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Journal of Economic Entomology, 117(2), 2024, 578–584
https://doi.org/10.1093/jee/toae001
Advance Access Publication Date: 18 January 2024
Research

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A fast and reliable larval sampling method for improving the monitoring of fruit flies in soft and stone fruits

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Subject Editor: Hannah Burrack

Received on 20 September 2023; revised on 12 December 2023; accepted on 16 January 2024

The spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), threatens both the soft-skinned and stone fruit industry in Asia, Europe, and America. Integrated pest management requires monitoring for infestation rates in real time. Although baited traps for adult *D. suzukii* are widely used for field monitoring, trap captures are weakly correlated to larval infestation rates. Thus, monitoring for larvae instead of adult flies represents the most reliable monitoring technique. Current methods for larval monitoring (e.g., sugar or salt floatation) are time-consuming and labor-intensive. In this study, we develop a new "sleeve method" for detecting larvae in strawberries through the inspection of individual fruits crushed within transparent plastic sleeves. Samples can be optionally frozen until further processing. Based on count data from non-expert observers, the estimation of larval infestation with the sleeve method is fast, precise, and highly repeatable within and among observers. Mean processing time is half the time compared to previous methods (33–80 s per sample depending on infestation levels). As the accuracy of the sleeve method decreases with infestation levels, we suggest ways to improve its accuracy by incubating fruits for 48 h and calibrating data using fruits with a known number of larvae. The method could also be used in other fruits, as it is easier to use, faster, and requires less equipment than previous monitoring methods. Finally, the method represents a promising tool for growers or researchers to effectively monitor and manage *D. suzukii* and other insect pests of soft and stone fruits.

Key words: spotted-wing drosophila, larval infestation, transparent plastic sleeve, integrated pest management, SWD

Introduction

The spotted-wing drosophila (SWD), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is a major insect pest of both soft and stone fruits (e.g., strawberries, raspberries, blackberries, blueberries, and cherries; Asplen et al. 2015). The serrated ovipositor of SWD females enables them to lay their eggs in ripening fruits, making them unmarketable. With regular integrated pest management (IPM), the rates of SWD infestation in soft fruits is heterogenous among crops, farms, and years, but typically range from 10% to 30% and result in important revenue losses for farmers (Knapp et al. 2021).

SWD management relies mostly on chemical control using broad-spectrum insecticides (Schetelig et al. 2018). Due to human health and environmental concerns with chemical insecticide use and the regulatory withdrawal of several active substances (Nicolopoulou-Stamati et al. 2016, Eggleton 2020), alternative biological control methods are currently under development (e.g., including deployment of natural enemies and sterile insect technique; Lee et al. 2019, Tait et al. 2021, Homem et al. 2022).

IPM programs for SWD require an early and accurate detection of presence in the crop to initiate chemical treatments (Tait et al. 2021, Yeh et al. 2023). Traps with baits and semiochemical lures are widely used for adult SWD field monitoring (Cloonan et al. 2018). However, trap captures with baits are either not correlated to or weakly correlated to infestation rates (Harris et al. 2014, Burrack et al. 2015). Thus, monitoring larvae in fruits remains the most reliable predictor of fruit damage (Van Timmeren et al. 2017, 2021).

Few methods are available to estimate the presence and number of SWD larvae in soft or stone fruits (Van Timmeren et al. 2017, 2021, Shaw et al. 2019, Babu et al. 2023). First, while the dissection of fruits allows the detection of larvae, this method can underestimate the number of larvae, even when using a stereomicroscope (Shaw et

al. 2019). Second, the extraction of larvae from fruit, using crushing with sugar or salt floatation and filtration allows for the detection of small larvae resulting in a more accurate estimation of both the presence and number of larvae than dissection methods (Van Timmeren et al. 2017, 2021). For field trials that require large sample sizes, floatation and filtration can be labor-intensive and require large quantities of liquid for floatation (i.e., either sugar, salt, or detergent solution; Van Timmeren et al. 2017). Although it does not require crushing fruits, an alternative method based on the vacuum extraction of larvae requires buying a vacuum pump (for approximately $250) and safe operation (Babu et al. 2023). As all these methods can be impractical for the daily inspection of fruit infestation by agronomists or fruit growers, more practical and economically viable methods are needed. Due to the fiber content of strawberry, the estimation of infestation rates in this fruit is more challenging than estimation in other fruits (blueberry, blackberry, raspberry, and cherry; Shaw et al. 2019) and hence was the focus of this study.

To estimate the number of SWD larvae in strawberry fruits, we aim to develop an easy-to-use method based on crushing one or several fruits within plastic sleeves and direct observation. Efficacy of the method was based on the rate of false negatives, the rate of false positives, speed, accuracy, precision, and within- and among-observer repeatability.

Materials and Methods

General Methods

All experiments were conducted at Centre Technique Interprofessionnel des Fruits et Légumes (CTIFL) in Bellegarde, France in 2021–2022. Additional details regarding the sleeve method are provided in Supplementary materials (Supplementary Fig. S1).

Sample preparation.

We first estimated the average time required to prepare samples using 99 commercial strawberry fruits. After removing the calyx, we placed each fruit in the middle of a transparent polypropylene sleeve (pack of 100 punched pockets—A4 Clear Transparent 50 Microns Polypropylene, 11 holes, 21 cm × 29.7 cm, Office depot product no: 0000033761, Fig. 1A), then softly squashed each fruit into a thin layer of puree with the hand palm without applying too much pressure that might damage larvae (Fig. 1B).

Larval count.

To estimate the performance of the sleeve method, we sampled 200 strawberries from a commercial glasshouse in the south of France (L'Isle-sur-la-Sorgues, France). The strawberries were split between 2 treatments: 100 strawberries were individually frozen at –20 °C for 4 days (control treatment), while the other 100 strawberries were individually incubated at 24 °C for 2 days (incubation treatment). The strawberries of this incubation treatment were then frozen for 2 days at –20 °C, which allowed us to count on a single day the larvae in strawberries from both treatments. In addition, freezing the sample kills the larvae and prevents them from moving across the sleeve, which facilitates counting. Each strawberry was allowed to thaw at room temperature, before being prepared according to the sleeve method. The exact number of larvae in each strawberry sample was counted by 2 expert observers (G. Z. and R. B.) and double-checked using a stereomicroscope. We then chose 15 strawberry samples in each of the 2 incubation treatments. Samples were carefully chosen to approximately match the number of larvae between the 2 incubation treatments (i.e., 5 samples did not include any larva, while the 10 remaining samples had between 1 and 32 larvae). We then asked 10 non-expert observers to count larvae in each of these 30 strawberry samples, presented in a random order. Among observers, 3 had never seen a SWD larva before (hereafter 'naive'), while the 7 others had seen SWD larvae in cherries at least once (hereafter 'experienced'). Importantly, only 2 experienced observers had previously counted SWD larvae once for a pilot of this study. Each observer counted with the naked eye the number of larvae in each of the 30 strawberry samples. To this end, observers placed each plastic sleeve on a transparent 1-cm grid paper laid above a square LED light (START Panel Backlit 600 IP65 36W 4400lm 840, Sylvania; Fig. 1C). After the first count of all 30 samples, a second count was performed in a random order which was different from the first (so that replicate counts can be considered independent), which resulted in 600 observations in total (30 samples × 10 observers × 2 replicates). To estimate the speed of the method, the observer recorded the time required for counting larvae in the fruit sample using a manual chronometer, for each of the 2 replicate counts separately.

Estimating the Speed, Accuracy, Precision, and Repeatability of the Sleeve Method

To assess the performance of the sleeve method, we used the dataset with 600 observations to estimate the rate of false negatives, the rate of false positives, the speed, accuracy, precision, and repeatability. To estimate the rate of false negatives and false positives across observers, we estimated the rate of failure to detect a larva and the rate of misidentification. We estimated the accuracy (bias relative to the exact number of larvae based on the repeated larval counts of each sample by the 2 experienced observers) and precision (magnitude of

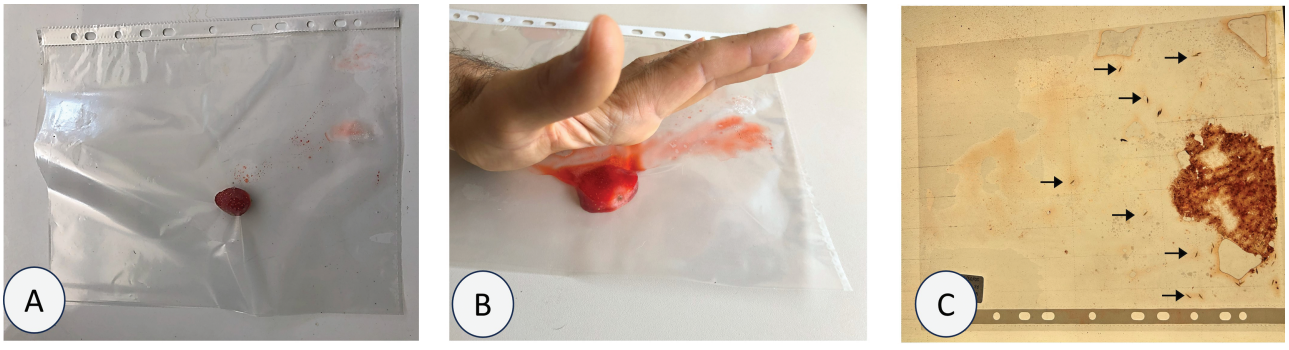

Fig. 1. Monitoring the prevalence and intensity of infestation of *D. suzukii* larvae in strawberries using the plastic sleeve method. A) Place one or more strawberry in the middle of a transparent plastic sleeve, B) squash the strawberry with hand palm, to count larvae, and C) place the sleeve against a light source. Larvae are indicated by arrows.

the standard deviation among observers) of each larval count. To control for the potential confounding effects of short-term experience (first vs. second replicate count of the same sample) and previous observation of SWD larvae (naive vs. experienced observers), we split the 600 observations into 4 different datasets depending on the number of the replicate and whether observers were naive or experienced. For each of the 30 samples in each dataset, we computed the bias of each larval count as the difference between the average of larval counts across observers and the exact number of larvae and we computed the standard deviation among larval counts from different observers (2 types of observers × 2 replicates × 30 samples = 120 bias/standard deviation estimates across the 4 datasets).

Finally, we also estimated the within- and among-observer repeatability of larval counts of the method. Repeatability "expresses the proportion of the total variation that is reproducible among repeated measurements of the same subject or group" (Nakagawa and Schielzeth 2010). The within-observer repeatability is the proportion of the total variation in larval counts that can be attributed to within-observer variation. For example, observers might make some random count errors, which will contribute to an increase in the total variation in larval counts. In contrast, the among-observer repeatability is the proportion of the total variation in larval counts that can be attributed to among-observer variation. For example, one observer might consistently count fewer larvae in each fruit than another observer, which will increase the total variation in larval counts.

Statistical Analyses

All statistical analyses were performed using R statistical software (R Core Team 2022). To estimate the speed, accuracy, precision, and repeatability of the sleeve method, we used separate linear mixed models (LMMs) with a Gaussian distribution. For the analyses of speed, accuracy, and precision, we tested for main effects and interactions using stepwise model selection with likelihood ratio tests (Bates et al. 2014). Finally, for each analysis, we visually checked for the normality of the residuals of the final model.

To investigate the effects that could affect the speed of the sleeve method, we fitted LMMs on the log-transformed time to count each sample. Fixed effects included the 48 h-incubation status ("yes" vs. "no"), previous observation of SWD larvae ("experienced" vs. "naive"), and the short-term experience of the observer ("first" vs. "second" replicate count) as factors. To test whether the speed varied depending on the number of larvae in the sample, we also included the exact number of larvae as a continuous covariate. To test whether the effect of incubation on speed varied depending on the number of larvae, the model also included an interaction between incubation status and exact number of larvae. To account for the non-independence among observations from the same fruit sample or from the same observer, all models included the identity of the strawberry and the identity of the observer as random effects.

To investigate the accuracy of the sleeve method, we fitted LMMs on the 120 bias estimates across the 4 datasets using the same 4 fixed effects as in the analysis above. To account for the non-independence among observations from the same fruit sample, all models included the identity of the strawberry as random effect (observations are averaged across observers so that a random observer effect is not necessary). To investigate the precision of the sleeve method, we fitted LMMs on the 120 standard deviation estimates using the same 4 fixed effects and the random effect as in the accuracy analysis above.

Finally, to estimate the within- and among-observer repeatability, we fitted a single LMM on the log-transformed count of larvae which included the identity of the strawberry sample and the identity of the observer as random effects (Eq. 11 in Nakagawa and Schielzeth 2010).

Results

Rates of False Negatives and False Positives

Across the 30 strawberry samples, larvae were present in 10 samples with incubation and 10 samples without incubation (i.e., 66% of the fruits) with 4.6 (SD = 6.94) larvae per strawberry fruit. Across these 20 samples, failure to detect an infestation was 11.2% (i.e., 45 in 400 counts with 9.0% in samples with incubation and 13.5% in samples without incubation). Across the other 10 samples with no larvae (non-infested), only 1 observer counted 1 larva in 1 of the 2 replicate counts. Hence, the rate of false detection of infestation was 0.5% (1 in 200 counts).

Speed of the Sleeve Method

The average time required to crush one strawberry in a plastic sleeve was 16 s (SD = 11 seconds). The average time required to count larvae in one strawberry sample was 25 s (SD = 15). Hence, the total time to process one strawberry sample was 41 s (SD = 19). The average time required to count larvae in each fruit increased with the number of larvae (from 17.5 seconds for fruits with fewer than 5 larvae to 63.0 s for fruits with more than 15 larvae; $\chi^2_1 = 29.52$; $P = 5.10^{-8}$; Fig. 2A; Supplementary Table S1). When accounting for this effect of the number of larvae on count time, incubating fruits for 48 h did not significantly decrease the amount of time required to count larvae in each fruit ($\chi^2_1 = 0.22$; $P = 0.64$; Fig. 2A). For each sample and observer, the time needed to count larvae was lower for the second than for the first count ($\chi^2_1 = 8.69$; $P = 0.003$; Fig. 2A), indicating a short-term effect of previous experience. Observers with previous experience in the observation of SWD larvae did not count faster than observers without any previous experience ($\chi^2_1 = 0.73$; $P = 0.39$), indicating that this variable did not conflate our results.

Accuracy of the Sleeve Method

The number of larvae per sample was consistently underestimated (negative bias) as the exact number of larvae per sample increased (Fig. 2B). The increase in this underestimation was significantly lower in incubated than in non-incubated samples (significant interaction between incubation status and exact number of larvae; $\chi^2_1 = 11.40$; $P = 0.0007$). We estimated that when the exact number of larvae increased by 10 larvae, the observed number of larvae increased by only 5 larvae when samples were not incubated or by 3 larvae when samples were incubated (dotted and solid lines in Fig. 2B). Importantly, this interaction remained significant when removing 1 non-incubated sample that included more than 30 larvae ($\chi^2_1 = 5.17$; $P = 0.02$). For each sample and observer, the accuracy of second count replicate was slightly and significantly higher than that of the first replicate ($\chi^2_1 = 7.22$; $P = 0.007$; red and green lines in Fig. 2B). Experienced observers did not count more accurately than naive observers ($\chi^2_1 = 0.68$; $P = 0.41$), indicating that this variable did not conflate the results on the method's accuracy.

Precision, Within- and Among-Observer Repeatability of the Sleeve Method

The precision of the method decreases as the exact number of larvae per sample increases, as expected with count data (increase in the

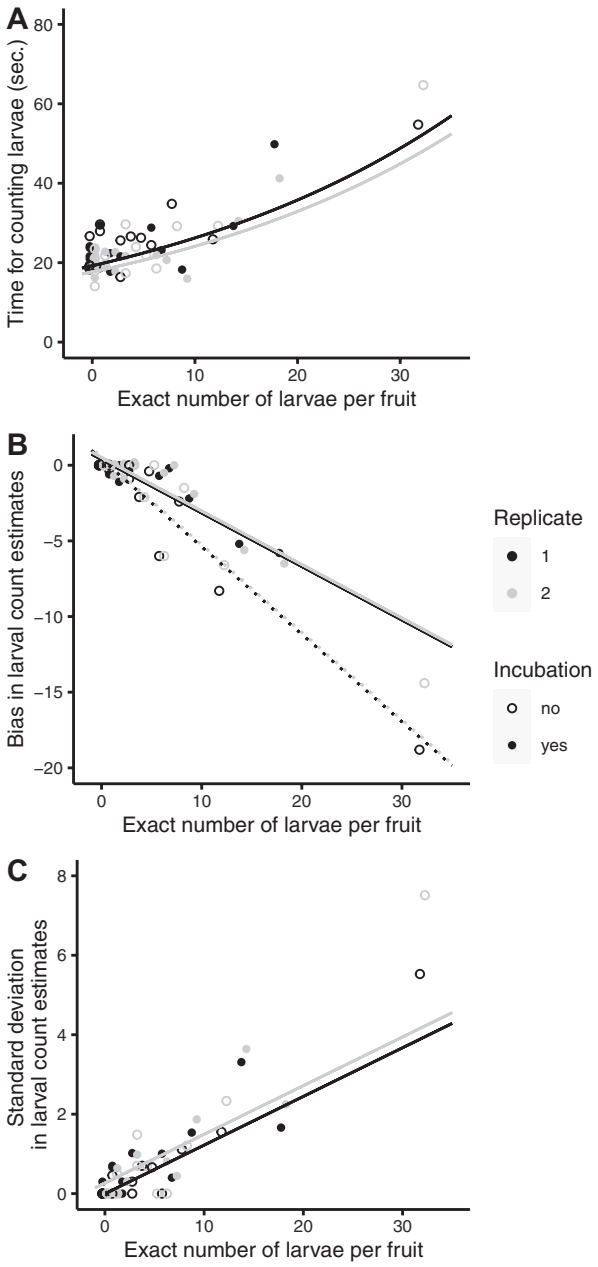

Fig. 2. The 3 parameters for the performance of the sleeve method, A) counting time, B) accuracy, and C) precision (i.e., inverse of the standard deviation among estimates), can depend on the number of larvae, on the 48-h incubation or not (filled and solid circles) and on the order of the 2 replicate counts of the same sample (black and grey symbols). Dotted and solid lines represent the fitted lines for samples, respectively, counted with or without a 48-h incubation (the 2 types of lines are superimposed in panels A and C).

standard deviation with the number of larvae in Fig. 2C). Sample incubation did not significantly affect the precision of counts ($\chi^2_1 = 0.65$; $P = 0.42$; superimposed solid and dotted lines in Fig. 2C). For each sample and observer, the precision of second count replicate was slightly and significantly lower than that of the first replicate ($\chi^2_1 = 5.93$; $P = 0.01$; red and green lines in Fig. 2C). However, the previous observation of SWD larvae did not affect the speed of counts ($\chi^2_1 = 0.24$; $P = 0.62$), indicating that the experience of observer did not conflate the results on the method's precision.

The variance in the number of larvae among samples ($V_{sample} = 0.81$) was large relative to the variance among observed ($V_{among} = 0.005$) and the residual within-observer variance ($V_{within} = 0.04$). Hence, within- and among-observer repeatabilities were greater than 94% (Supplementary Fig. S1).

Discussion

We developed a new easy-to-use method for monitoring SWD infestation in strawberries by non-expert observers. By crushing fruits within a transparent plastic sleeve, the method provided satisfactory results for all performance criteria (failure to detect an infestation, false detection of an infestation, speed, precision, within- and among-observer repeatability), but accuracy at high infestation levels (Fig. 2B). Overall, the sleeve method allows for the fast detection of larvae with the naked eye or with a magnifier. The thorough experimental design and robust statistical framework used to assess the performance of the sleeve method are readily transferable to assess the performance of similar methods to count the larvae of other insect pests.

Advantages of the Sleeve Method Compared to Other Methods

The underestimation of the number of larvae at high infestation levels is likely due to observers losing track of the larvae they already counted and might be independent of the fruit preparation method. If intensities of infestation are to be estimated, we recommend counting the exact number of larvae on a fraction of the samples to perform a calibration. As a rule of thumb based on calibration from Fig. 2B, we estimate that the observed number of larvae should be multiplied by 1.5 (respectively by 2) to estimate the exact number of larvae in the presence (respectively in the absence) of incubation.

The sleeve method allows higher recovery rates than previous methods based on salt or sugar floatation or vacuum extraction. These studies have acknowledged the risk of larval recoveries lower than 100% (as larvae can remain within the fruits and remain undetected; Van Timmeren et al. 2021, Babu et al. 2023). With the sleeve method, accuracy is not affected by larval recovery, as 100% of the larvae end up in the sleeve. In case of doubt regarding the presence of larvae overlapping with or remaining under fruit material both sides of the sleeve can be checked. Furthermore, total processing time per fruit with other methods is typically more than 120 s (Van Timmeren et al. 2017, 2021, Babu et al. 2023). With the sleeve method, total processing time per fruit (to prepare samples and count larvae) is 41 s for infestation intensities ranging from 5 to 10 larvae, which results in a high throughput of ~100 fruits per hour. Although freezing unincubated fruits can ease their squashing, this step remains optional (incubated fruits are easy to squash and do not require freezing). Considering the short time needed to process fruit samples and count larvae with our method, we presume that the sleeve method may perform better than previous monitoring methods. Nevertheless, this presumption needs to be confirmed by a direct comparison between our and other methods by the same observers. Finally, the sleeve method requires less consumables than other methods, as transparent plastic sleeves are reusable many times (Table 1). In contrast with other methods, the sleeve method is versatile as both unhatched eggs and larvae can be counted with the naked eye or with a stereomicroscope (the latter allows for a higher detection rate of eggs and small first- or second-instar larvae).

Table 1. Comparison of the different methods available to estimate the presence and number of SWD larvae in strawberry fruits. Optional steps are indicated between parentheses. We assume that count error is low and equal among the different methods

Reference	Method	Mean/variance in presence and number of SWD larvae	Larval recovery	High-throughput	Stereomicroscope	Material required	Potential freezing for later detection
Shaw et al. 2019	Dissection	Mean and variance	100%	–	No/Yes	Dissection forceps	Yes
Shaw et al. 2019	Crush + floatation	Mean and variance	≤100%	–	No	Plastic funnel, cloth, floatation solution	No
Van Timmeren et al. 2017, Van Timmeren et al. 2021	Crush + floatation + filtering	Mean and variance	≤100%	+	No/Yes	Plastic funnel, cloth, reusable coffee filter, floatation solution	No
Babu et al. 2023	Vacuum + rinsing + filtering	Mean only	≤83%	++	No/Yes	Vacuum pump, reusable coffee filter	No
This study	(Incubate+) (Freeze+) crush	Mean and variance	100%	++	No/Yes	Plastic sleeve (remain transparent even after being properly rinsed with water and reused more than 5 times)	Yes

Advantages of the Short-Term Experience of Observers

Although not investigated in other methods, previous short-term experience of observers allows faster and more accurate counts. Indeed, the accuracy of the sleeve method was on average higher in the second than in the first replicate count (Fig. 2B). This suggests that the skills of observers to count larvae could be improved by a quick preliminary session (e.g., by counting mock samples with known numbers of larvae).

Advantages and Drawbacks of Incubation in the Sleeve Method

In the sleeve method, incubating strawberries at 24 °C for 2 days allows for more accurate estimations of the number of larvae in each fruit, but not faster or more precise counts. Incubation likely allows the detection of second- and third-instar larvae initially present as eggs or first instar. Incubation is particularly advantageous for estimating the number of larvae per fruit when infestation intensities are high, as it reduces the underestimation of the number of larvae (Fig. 2B). Everything else being equal, incubation does not affect larval recovery, as third-instar larvae within fruits are counted as pupae after 2 days of incubation at ~20 °C. The main drawback of incubation is a potential underestimation of prevalence and intensity of fruit infestation due to larval mortality. For example, microbial development during incubation could decrease larval survival when using incubation in the sleeve method. The relative drawbacks of incubation due to larval mortality remain to be quantitatively assessed.

From a more practical perspective, fruit incubation may help growers determine their treatment plans such as pre-harvest insecticide application or post-harvest cold storage. In contrast, incubation might not be required by growers who wish to detect larval infestation to decide whether their fruit can be marketed (in particular, if fruits are always stored cold prior to marketing).

Advantage for Fruit Growers and Academic Researchers

Fruit growers, academic researchers, and agronomists can easily use the sleeve method. Fruit growers are interested in assessing the presence of larvae in real time to make management choices and make decisions on fruit marketability or insecticide applications. In this case, the incubation and freezing steps of the sleeve method can be omitted.

In contrast, academic researchers are often interested in assessing both the mean and variance of the prevalence and intensity of fruit infestation (Table 1; McIntosh et al. 2022). In this case, incubation can allow for a more accurate estimation of infestation intensity. In addition, an important advantage of the method is that samples can be stored frozen until further processing, which allows for experiments with large sample sizes. The sleeve method will be particularly useful for the development of innovative pest control strategies by allowing the accurate estimation of their efficiency in both laboratory and field settings (e.g., for the development of the sterile insect technique).

Based on data from non-expert observers, the sleeve method presents advances over previous methods. Incubating fruits improves the accuracy of the method. This study represents a first proof of principle of the utility of this new method for the estimation of the prevalence and intensity of SWD infestation of soft and stone fruits. As strawberries are among the fruits where the detection of infestation is the most difficult (Shaw et al. 2019), the sleeve method is likely to easily be transferable to other fruits.

The thorough experimental design and robust statistical framework used here will allow for additional development of the sleeve method to detect SWD larvae in other fruits or to detect the larvae of other insect pests that infest soft or stone fruits. It thus represents a promising alternative to floating techniques currently used to detect the larvae of other pest insects such as tephritids (*Bactrocera tryoni*, *Ceratitis capitata*, or *Rhagoletis* sp.; Yee 2014, Balagawi et al. 2022).

Finally, further research on the comparison of different fruit sampling strategies (i.e., varying the spatial and temporal distribution of sampling sites within the field, number of fruit samples, etc.) will advance the utility of the sleeve method in monitoring infestations. We hope that the ease of this new method will encourage fruit growers to incorporate direct monitoring of fruit samples in their IPM program. The accurate detection of larvae and other insect pests will likely help them make informed decisions and improve the timing of control methods.

Acknowledgments

We are grateful to the volunteers for their participation in the study and to Frédéric Soula for providing strawberries for the experiment. We also thank Joël Escaich, Isaack Wanko, and Antoine Escamez for their help. We also thank Hannah Burrack and 3 anonymous reviewers for their comments.

Funding

This research was funded by the CTIFL (project " Élevage TIS"). L.T. B. and P. P. were supported by funding from the Agence Nationale de la Recherche (ANR-21-ECOM-0002).

Author Contributions

Ghais Zriki (Conceptualization [lead], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Project administration [equal], Supervision [equal], Visualization [equal], Writing—original draft [lead], Writing—review & editing [equal]), Rémy Belois (Data curation [lead], Investigation [equal], Methodology [equal], Resources [equal], Writing—review & editing [supporting]), Christine Fournier (Data curation [equal], Investigation [equal], Resources [equal], Writing—review & editing [supporting]), Léa Tergoat-Bertrand (Data curation [equal], Methodology [equal], Writing—review & editing [supporting]), Pierre-Yves Poupart (Data curation [equal], Methodology [equal]), Amélie Bardel (Investigation [supporting], Methodology [equal], Writing—review & editing [supporting]), Benjamin Gard (Methodology [equal], Writing—review & editing [equal]), and Nicolas Rode (Conceptualization [equal], Methodology [equal], Software [equal], Supervision [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [lead])

Data availability

The data and R scripts to analyze the performance of the sleeve method are available at: https://github.com/nrode/Y2023.LarvCount

Supplementary material

Supplementary material is available at *Journal of Economic Entomology* online.

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