $p = 0.007$). After 4 hours, treatments were statistically similar to each other and to the no-treatment group (Figure 2; Table 1). After 24 h, the mean concentration for the cold storage treatment

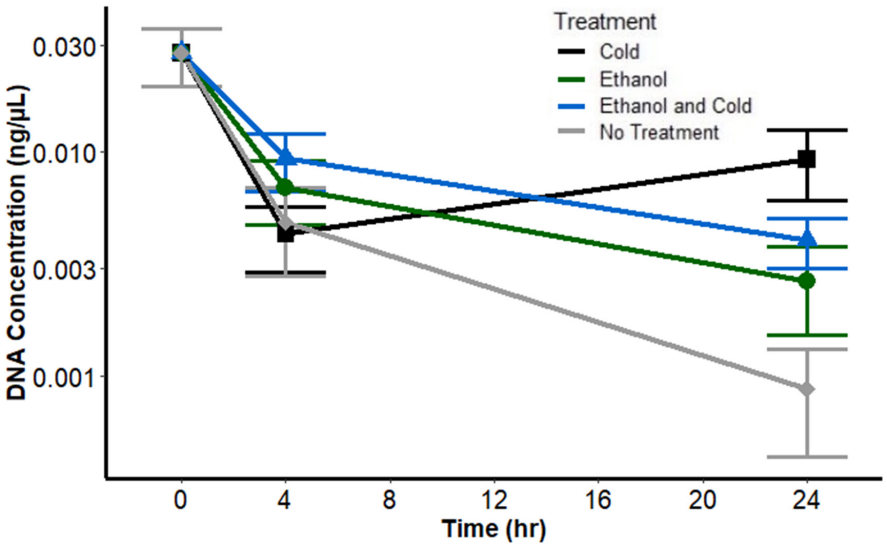

FIGURE 2 Mean degradation of spotted lanternfly (*Lycorma delicatula*) eDNA (ng/μl ± SE) over 24 h on rollers that were preserved in cold storage, via ethanol application, or a combination of both

TABLE 1 Mean concentration (ng/μl ± SE) of spotted lanternfly (*Lycorma delicatula*) eDNA on rollers, percent change in mean concentration, and statistical significance of treatment and time of a 24-h degradation laboratory experiment

Time (h)	Treatment	eDNA concentration (ng/μl ± SE)	Percent (%) change from immediate filtration	*N*	Significance[a]
0	Immediate filter	0.0276 ± 0.0081	0	10	A
4	Ethanol and cold	0.0093 ± 0.0027	−66.3	10	AB
4	Ethanol	0.0069 ± 0.0022	−75.0	10	AB
4	Cold	0.0043 ± 0.0014	−84.4	10	B
4	No treatment	0.0048 ± 0.0020	−82.6	8	B
24	Ethanol and cold	0.0040 ± 0.0010	−85.5	9	B
24	Ethanol	0.0026 ± 0.0011	−90.6	10	BC
24	Cold	0.0093 ± 0.0031	−66.3	10	AB
24	No treatment	0.0009 ± 0.0004	−96.7	8	C

[a]Treatment groups with different letters indicate significant difference.

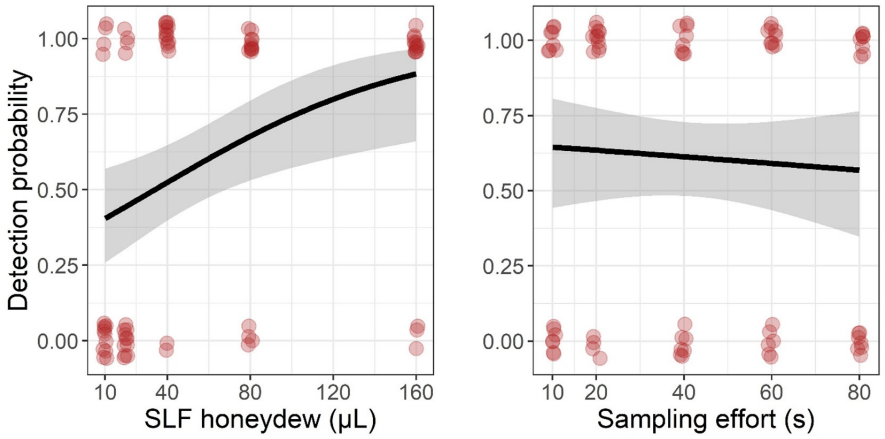

FIGURE 3 Detection and non-detection data (red dots, jittered vertically from 0 or 1 and horizontally from treatment level for visualization purposes) of spotted lanternfly (*Lycorma delicatula*) eDNA and modeled detection probability (black lines) across (a) the amount of time (s) spent sampling and (b) the amount of eDNA (volume of diluted honeydew) applied on individual trees. Shaded gray area represents 95% confidence intervals of generalized linear models

(0.0093 ± 0.0031 ng/μl) and that for ethanol and cold storage combined (0.0040 ± 0.0010 ng/μl) outperformed the no-treatment group (0.0009 ± 0.0004; Figure 2; Table 1). Mean concentrations for those storage methods represent a 66–86% decrease in DNA concentration from the immediate filtration group after 24 h; meanwhile, the no-treatment group declined by an average of 97% (Table 1). Although the mean concentration for the no-treatment group was lowest, it was still ~136 times higher than the 95% lower limit of detection for the assay (Allen et al., 2021).

In Experiment 2, we detected SLF eDNA on 45 (60%) of the 75 replicate peach trees. The logistic model describing the relationship between detection probability, honeydew density, and per-tree sampling effort was (Figure 3):

$$p = \frac{1}{1 + e^{-0.3528 + (0.0161 \times \text{density}) - (0.0046 \times \text{effort})}}$$

This relationship revealed a strong influence of honeydew density on detection probability (odds ratio = 1.016, 95% CI = [1.006, 1.029]; Figure 3a), and a negligible effect of per-tree sampling effort (odds ratio = 0.9954, 95% CI = [0.976, 1.015]; Figure 3b). The model predicts 40% detection probability (95% CI = [26%, 57%]) at the lowest SLF honeydew density evaluated (10 μl of 1/10 diluted honeydew), and 88% detection probability (95% CI = [66%, 97%]) at the highest honeydew density (160 μl of 1/10 diluted honeydew), assuming a mean sampling effort of 60 s per tree (Figure 3a). Thus, the lowest per-tree sampling effort we tested (10 s) was sufficient to maximize detection probability across all SLF honeydew densities tested.

In Experiment 3, we found that the concentration of eDNA on rollers significantly declined with an increase in the number of trees sampled after the initial encounter with SLF honeydew on the first tree ($F = 4.00$; $df = 3,17$; $p = 0.025$; Figure 4). The sampling of one peach tree (the one with honeydew) produced the highest eDNA concentration (mean: 0.00226 ng/μl), which was a 9 and 11-fold higher concentration than on rollers used to sample a total of and five (mean: 0.00026 ng/μl) and seven (mean: 0.00021 ng/μl) trees, respectively. The sampling of three trees after initial collection of honeydew onto a roller from the first tree produced intermediate concentrations of eDNA (mean: 0.00066 ng/μl). If we assume exponential decay, then concentrations do not fall below the limit of detection until after sampling 15 trees with one roller (Figure 5).

In Experiment 4, we tested whether a roller encountering SLF honeydew after being used to sample trees without honeydew prior would show decreased eDNA concentrations. In this case, the sampling of three (mean: 0.00101 ng/μl), five (mean: 0.00034 ng/μl), and

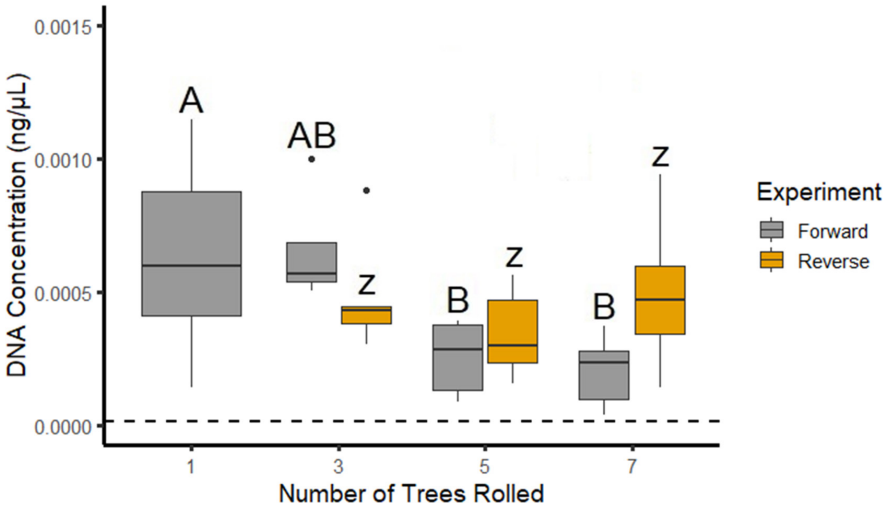

FIGURE 4 Concentration of spotted lanternfly (*Lycorma delicatula*) eDNA (ng/µl) recovered from the first tree ("forward" experiment) and last tree ("reverse" experiment) of up to seven sampled peach trees. Letters indicate statistical significance in difference in eDNA recovered among number of trees sampled within the forward experiment (uppercase letters) and the reverse experiment (lowercase)

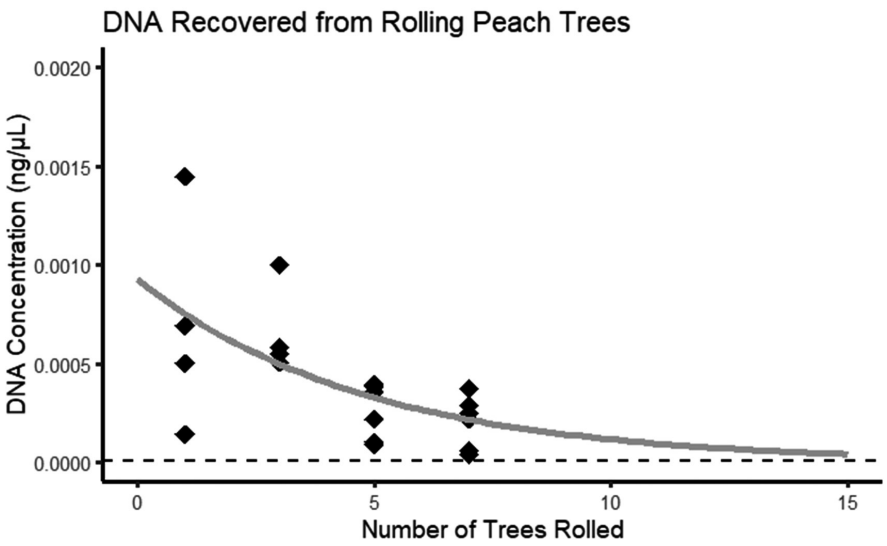

FIGURE 5 Relationship of spotted lanternfly (*Lycorma delicatula*) eDNA concentration (ng/µl) recovered and number of trees sampled assuming exponential decay (black) regression line. Black diamonds are concentrations recovered from one, three, five, and seven trees from the forward experiment (see Figure 4). *The highest value from the first group is not displayed to clearly display the other data

seven (mean: 0.00050 ng/µl) trees resulted in similar eDNA concentrations on the roller ($F = 1.51$; $df = 2,14$; $p = 0.254$; Figure 4).

We found evidence of contamination from field, equipment, extraction, and template negative controls. All field positive controls successfully amplified SLF DNA indicating no issues with DNA extraction or amplification.

4 | DISCUSSION

Invasive pests threaten forests around the world and early detection of these species is crucial to their eradication or successful control (Bradshaw et al., 2016; Martinez et al., 2020; NISC, 2016). In the past decade, eDNA-based surveys have emerged as useful tools in this global effort (Eiler et al., 2018; Jarman, Berry, & Bunce, 2018; Jerde et al., 2011; Valentin et al., 2020), while surface eDNA methods have emerged as a promising new technique to survey for forest pest insects (Allen et al., 2021; Valentin et al., 2020). Our work clarifies the logistical flexibilities and constraints of the roller surface eDNA method, aids in sampling designs, and progresses this method toward operational use. We found that easy-to-use preservation methods (cold storage or ethanol application) were effective at decreasing eDNA degradation rates on rollers that capture realistic amounts of spotted lanternfly eDNA when the species is present in very low abundance (equivalent to one lanternfly excreting honeydew for ~1 h in a single location). We also found that DNA concentrations were maximized by sampling less surface area (one peach tree) per roller, but sampling up to 15 trees per roller will likely detect lanternfly eDNA when present. Finally, we found that detection rates were maximized at a relatively low amount of sampling effort per peach tree, with no improvement gained by increasing this rate. Together, these results suggest that roller eDNA surveys are flexible enough to serve as a practical pest insect surveillance and monitoring tool that can be deployed across a wide range of forest contexts (remote, urban and agricultural).

Extensive research within aquatic systems indicates that post-collection handling of water samples can have substantial impacts on the amount of eDNA lost before extraction and qPCR testing

and can be an important limiting step in the detection of rare species (Goldberg et al., 2016, 2018; Pilliod et al., 2014; Strickler et al., 2015). Cold storage of water samples in the field is a common method of preserving eDNA within samples for longer timeframes before filtration and extraction (Strickler et al., 2015; Tsuji et al., 2017), while ethanol and other chemical preservatives have also been used effectively to slow degradation rates (Minamoto et al., 2016; Renshaw et al., 2015; Spens et al., 2017). Although our study involved the collection of eDNA from dry surfaces, once the DNA is transferred onto damp rollers, many of the same principles of DNA degradation in aquatic systems likely apply (Barnes et al., 2014). We found that cold storage or ethanol application reduced loss of captured eDNA by 3- to 10-fold, while the no-treatment samples lost ~97% of captured DNA after 24 h. This result agrees with aquatic eDNA research that has found approximately double the detection rate for water samples stored at 5°C compared with 25–30°C after initial collection (Goldberg et al., 2018). Even after 24 hours and no post-collection preservation treatment, however, we found that rollers retained enough eDNA to likely detect a single lanternfly after an hour of active honeydew deposition. With cold or ethanol preservation techniques, we suggest that field crews can forego filtering samples in the field, saving time/money, and eliminating the need to carry heavy items like water and pumps for filtration into remote locations. Our results also suggest that the use of cold or ethanol preservation methods allows stakeholders or citizen scientists to sample with rollers at field sites and ship those rollers overnight to a specific laboratory for DNA extraction, qPCR processing, and data analysis. Further research should explore whether ethanol application to rollers with different volumes or the use of other chemical preservation may better preserve DNA (e.g., Renshaw et al., 2015).

In our field trials, the probability of detecting lanternfly eDNA on individual peach trees can be considered the product of two components: the probability of encountering DNA (i.e., of rolling over the honeydew; p_e) and the probability of recovering detectable quantities of DNA given that it is encountered (p_r). Given that all replicates had four honeydew locations, our DNA density treatment should affect only p_r, while our sampling effort treatment could, in theory, affect both rates. Predictably, we found that per-tree detection probability increased with higher quantities of eDNA on that tree. However, per-tree detection probability remained unchanged regardless of the time spent sampling that tree, even when controlling for eDNA density. This result suggests that per-tree eDNA encounter and recovery (i.e., $p_e \times p_r$) by the rollers were already maximized at our lowest sampling effort of 10 s, and that, at least in systems with similar bark textures and surface area per replicate, any extra effort spent per tree would be superfluous. Less time spent sampling any single tree leads to shorter overall time on survey execution, saving on labor costs and enabling the sampling of larger areas within a given budget (Hinlo et al., 2017).

In aquatic eDNA studies, increasing the water volumes collected per sample can increase the likelihood of detecting a target organism (Lopes et al., 2017; Mächler et al., 2016). The analogous conclusion for rollers would be that detection probability is increased by sampling as much surface area as possible with each roller. However, we found that, when only the first tree the roller encountered had lanternfly eDNA, increasing the number of total trees sampled to more than three diminished the concentration of recovered eDNA. This pattern was likely due to loss of initially captured eDNA from the roller as it encountered more trees rather than to inhibitor build-up given the results of our Experiment 4 (discussed below). Thus, if the goal of a survey is to capture the highest concentrations of eDNA possible, particularly when detection at low densities is critical (Martinez et al., 2020; NISC, 2016), our results suggest that only a few trees should be sampled per roller. However, even with the relatively low amounts of eDNA used in our experiment, we estimated that potentially up to 15 could be sampled before spotted lanternfly eDNA concentrations dropped below the limit of detection. However, these findings regarding the optimal number of trees to sample deserve further investigation. The influence of environmental factors like PCR inhibitors can change in different survey contexts, as can other factors that we did not evaluate in this study, including bark texture, the amount and source of eDNA (excrement, exuvia, saliva etc.), and the eDNA assay in use. Thus, small-scale field experiments, such as ours, will be consistently needed to identify the situation-specific tradeoffs between lowering survey costs, simplifying sampling logistics, and maximizing detection probability (Hinlo et al., 2017).

Tree surfaces likely produce several compounds that can inhibit PCR reactions including, among others, pectins, polyphenols, polysaccharides, xylan, humus, or sediments (Opel, Chung, & McCord, 2010; Schrader, Schielke, Ellerbroek, & Johne, 2012; Stoeckle et al., 2017). If these compounds are common, we should expect that a roller will accumulate these compounds as it is used to sample more trees. In addition, as a single roller is applied to increasing numbers of trees, it may become clogged with debris, which could reduce its effectiveness at transferring and retaining any eDNA it encounters. No matter the mechanism, it is possible that with more trees sampled by a single roller before encountering target species eDNA, DNA concentrations as measured by qPCR will decline. We did not find evidence for this effect, at least up to seven trees sampled, suggesting that this issue is less concerning in regard to maximizing probability of detection than the back-transfer of captured eDNA (above). We note, however, that the amount of inhibitor compounds can vary between tree species, and peach trees may not be representative of the conditions expected in other situations (John, 1992; Singh & Singh, 1996; Singh, Nie, Singh, Coffin, & Duplessis, 2002). Further investigation should broaden the range of tree species examined and include direct, experimental evaluation of PCR inhibition (McKee, Spear, & Pierson, 2015; Opel et al., 2010).

Given the high environmental and economic costs of forest insect pests, any survey tool that can substantially increase sample detection rates is attractive (Tobin et al., 2014). Ideally, however, the tool should also be practical, and fit within the logistical constraints of fieldwork presented by the system. We provide

relevant information to hone the sampling design of a novel surface eDNA tool, defining the boundaries of use, and its strengths and limitations, for the monitoring of insect pests within forested habitats. We identified factors that should be evaluated within species- and habitat-specific contexts, and show that there is reasonable flexibility to overcome logistical hurdles for widespread field deployment.

AUTHOR CONTRIBUTIONS

DLP, MCA, AV, and JLL conceived the study; DLP, MCA, and AV contributed to fieldwork; JLL secured funding; DLP drafted the manuscript; and all authors contributed substantially to revisions.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

DATA AVALIABILITY STATEMENT

Raw data from this study were deposited online with Center for Open Science, Inc. and is available via the link: https://osf.io/apfub/?view_only=629aa1b64ee84123964cf5baa8d00375

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