Research Article

Received: 23 June 2021 Revised: 10 September 2021 Accepted article published: 16 September 2021 Published online in Wiley Online Library: 21 October 2021

(wileyonlinelibrary.com) DOI 10.1002/ps.6645

Development of a qPCR-based method for counting overwintering spruce budworm (*Choristoneura fumiferana*) larvae collected during fall surveys and for assessing their natural enemy load: a proof-of-concept study

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Abstract

BACKGROUND: In eastern Canada, surveys of overwintering 2nd instar spruce budworm (*Choristoneura fumiferana*) larvae ('L2s') are carried out each fall to guide insecticide application decisions in the following spring. These surveys involve the collection of fir and spruce branches in selected stands, followed by the mechanical/chemical removal of larvae. The latter then are counted manually on filter papers, using a stereomicroscope. Considering the significant effort and difficulties which this manual counting entails, we developed a quantitative (q)PCR-based 'molecular counting' approach designed to make this step less tedious.

RESULTS: Using the *C. fumiferana* mitochondrial *cytochrome c oxidase 1* (COI) gene as a target for qPCR DNA quantification, we show that the amount of DNA in a larval extract is strongly correlated with the number of larvae used to generate that extract, and that molecular estimates of L2 counts are comparable to those generated using the manual approach. In addition, we used the same DNA extracts to monitor the microsporidian pathogen *Nosema fumiferanae*, and the hymenopteran parasitoids *Glypta fumiferanae* and *Apanteles fumiferanae* in overwintering L2s employing a subset of a TaqMan assay developed by Nisole *et al.* (2020) for the identification of budworm natural enemies. We show that the proportion of individuals affected by each natural enemy in samples containing a known number of larvae can be estimated from presence/absence data through the binomial probability distribution.

CONCLUSION: The present proof-of-principle study shows that a molecular approach for counting L2s and assessing their natural enemy load is clearly possible and is expected to generate reliable results.

Supporting information may be found in the online version of this article.

Keywords: spruce budworm; *Choristoneura fumiferana*; overwintering; qPCR; molecular counting; natural enemies

1 INTRODUCTION

In eastern Canada, populations of the spruce budworm [SBW; *Choristoneura fumiferana* (Clemens)] reach outbreak levels every 35–45 years, causing widespread reduction in growth and mortality in spruce and balsam fir stands.[1] Pest management efforts deployed to combat this insect include fall surveys of overwintering larvae (2nd instars, commonly referred to as 'L2s'), which are used to monitor annual progress of outbreaks and guide insecticide application decisions in the following spring. For instance, in Quebec, where SBW management focuses on foliage protection in high-value stands, spray decisions are based on both defoliation severity

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estimates and L2 counts.[2] In New Brunswick, where an early intervention strategy is currently being implemented in an effort to stop or slow outbreak development, spray decisions are based primarily on an L2 count threshold of seven larvae/branch.[1,3]

In order to estimate SBW population densities from L2s, branches are collected in the mid-canopy of balsam fir and spruce trees at designated sites (three per site) and processed for the mechanical/chemical removal of larvae – a procedure sometimes referred to as 'branch washing', whereby branches are cut in smaller pieces and placed in a solution of sodium hydroxide (NaOH) to break down the silk hibernacula that protect overwintering larvae. The branches then are washed through several large sieves to remove the coarser material. At the end of this process, an L2-laden organic phase is decanted onto a filter paper placed over a vacuum funnel to remove residual liquids; the filter papers are then stored for later counting of larvae under a stereomicroscope.[4,5] Considering that thousands of such filters must be processed manually every year, the latter step proves to be very labor-intensive. In addition, although the majority of plant debris separates into the final aqueous phase, varying proportions of it typically remain in the organic phase and find their way onto the filter papers, hindering the recognition of L2s among plant debris and possibly resulting in counting errors (Fig. 1).

In an effort to improve the L2 counting process and make it more efficient, we set out to develop a quantitative (q)PCR-based counting method. This approach involves the qPCR amplification of a SBW gene (*cytochrome c oxidase 1*; 'COI') in DNA extracts obtained from material collected in the above-described organic phase. Larval counts then are estimated based on the linear correlation observed between the number of COI copies and the number of larvae. Here, we present data confirming the validity of the molecular method's underlying principle as well as results of an experiment aimed at validating the qPCR-based approach through a side-by-side comparison of the two procedures using field-collected samples. In addition, using components of a TaqMan® assay we recently developed for the molecular identification of spruce budworm natural enemies,[6] we show that the same DNA extracts can be utilized for detection and estimation of the relative frequency of the transovarially transmitted microsporidian pathogen *Nosema fumiferanae* (Thomson)[7] and of two hymenopteran parasitoids that lay their eggs in pre-diapause 1st and 2nd instar hosts: *Glypta fumiferanae* Viereck[8] and *Apanteles fumiferanae* Viereck.[9] These three species are among the numerous natural enemies known to play a role in regulating SBW populations.[10] A capability to assess their prevalence in overwintering

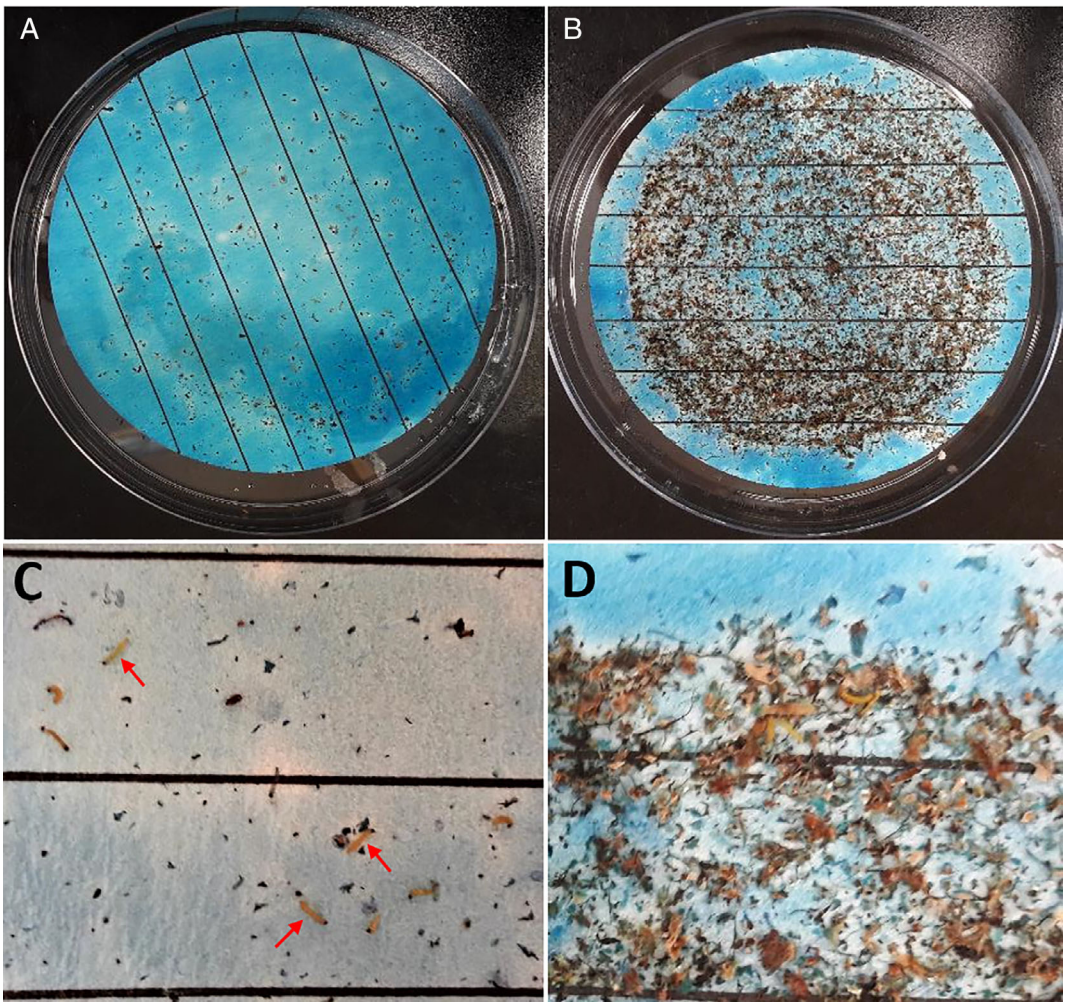

Figure 1. Examples of L2 extracts on filter paper with little (A, C) and abundant (B, D) branch debris. (C) and (D) show a portion of (A) and (B), respectively, at higher magnification. L2s are easy to discern (arrows) on the filter shown on picture C, but their recognition is challenging on the filter shown in picture D.

Pest Manag Sci 2022; **78**: 336–343

SBW larvae would enhance our ability to forecast outbreak severity in the following summer.

2 MATERIALS AND METHODS

2.1 Real-time qPCR approach

Design of qPCR primers (Table 1) targeting the *C. fumiferana* COI gene (GenBank JF702967) was performed using Oligo Explorer v1.2 and Oligo Analyzer v1.2 (Gene Link, NY, USA). Primers were designed to ensure short amplicon length (97 bp) in an effort to maximize the chances of positive amplification in a context where the DNA could be partially degraded after larval extraction from the foliage.

qPCR analysis was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermofisher, Waltham, MA, USA). All reactions were performed in a final volume of 10 μL and contained 1x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germantown, MD, USA), 0.5 μM of each of the two primers, and 1 μL template DNA. Real-time PCR thermocycling conditions were set at 95 °C for 15 min, followed by 50 cycles at 95 °C for 15 s, 58 °C for 30s and 65 °C for 60s. For quantification, we used the LRE method of Rutledge.[11]

2.2 Assessing the relationship between larval counts and COI copy number

Before undertaking the development of the method reported here, we needed to assess the strength of the correlation between COI copy number and larval counts. To this end, we first used live post-diapause L2s obtained from Insect Production Services (Great Lakes Forestry Centre, Sault Ste. Marie, ON, Canada). Larvae were allowed to emerge from their hibernacula at room temperature and were transferred to individual 2-mL microfuge tubes, either individually or in groups of 10, 20, 30, 40, 50, 100, 150 or 200 larvae (prepared in triplicates), using a fine artist paintbrush. Before DNA extraction, a 3-mm tungsten bead (Qiagen-) was added to each sample to allow grinding of larvae. These were snap frozen in liquid N_2 and ground using a Mixermill MM 300 (Retsch, Haan, Germany) at 30 Hz for 1.5 min. Samples then were centrifuged 30 s at 13 000 rpm to pellet the material. For DNA extraction, 180 μL ATL lysis buffer (Qiagen) and 20 μL proteinase K (20 $\mu g \cdot \mu L^{-1}$, Qiagen) were added to each sample. Tubes then were vortexed vigorously and incubated at 56 °C overnight with shacking (600 rpm). On the next day, lysates were centrifuged at 13 000 rpm for 5 min, and 1 μL of each supernatant, containing the raw soluble extract, was directly diluted ×100 with sterile distilled water for qPCR quantification.

Once the existence of a strong linear relationship between COI copy number and larval counts was established for live L2s (see Results, Section 3), we assessed the strength of that relationship using post-processing (dead) L2s. In a first test, larvae were collected individually on dried filter papers [held at 4 °C for various periods; obtained from the Quebec Ministry of Forests, Fauna and Parks (QMFFP)] under a stereomicroscope, using fine forceps, and processed as described above in groups of 1, 10, 20 and 30 larvae, with six biological replicates. These samples were free of plant debris. As a second approach, L2s were counted under a stereomicroscope immediately after decantation of the organic phase (heptane) onto a filter paper, after which larvae were transferred promptly to a 50-mL polypropylene tube by washing the filter with distilled water (done by QMFFP personnel). Larvae and plant debris were left to sediment at 4 °C, after which water was carefully decanted. ATL buffer (1080 μL instead of 180 μL) was then added to the sediment, the volume of ATL being adjusted here to take into account the presence of significant amounts of plant debris. The resulting solution was held at 55 °C for 5 min to facilitate pipetting (using 1-mL pipette tips with the cut-off ends) and transferred to a 2-mL tube. After addition of a bead and direct grinding using a Mixermill MM 300, 120 μL proteinase K was added to each tube, followed by vigorous vortexing and incubation at 56 °C, as described above. qPCR amplification was carried out directly on 1 μL lysate, diluted ×100 or ×1000 (see Supplementary Information Appendix S1) in sterile distilled water. The number of larvae counted on each filter paper ($N = 76$) varied between 2 and 50. A fourth assessment of the strength of the relationship was conducted in the context of the experiment described below.

2.3 Side-by-side comparison of the conventional and molecular counting methods

To begin assessing the reliability of the molecular counting approach under operational conditions, we conducted a comparison of paired samples where one member of each pair was processed using the conventional visual counting method while the other member was submitted to molecular counting. In the fall of 2020, branches were sampled at five locations in northern New Brunswick, in an area known to have relatively high numbers of budworm larvae and observable current-year defoliation. At each location, five plots were selected every 50 m along a transect through the stand. In each plot, one 75 cm-long mid-crown branch was collected from each of two randomly selected trees – either balsam fir or white spruce, but the same host species within each paired sample – for a total of 50 branches. To avoid important discrepancies in actual larval densities between samples within a pair, the two paired branches (foliage and stems) were carefully broken into smaller pieces and mixed so that one-half of each cut branch, in each pair, would be represented in each of the final paired samples. Evenness was ensured by weighing each mixed sample within a pair. Extraction of larvae from the branch material ('branch washing') was conducted manually using the method of Sanders,[4] a process involving soaking branches in a NaOH solution (to release larvae from their hibernacula) and washing them through several large sieves to remove the coarser material. Each resulting filtrate, containing both larvae and small debris, was transferred to a separatory funnel, along

Table 1. qPCR primers used to assess COI copy number in *C. fumiferana* larvae

Primer name	Primer sequence	Primer length	Primer Tm*	Amplicon length
Cf COI F379-398	ACAGTAGGAGGCTTAACAGG	20 bp	60.5 °C	97 bp
Cf COI R452-476	AGAACATAATGGAAATGGGCAACTA	25 bp	59.6 °C	

*Melting temperature.

with hexane, where the larvae partitioned into the upper hexane phase, near the water–hexane interface, while debris partitioned into the lower water phase. After draining a large portion of the bottom water phase, the remaining material was either carefully poured over a filter paper sitting in a vacuum funnel to remove residual water and hexane (for subsequent manual counting under a stereomicroscope) or transferred to a disposable 250-mL polypropylene tube (for subsequent processing for molecular counting). The latter were processed for DNA extraction within a week of their collection (see Appendix S2).

Before extracting DNA from larvae in the hexane/water samples, a large portion of the water phase was removed using a 25-mL pipette. The remaining interphase (where larvae tend to collect) and a portion of upper hexane phase (total volume of ~25 mL) were transferred to a 50-mL polypropylene tube, to which 25 mL distilled water was added, followed by centrifugation at 4000 rpm for 5 min to pellet the remaining plant debris. The resulting interphase was collected using a 1-mL pipette tip with cut-off end and the larvae it contained transferred to a 2-mL tube. For DNA extraction, a tungsten bead was added to each tube, followed by freezing in liquid N_2 and grinding in a Mixermill MM 300 at 30 Hz for 1.5 min. A total of 540 µL ATL buffer and 60 µL proteinase K were added to the tube, followed by vortexing and overnight incubation at 56 °C on a rotary shaker (600 rpm). After centrifugation at 13 000 rpm for 5 min, 1 µL supernatant was diluted ×1000 in sterile distilled water and used for qPCR.

For assessing the number of larvae in each sample, based on COI copy number, we first generated a standard curve using larvae processed with hexane (as opposed to heptane, used in prior tests). To this end, we sacrificed two field-collected samples that appeared to have a particularly high content in budworm larvae, based on a visual inspection of the final interphase used for DNA extraction. The latter was poured into a petri dish, where larvae were manually picked out using a 1-mL pipette tip with cut-off end and transferred individually to one of 11 tubes to generate the following larval counts: one (three tubes), five (two tubes), ten (two tubes), and one tube each of 20, 35, 50 and 70 larvae. DNA extraction and qPCR quantification were conducted as described above. The regression equation describing the relationship between larval counts and COI copy numbers was used to estimate the number of L2s in the 23 remaining samples. For each site, a Student's *t*-test was used to compare the number of L2s estimated using the visual and molecular methods. The package R/GGPUBR (https://cran.r-project.org/web/packages/ggpubr/index.html) was used to perform statistical analysis and generate the box-plots.

2.4 Detection of natural enemies in L2 DNA extracts

In order to determine whether *N. fumiferanae*, *G. fumiferanae* and *A. fumiferanae* could be detected in DNA extracts prepared for the purpose of counting larvae, we used qPCR primers and TaqMan probes developed for each species in the context of an earlier study.[6] Following the procedure developed by these authors, we processed 76 and 23 samples from Quebec and New Brunswick, respectively (i.e. samples employed to develop the molecular counting procedure), using 2 µL aliquots of a ×1000 dilution of

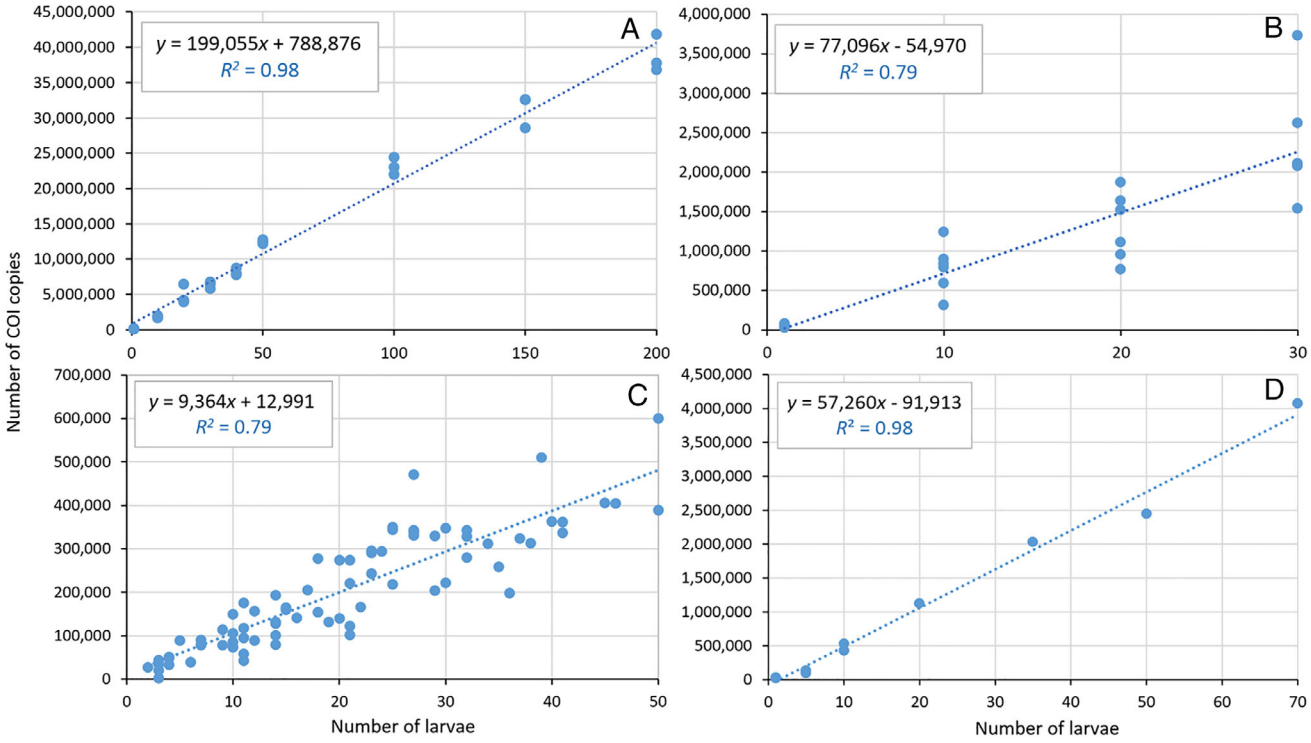

Figure 2. Relationship between COI copy number, as quantified by qPCR, and the number of 2nd instar larvae. (A) Test conducted with live post-diapause L2s; number of larvae tested (in triplicates): 1, 10, 20, 30, 40, 50, 100, 150 or 200. (B) Test conducted using post-processing (i.e. after branch washing; organic phase: heptane) dead L2s manually removed from dried filter papers and processed for DNA extraction in batches of 1, 10, 20 and 30 ($N = 6$ per batch). (C) Test conducted using post-processing (i.e. after branch washing; organic phase: heptane) dead L2s washed off filter papers (along with plant debris) with distilled water immediately after counting larvae under a stereo-microscope; number of larvae per test: 2–50; $N = 76$. (D) Test conducted using post-processing dead L2s collected individually from the hexane phase within one week after branch washing; number of larvae per test (no. tests): one (three), five (two), ten (two), 20, 35, 50 and 70 (one each).

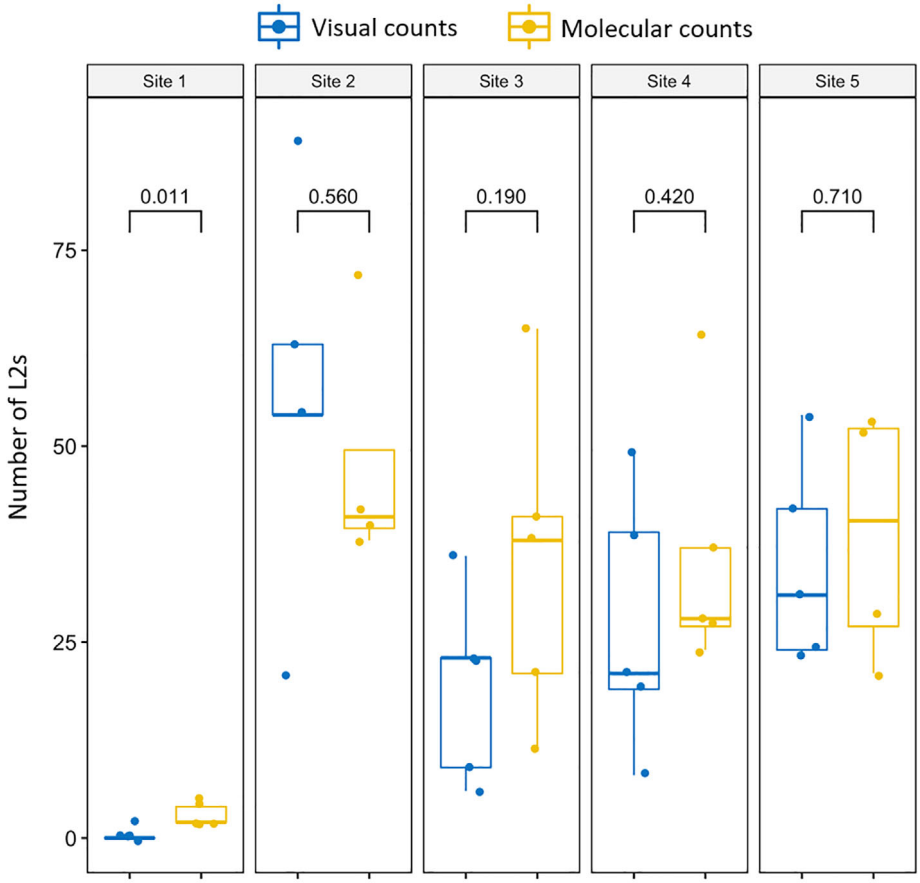

Figure 3. Box-plots comparing L2 counts (no. larvae/branch) estimated using the conventional (visual counts) and qPCR-based methods, following extraction of larvae from balsam fir and white spruce branches collected in pairs at five different sites in northern New Brunswick in the fall of 2020 (see Materials and methods for details). Molecular count values were computed using the following equation: $y = 57\,260x - 91\,913$, where y is qPCR-based COI copy number and x is no. of larvae [see Fig. 2(D)]. Molecular counts at sites 2 and 5 are based on four samples; for all other samples $N = 5$. Values shown in the upper portion of each box represent the level of significance of a Student's *t*-test.

Table 2. Proportion of L2 samples (DNA extracts) from Quebec and New Brunswick in which the microsporidian pathogen *N. fumiferanae* and the hymenopteran endoparasitoids *A. fumiferanae* and *G. fumiferanae* were detected using a triplex TaqMan® assay[6]

Sample origin	No. of samples	% positive for *Nosema*	% positive for *Glypta*	% positive for *Apanteles*
Quebec	76	59% (4.9 ± 0.8%)	61% (5.9 ± 0.9)	62% (5.4 ± 0.8%)
New Brunswick	23	48% (2.7 ± 0.9%)	65% (4.2 ± 1.2%)	83% (8.8 ± 2.7%)

Values in brackets: relative frequency of natural enemy presence ($\kappa \pm SE_\kappa$), estimated by logistic regression with Eqns (1) and (2).

each DNA extract. The assay we ran was a small subset of the Nisole *et al.*[6] suite of assays, where the *Nosema*-specific probe targets the SSU rDNA gene (Fam fluorophore), while the *Glypta*- and *Apanteles*-specific probes target the 28S rDNA (Texas Red fluorophore) and COI (Cy5 fluorophore) genes, respectively. The assay was run in triplex format, with two technical replicates per sample.

As each sample contained varying (but known) numbers of larvae, we estimated the proportion of individuals affected by each natural enemy ('relative frequency') from presence/absence data through the binomial probability distribution, assuming that all larvae were sampled at random with respect to the occurrence of these enemies and that the frequency to be estimated was the same in all samples from each province. Because the detection data record presence/absence in a sample of L larvae, rather than the number of individuals n in the sample that contain a given natural enemy, we used the probability of absence ($n = 0$) or presence ($n > 0$) to estimate the relative frequency of the natural enemy κ, with:

$$P(n=0) = (1-\kappa)^L \quad (1)$$

$$P(n>0) = 1-(1-\kappa)^L \quad (2)$$

The relative frequencies of all three natural enemies (κ_N for *Nosema*, κ_G for *Glypta* and κ_A for *Apanteles*) were estimated

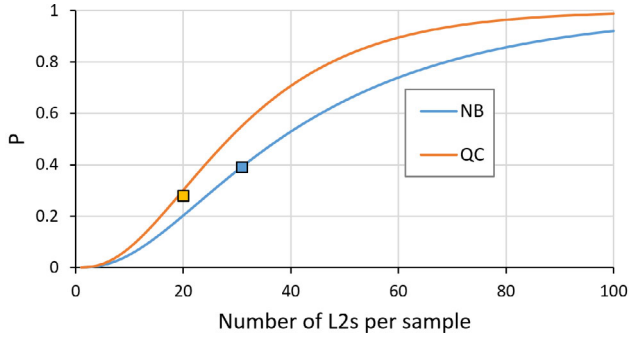

Figure 4. Relationship between the number of L2/sample and the proportion of samples (P) containing all three natural enemies using a TaqMan assay [lines: Eqn (3) with estimates of relative frequencies in Table 2; points: proportion of samples actually containing all three and the corresponding average number of L2 per sample]. Blue, New Brunswick; orange, Quebec.

by maximum-likelihood, using binomial regression with the SAS procedure NLMIXED (SAS Institute Inc., Cary, NC, USA).

Under the assumption of independence between the three natural enemies (no positive or negative interactions), the probability of detecting all three in a sample can be calculated with Eqn (2). This probability depends solely on the number of larvae (L) in the sample and on the relative frequency of each natural enemy (κ_N, κ_A and κ_G) in the larvae:

$$P(\text{all three}) = \left[1-(1-\kappa_N)^L\right]\left[1-(1-\kappa_A)^L\right]\left[1-(1-\kappa_G)^L\right] \quad (3)$$

We compared this proportion of samples from New Brunswick and Quebec in which all three natural enemies were detected with the value expected in a sample containing the average number of larvae from each province.

In order to verify that the assumption of independence was valid, and that there were no discernible interactions between these three natural enemies, we compared by χ^2 test the observed and expected frequencies of joint presence/absence in the larval samples from New Brunswick and from Quebec. Under the assumption that occurrence of one natural enemy is independent of occurrence of the others, the expected number of samples (E) with presence or absence of all three natural enemies larvae can be obtained from Eqns (1) and (2). There are eight distinct possible outcomes: {(Nf, Af, Gf), ($\neg Nf$, Af, Gf), …, ($\neg Nf$, $\neg Af$, $\neg Gf$)}, where $\neg$ represents the logical 'not' (for absence). The expected number of samples with each outcome is given by:

$$\begin{aligned}
E(Nf,Gf,Af) &= \sum_{i=1}^{n}\left[1-(1-\kappa_N)^{L_i}\right]\left[1-(1-\kappa_A)^{L_i}\right]\left[1-(1-\kappa_G)^{L_i}\right]\\
E(\neg Nf,Gf,Af) &= \sum_{i=1}^{n}\left[(1-\kappa_N)^{L_i}\right]\left[1-(1-\kappa_A)^{L_i}\right]\left[1-(1-\kappa_G)^{L_i}\right]\\
&\vdots\\
E(\neg Nf,\neg Gf,\neg Af) &= \sum_{i=1}^{n}\left[(1-\kappa_N)^{L_i}\right]\left[(1-\kappa_A)^{L_i}\right]\left[(1-\kappa_G)^{L_i}\right]
\end{aligned} \quad (4)$$

3 RESULTS

3.1 Relationship between larval counts and COI copy number

Using fresh post-diapause L2s, the linear relationship between the number of larvae and the number of COI copies was very strong, with an R^2 of 0.98 and ~250 000 COI copies per larva [Fig. 2(A)]. In comparison, post-processing larvae picked individually on filter papers yielded a somewhat lower R^2 of 0.79 [Fig. 2(B)]. Not surprisingly, a reduced number of COI copies (~77 500/L2; mean of values obtained for ten larvae) was observed in these dead larvae, likely as a result of the treatment to which larvae were subjected during their extraction from the foliage, plus storage time and conditions before sample processing. When postextraction larvae were obtained by washing each filter paper into a 50-mL polypropylene tube [Fig. 2(C)], the relationship between the number of larvae and the number of COI copies remained strong (R^2 = 0.79), despite the presence of plant debris in the material processed for DNA extraction. Lastly, within the context of the experiment described below, we generated a standard curve using dead larvae picked out individually from the hexane phase shortly after branch washing. Surprisingly, the linear relationship between the number of larvae and the number of COI copies [Fig. 2(D)] was as strong as when we used live L2s [Fig. 2(A)].

3.2 Comparison of the conventional and molecular counting methods

In an effort to benchmark the present L2 molecular counting approach against the manual procedure, counts were generated using both methods for paired samples collected at each of five field sites in New Brunswick. Although the two members of each pair were not expected to contain the exact same number of larvae (see Materials and methods for details), no significant differences were observed between the two counting methods except for Site 1, where larval densities were much lower than at the

Table 3. χ^2 test of interactions between three natural enemies of overwintering L2 spruce budworm larvae

Presence(1)–absence(0) of *Nosema–Glypta–Apanteles*	1 0 0	0 1 0	0 0 1	1 1 0	1 0 1	0 1 1	1 1 1	0 0 0	Total	*P*
New-Brunswick										
Observed	0	1	5	1	1	4	9	2	23	
Expected	0.4	0.8	2.6	0.4	2.1	4.6	8.7	3.4	23	
χ^2	0.435	0.077	2.149	1.064	0.585	0.077	0.013	0.602	5.00	0.66
Quebec										
Observed	6	3	4	9	9	13	21	11	76	
Expected	5.1	6.8	5.9	8.0	6.8	9.7	23.7	10.0	76	
χ^2	0.157	2.154	0.640	0.121	0.733	1.160	0.299	0.099	5.36	0.62

other sites and where the molecular approach detected significantly more larvae than the manual counting method (Fig. 3). Overall, counts obtained for paired samples by each method displayed a strong correlation ($R^2 = 0.64$; Fig. S3). Importantly, the same insecticide application decisions (based on an L2 count threshold of seven larvae/branch) would have been made at all five sites using either method.

3.3 Natural enemies in L2 DNA extracts

Finally, DNA extracts generated from field-collected samples were used to determine whether a triplex TaqMan assay could detect three key SBW natural enemies whose presence in overwintering larvae is expected to be common, particularly in outbreaking populations. Results show that all three organisms were detected in larval samples (Table 2). From the presence/absence data we estimated the relative frequency of each natural enemy in the two sets of samples with Eqns (1) and (2) (Table 2); both the lowest (2.7%, *Nosema*) and highest (8.8%, *Apanteles*) predicted frequencies were observed in the New Brunswick samples. Of course, the DNA extracts used in these tests were derived from samples with varying larval counts, a parameter that affects the probability of detection [see Eqns (1) and (2)]. Indeed, samples with high larval counts will test positive for all three species more often and, conversely, those with low larval counts are more likely to test negative for one or more of the targeted species, as per Eqn (3). There was an excellent correspondence between the expected and observed frequencies of detection of the three natural enemies in samples from both provinces (Fig. 4). We found no evidence of interactions between these three natural enemies (Table 3).

4 DISCUSSION

The present study was aimed primarily at developing a qPCR-based method for counting overwintering SBW larvae collected during fall surveys. Our work clearly shows that it is possible to assess the number of 2nd instar larvae in SBW samples using a qPCR approach that estimates the number of COI copies present in a DNA extract derived from the sample. Not surprisingly, the strength of the relationship between the number of L2s and the number of COI copies was very high in situations where fresh larvae were used to generate the DNA extract [Fig. 2(A)]. The harsh NaOH treatment employed to remove L2s from their hibernacula and foliage following field collection[4,5] was expected to have a negative impact on the quality of DNA recovered from such larvae, thereby affecting both the number of COI copies recovered and, possibly, the relationship between COI copy number and larval counts. Although this seems to have been the case in the first two series of tests we conducted, resulting in increased variability in COI copy numbers, the R^2 estimated for such relationships remained relatively high for both plant debris-free and plant debris-laden samples [$R^2 = 0.79$; Fig. 2(B),(C)]. Surprisingly, however, when we assessed the strength of the relationship between the number of larvae and the number of COI copies using dead larvae picked out individually from the hexane phase shortly after branch washing [Fig. 2(D)], the R^2 was as high as that obtained using fresh larvae [Fig. 2(A)]. These results strongly suggest that the presence of plant debris in the material used for extraction has an impact on the accuracy of the qPCR measurements made, likely because plant-debris-derived contaminants in the crude DNA extract have an inhibitory effect on the qPCR reaction (see Appendix S1). In this context, the variability in COI copy number observed when larvae were picked individually from filter papers [Fig. 2(B)] likely resulted from uneven DNA degradation during pre-processing storage at 4 °C. Altogether, the present results suggest that efforts to improve the method of larval removal from foliage with a view to minimizing the amount of plant debris that collect in the final heptane/hexane phase would likely result in greater accuracy of the qPCR-based assessments of COI copy numbers, through both a reduction in debris-related inhibitory effects on the PCR reaction and an improvement in the efficiency of the grinding process during DNA extraction from larvae.

We conducted a side-by-side comparison of the two larval counting methods using pairs of field-collected branch samples. On the one hand, although such paired samples were not expected to contain the exact same number of larvae, the counts obtained using each method were not significantly different at four of the five study sites; the only statistically significant difference was observed at Site 1, where larval densities were very low, which increased the probability of discrepancy in larval content in paired branch samples. On the other hand, it also is possible that the molecular counting method was more effective in detecting rare larvae when plant debris were abundant (e.g. Figure 1). Importantly, on the basis of the data presented in Fig. 3, the same insecticidal application decisions would have been made using either counting approach.

For the side-by-side comparison of the two counting methods, L2s were recovered directly from the hexane–water interphase, a laborious process that would certainly prove impractical under operational conditions. In this context, we think that the most effective approach for recovering larvae would be to wash them off the filter into a tube, using a gentle jet of water, immediately after the vacuum-driven removal of residual liquids from the filter. Such samples could then be stored frozen until DNA extraction.

The present work also shows that crude DNA extracts generated for counting larvae can be employed for estimating the prevalence of three SBW natural enemies often encountered in overwintering larvae (Table 2). The presence/absence data obtained for each natural enemy in each sample, combined with the estimated number of larvae in these samples, can be used to assess the relative frequency of each natural enemy in a given population. A gradual loss in the accuracy of such assessments is to be expected with increasing prevalence of a natural enemy; predictions based on the binomial distribution ultimately become impractical if the natural enemy is detected in all samples (an outcome that also will be affected by the number of host larvae in the samples). In such situations, the assay could be run on small subsamples or even on individual larvae, which would then provide a direct assessment of natural enemy prevalence. Interannual comparisons of the relative frequency of the three L2 natural enemies considered here may help predict whether a given outbreaking SBW population is expected to collapse in the following growing season. In the work reported here, we established clearly the absence of significant interactions (either positive or negative) between these three prevalent natural enemies in overwintering SBW larvae: presence of one does not increase or decrease the probability of finding another.

5 CONCLUSIONS

The present proof-of-principle study shows that a molecular approach for counting L2s and assessing their natural enemy load is clearly possible and is expected to generate reliable results. Of course, various technical aspects of the method presented here will need to be optimized in order to scale-up the procedure and improve its capacity to provide larval counts and relative

frequencies of natural enemies rapidly in an operational setting. For example, the DNA extraction step likely could be shortened and simplified, possibly by skipping the grinding of larvae and reducing incubation time at 56 °C; preliminary assessments of such modifications have provided encouraging results. Additionally, qPCR quantification conditions could be optimized to reduce reaction time, and the use of a qPCR instrument equipped with a block for running 384-well plates (as opposed to 96-well plates) would lead to a substantial improvement in processing efficiency, with an output equivalent to 384 filters being counted in a few hours. Finally, to maximize the reliability of the proposed counting approach, we suggest that new standard curves be established, using multiple replicates, whenever changes are made to the branch-washing procedure or when branches are collected at sites showing important differences in stand composition and/or geographical location relative to previously surveyed sites. Clearly, the proposed qPCR-based counting method shows promise as a means of eliminating the manual larval counting step during fall surveys of overwintering SBW larvae. Moreover, the approach we propose for monitoring natural enemies in L2s could enable a reduction in insecticide applications insofar as high levels of parasitism or infection may signal a decline in outbreak severity.

ACKNOWLEDGEMENTS

We thank personnel at the Quebec Ministry of Forests, Fauna and Parks, Quebec City, and at the New Brunswick Department of Natural Resources and Energy Development, Fredericton, for providing the post-branch-washing larval samples used in this study. We also thank É. Gauthier, NRCan-Quebec City, for ideas and discussions on the basis of which this research was launched. Funding for this work was provided by grants from the "Assistant Deputy Minister Innovation Fund" (Canadian Forest Service) and the Atlantic Canada Opportunities Agency and Natural Resources Canada (Spruce Budworm Early Intervention Strategy Program).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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