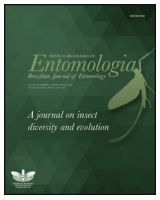
Revista Brasileira de Entomologia 68(1):e20230026, 2024

SOCIEDADE BRASILEIRA DE ENTOMOLOGIA
FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution



Evaluation of water-soluble dyes to mark internal structures of Lepidoptera via larval feeding

Gabriel Fernandes Rezende[1], Odair Aparecido Fernandes[1*]

[1]*Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brasil.*

ARTICLE INFO

Article history:
Received 08 May 2023
Accepted 29 January 2024
Available online 19 April 2024
Associate Editor: José Serrão

Keywords:
Insect movement
Gene flow
Mark-capture technique
Spatial distribution

ABSTRACT

Understanding aspects of insect ecology is a key component for the applicability of control methods or pest resistance management. For instance, the comprehension of insect dispersal is crucial to determining insect gene flow and the maximum distance between refuge areas in *Bt* (*Bacillus thuringiensis*) crops. But, for such studies, insects need to be marked prior to release. Seeking to refine the technique of dispersion studies of the genetic material of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), this study aimed to determine the efficiency of water-soluble dyes for the internal marking of *S. frugiperda* immatures and adults via larval feeding, with emphasis on marking the spermatophore. Rhodamine B, Methylene Blue, Ponceau, Coomassie, and Eosin-Nigrosin were added to the larval artificial diet at concentrations of 0, 100, 200, 400, 600, 800, and 1000 ppm. Mortality and duration of the larval stage as well as the efficiency of marking internal structures of larvae and adults were evaluated. Rhodamine B and Methylene Blue increased the duration of the larval period, but the former caused higher mortality. The staining of the gut, hemolymph, and imaginal discs, in the larval phase, was observed for Rhodamine B, Methylene Blue, and Coomassie dyes. However, none of the dyes were efficient for marking spermatophores. Thus, the addition of these water-soluble dyes to the larval diet of *S. frugiperda* can mark internal structures of the larva, but not the adult. Therefore, these dyes have limited application once they may be used only for marking larvae.

Introduction

Plant resistance through the expression of insecticidal proteins (*Bt* plants) represents currently one of the main strategies for managing agricultural insect pests, with increasing adoption worldwide (ISAAA, 2019). However, the limited array of insecticidal proteins and the widespread use of *Bt* plants have caused the development of insect populations resistant to this control tactic (Bentivenha et al., 2019). This was the case observed in the fall armyworm - FAW, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), whose population developed resistance to the *Bacillus thuringiensis* Cry1F protein expressed in maize hybrids in just four years after the release of the technology (Farias et al., 2014; Monnerat et al., 2015). Therefore, the sustainability of the *Bt* crops should include management strategies for resistant insect populations within an integrated pest management program.

In order to minimize the development of resistant populations, some management strategies were established such as the adoption of refuge areas, expression of high doses (capable of controlling > 95% of heterozygous individuals), and the pyramiding of proteins (Fatoretto et al., 2017; Tabashnik and Carrière, 2017; Gilreath et al., 2021). Refuge areas allow the development of Bt-insecticidal protein susceptible insects. These insects disperse and mate with resistant ones in Bt crops, resulting in susceptible heterozygotes (Fatoretto et al., 2017). However, for this strategy to be effective, refuge areas must be near to *Bt* crops ensuring a higher mating rate between resistant and susceptible insects. The maximum distance established between refuge areas and *Bt* maize plants is 800 m for both *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) in the United States (Showers et al., 2001) and *S. frugiperda* in Brazil (Vilarinho et al., 2011).

Determining the maximum distance between refuge areas and the farthest point in the *Bt* crop considers insect dispersal capabilities, usually assessed through a combination of insect marking techniques and Marking-Release-Capture methods. In this process, laboratory-reared marked insects are released into the field and captured at varying distances, enabling the determination of their movement range. Marking can be performed externally or internally and ensures the differentiation of the released insects from wild ones (Gangwere et al., 1964; Southwood, 1978; Hagler and Jackson, 2001).

Internal marking is highly recommended for field studies, as it avoids transfer between insects and is less susceptible to losses due to factors such as rain or wind (Southwood, 1978). However, efficient

*Corresponding author:
E-mail: odair.fernandes@unesp.br (O.A. Fernandes).

https://doi.org/10.1590/1806-9665-RBENT-2023-0026

internal marking poses challenges, demanding dyes that provide easy marking and evaluation of numerous insects without sophisticated equipment. Additionally, marking should require minimal insect manipulation, be distinguishable from wild individuals, persist for the period of study, and cause no impact on insect biology or behavior (Gangwere et al., 1964; Hagler and Jackson, 2001). Dyes with lower excretion rates are also preferable (Nijhout, 1975; Southwood, 1978; Hagler and Jackson, 2001).

Marking internal structures of adults provides vital insights into genetic material dispersal. Marking structures such as the spermatophore, transferred from males to females during mating, enables identification of male groups. Such a technique facilitates gene flow distances once the release point is established. Although previous studies on *S. frugiperda* adult dispersion via internal marking with liposoluble dyes successfully determined the species dispersal capacity (Vilarinho et al., 2011), spermatophores were not assessed, leaving genetic material dispersion unexplored.

Due to the important implications of marked spermatophores, this technique could remodel structured refuge areas in commercial maize and other *Bt* crops. The development of a technique to identify the origin of a male sexual content (spermatophores) within a female would allow to track down the distance moved from the site of release of the male insect. Moreover, movement of unmated and mated insects is key for assure gene flow of susceptible insect to and from refuge (conventional crop) and Bt-crops and, therefore, for management of resistance. Consequently, this study aimed to evaluate the efficiency of water-soluble dyes in marking the internal structures of *S. frugiperda* larvae and adults, especially spermatophores, by incorporating these dyes into the artificial diet during the larval phase.

Material and methods

Insects

Egg masses of *S. frugiperda* were obtained from a laboratory colony. This population, originated from maize, has been maintained for ca. 20 years without reintroduction of field insects, ensuring the absence of resistance alleles to insecticidal proteins (unpublished data). The egg masses were cleaned using a sequence of aqueous solutions: 10% formalin, distilled water, and 1% copper sulfate. After drying out in room temperature for approximately 30 min, egg masses were placed in a 100 mL plastic cup (COPAZA®, Içara, SC, Brazil) with lid. Upon hatching, larvae were fed a 4 cm^3 of an artificial bean-based diet (Vilela et al., 2014) and reared under controlled conditions (25 ± 2°C; RH 70 ± 10%; Photophase 12h).

Insect marking and evaluation

Water-soluble dyes (Table 1) were incorporated into the standard diet (mentioned above) and were selected based on their efficacy for marking proteins, the main constituent of a spermatophore (Heller et al., 1998). This strategy was proposed to facilitate marking a larger number of insects and to enable field studies with newly emerged adults. Powder dyes were diluted in 10 mL of distilled water to provide better incorporation into the diet, while liquid dyes were added directly into the diet without previous dilution. Concentrations used were 0, 100, 200, 400, 600, 800 and 1000 ppm. This range was established based on Sparks and Cheatham (1973) and Vilarinho et al. (2006), whose studies enabled to mark insects without affecting the biological aspects of FAW at a concentration of 1000 ppm (injection into the adult) and 400 ppm (through larval feeding), respectively. Upon incorporation of the dye, the mixture was homogenized using a spatula, in circular movements until reaching a homogeneous color. Subsequently, the diet was kept under UV light for 60 min at room temperature for sterilization and transferred to a refrigerator (5 °C) for solidification.

Third-instar larvae were used to evaluate the dyes. This instar was adopted due to the higher mortality of neonates observed in a preliminary study. The larvae were individualized in Petri dishes (6 cm diameter x 2 cm height) containing 4 cm^3 of dye-containing diet, according to the established treatment (dye and concentration). Larvae were observed daily, and confirmed dead insects were registered and removed from the experiment.

A completely randomized design was used with 100 replications per treatment (concentration). Each experimental plot was represented by a Petri dish containing one larva (experimental unit). The insects were kept in a climate-controlled room (25 ± 2°C; RH 70 ± 10%; Photophase 12h). The dyes were evaluated separately, leading to five distinct experiments.

After pupation, insects from the same treatment were maintained in a single mating cage, consisting of a polyvinyl chloride (PVC) tube (20 cm high x 10 cm diameter) covered internally with bond white paper, which was used as a substrate for oviposition. Once emerged, adults were kept in a cage for seven days, when the internal marking was evaluated. This period allows visual confirmation of mating and observation of the first egg masses, ensuring the transfer of spermatophores to females (Wang et al., 2019; Ge et al., 2021). Small plastic containers (10 mL) with hydrophilic cotton swabs soaked in a solution of distilled water and honey (10%) were placed on top of each cage for adult feeding.

All larvae were handled ventrally for confirmation of marking by directly observing color changes without dissections. Five sixth-instar

Table 1
Dyes used to mark internally *Spodoptera frugiperda* larvae and adults.

No.	Dye	Source	Color	Information	Reference
1	Rhodamine B	Sigma-Aldrich	Red	Efficient for internal labeling of *Heliothis virescens* (Fabricius, 1781) (Lepidoptera: Noctuidae), *Aedes aegypti* (Linnaeus, 1762) and *Anopheles gambiae* s.l. (Diptera: Culicidae), and *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae) adults	Sparks and Cheatham (1973), Blanco et al. (2006), Johnson et al. (2017), Aviles et al. (2020)
2	Methylene blue	Sigma-Aldrich	Blue	Efficient for the internal marking of *M. sexta* adults of and *Norileca indica* (H. Milne Edwards, 1840) (Crustacea: Isopoda, Cymothoidae)	Sparks and Cheatham (1973), Kottarathil and Kappalli (2019)
3	Eosin – Nigrosin	Botupharma	Dark Violet	Not used for marking insects, but it was efficient for marking spermatozoa of *Pecari tajacu* (Linnaeus, 1758) (Artiodactyla, Tayassuidae) and *Mus* spp. (Rodentia: Muridae)	Sousa et al. (2013), Mazaheri et al. (2020)
4	Ponceau Red	Sigma-Aldrich	Red	It was used for marking insects, such as the brain of *Apis mellifera* (Linnaeus, 1758) (Hymenoptera: Apidae) and protein bands	Venkatesha et al. (2013)
5	Coomassie	Sigma-Aldrich	Blue	Widely used in gel electrophoresis	Noaman and Coorssen (2018)

larvae from each treatment were dissected to evaluate color change of internal organs, including the imaginal discs. Adult females were dissected using tweezers to remove the final portion of the abdomen to evaluate the bursa copulatrix, where the spermatophores are stored in mated females. Observations were made using a stereomicroscope at 10x magnification (Carl Zeiss, Jena, Germany). The marking of spermatophores was determined by visual analysis and comparison with spermatophores from the control (no dye).

Statistical analysis

Mortality data and larval stage duration for the tested dyes were submitted to analysis of variance (ANOVA – ExpDes.pt Package). Abbott's formula was used to correct the natural mortality observed in the control (without dye addition). Mean comparisons were conducted using Tukey's test at 0.05 significance using the package agricolae (R Core Team, 2020).

Results

Ponceau, Methylene Blue, Rhodamine B, and Coomassiedyes evidently marked the gut when observed through the ventral part of the insect, avoiding the need for dissection. Hemolymph of larvae was distinctly marked by Rhodamine B, displaying an intense red color. Conversely, Methylene Blue and Coomassie Blue resulted in lighter hemolymph marking. Ponceau and Eosin- Nigrosin dyes did not stain the hemolymph.

Imaginal discs exhibited limited marking when larvae were fed diets containing Methylene Blue, Rhodamine B, and Coomassie dyes (Table 2). Larvae fed with these dyes were easily identifiable through external observation due to distinct coloring. Marking was considered evident when marked insects were distinguishable from the control. The clearer the distinction from non-marked insects, the more evident the marking was considered.

During the dissection of adult females, internal structures such as gut, fat body, and ovaries showed no marking for any tested dyes. Similarly, no marking was observed on spermatophores obtained from mated females (Table 2, Figure 1). The efficiency of spermatophore marking was visually determined by comparing their coloration with spermatophores from the control treatment (Figure 1A), which exhibited varying colors, from light and translucent to black and opaque.

Blue and red dyes generally produced light-colored spermatophores, indistinguishable from the pattern observed in the control (Figure 1B-E). Spermatophores from Eosin-Nigrosin dye were black (Figure 1F). Despite aligning with the natural color of the dye, these spermatophores were

Table 2
Visual response of marking internal structures of *S. frugiperda* larvae and adults via ingestion of dye-containing artificial diet during larval stage.

Dye	Larva			Adult		
	gut	hemolymph	imaginal discs	fat body	ovary	spermatophore
Ponceau	+++	-	-	-	-	-
Methylene blue	+++	++	+	-	-	-
Rhodamine B	+++	+++	+	-	-	-
Eosin – Nigrosin	-	-	-	-	-	-
Coomassie	+++	++	+	-	-	-

- marking not observed; + little obvious marking; ++ evident marking; +++ very evident marking.

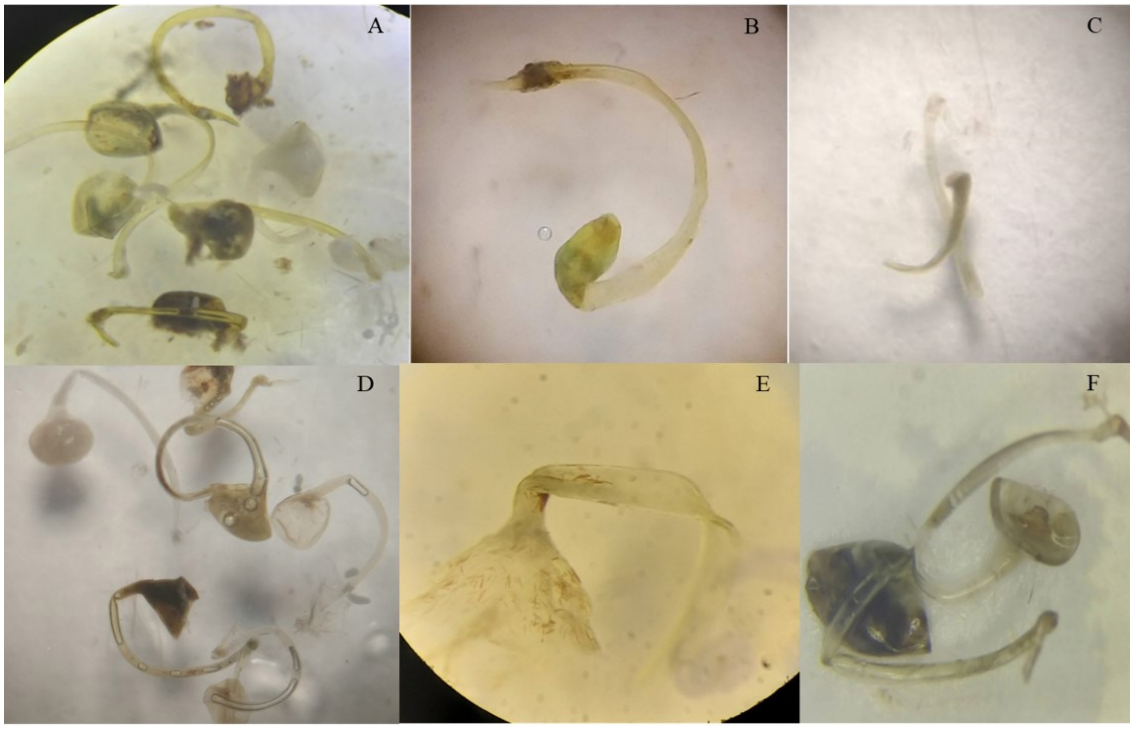


Figure 1 Spermatophores retrieved from *S. frugiperda* mated adult females whose larvae were fed with water-soluble dyes. (A) control insect (without dye); (B) Methylene Blue; (C) Coomassie; (D) Ponceau; (E) Rhodamine B; (F) Eosin - Nigrosin.

not considered marked, lacking a clear distinction from those taken from control insects, which also presented black-colored spermatophores.

The addition of Ponceau ($F_{5,6}$ = 0.58; P = 0.71), Methylene Blue ($F_{5,6}$ = 0.58; P = 0.72), Eosin-Nigrosin ($F_{5,6}$ = 0.61; P = 0.69), and Coomassie ($F_{5,6}$ = 0.99; P = 0.48) dyes into the artificial diet did not increase the mortality of *S. frugiperda* larvae compared to the control treatment. In all tests, mortality in the control treatment (diet without dye) remained below 10%. In contrast, Rhodamine B dye led to significant larval mortality ($F_{5,6}$ = 73.57; P < 0.0001), with the highest mortality observed at high concentrations, starting from the 600 ppm dose (Figure 2). Larval mortality mainly occurred during the pre-pupal stage when larvae ceased feeding and began molting to the pupal stage.

Larval development was not affected by diets containing Ponceau ($F_{6,7}$ = 1.94; P = 0.20), Eosin–Nigrosin ($F_{6,7}$ = 0.27; P = 0.94), and Coomassie ($F_{6,7}$ = 0.02; P = 0.99) dyes. In these essays, the larval periods in the control were 13.20; 15.30, and 17.00 days, respectively, with an increase of 8.9% for Ponceau and 3.1% for Eosin-Nigrosin in the highest concentrations. In contrast, Methylene Blue ($F_{6,7}$ = 18.17; P < 0.001) and Rhodamine B ($F_{6,7}$ = 5.69; P = 0.02) dyes increased larval development, particularly at a dose of 1000 ppm, with increases of 26.3% and 19.4%, respectively, compared to the control (Figure 3).

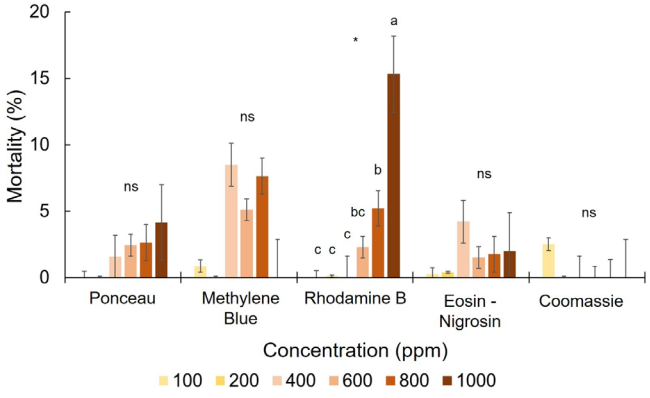


Figure 2 Mortality (%) of *S. frugiperda* larvae fed an artificial diet containing water-soluble dyes (ns indicates that the treatments did not differ from each other; bars followed by the same letter within the same dye are not significantly different by Tukey 's test at 0.05 significance). Data corrected by Abbott 's formula.

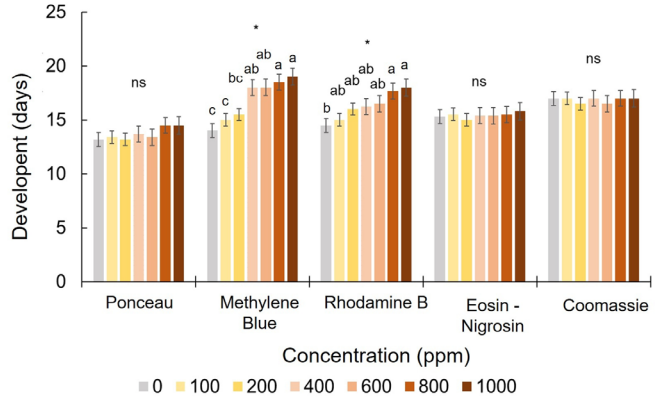


Figure 3 Development (days) of *S. frugiperda* larvae fed an artificial diet containing Ponceau, Methylene Blue, Rhodamine B, Eosin- Nigrosine and Coomassie dyes. (ns indicates that treatments did not differ from each other; bars followed by the same letter within the same dye are not significantly different by Tukey 's test at 0.05 significance).

Discussion

Marking internal structures of insects such as the gut, hemolymph, and imaginal discs in the larval stage was obtained using Rhodamine B, Methylene Blue, and Coomassie dyes incorporated into the diet. Conversely, Eosin-Nigrosin was not able to distinctly mark internal structures. The difference in staining intensity between these dyes was observed only in the hemolymph, with Rhodamine B marking the hemolymph very clearly in red and Methylene Blue and Coomassie staining the hemolymph in blue. The success in marking the internal structures is due, in part, to the constant re-entry of dyes via food, offered *ad libitum*, since dyes, like any other xenobiotic substances, are excreted both by the midgut and by the Malpighian tubules (Nijhout, 1975). Thus, larvae partially fed with dye-containing diets may not show the marking.

Ponceau dye was efficient for staining the gut resulting in an intense red color that could be observed regardless of the concentration used and even without dissection of the insects. This dye presented no effect on insect development. However, it failed to mark the hemolymph and imaginal discs. This dye was not able to cross the peritrophic membrane which is responsible for the largest absorption in the digestive tract. The peritrophic membrane of insects contains small pores, measuring between 7.5 and 8.0 nm (Terra, 2001), whereas Ponceau dye molecules exceed 30 nm. Venkatesha et al. (2013) demonstrated that this dye is retained when a membrane with 30 nm pores is used.

The excretion of dyes can also play an important role in eliminating dyes and, therefore, reducing insect marking efficacy. Dyes are xenobiotic compounds and may affect the insect's physiochemical dynamics, leading to biochemical responses toward excretion due to their toxicity (Fondren Junior and Heitz, 1979). In adult Lepidoptera, dyes are broken down in different places of the insect's excretory system and this excretion is intricately related to the pH of the dyes. Acidic dyes, such as Ponceau and Rhodamine B, are excreted in the midgut and middle section of the Malpighian tubules. In contrast, basic dyes, such as Methylene Blue, are excreted in the middle and proximal sections of the Malpighian tubules (Nijhout, 1975). Despite the observed excretion of dyes in our study, the constant reintroduction of the dye ensured its presence in sufficient quantities to guarantee effective marking (Barbosa and Peters, 1971).

Mortality of larvae exposed to Rhodamine B dye was likely due to its inherent toxicity because amine components of the dye replace oxygen molecules in the insect's body (Fondren Junior and Heitz, 1979). Increased dye concentration in the diet elevates toxicity overwhelming the excretory system and, consequently, negatively affecting insect development and survivorship. Despite its toxicity, Rhodamine B was used for marking spermatophores of insects, but it was efficient only when adults were fed upon or injected with the dye (Vail et al., 1966; Sparks and Cheatham, 1973; Van Der Reijden et al., 1997; South et al., 2008)

The Methylene Blue Eosin dye, one of the components of the Eosin-Nigrosin dye, demonstrated low mortality of the dyes at 0% and 2% concentrations when the adults of *Musca domestica* (Linnaeus) (Diptera: Muscidae) were fed with the dyes. (Yoho et al., 1973). Similarly, Robinson (1983) reported low mortality of *M. domestica* adults exposed to Methylene Blue whereas Eosin resulted in high mortality. The study emphasized the mortality concentration dependence, which, in our research was not evident.

Ponceau and Comassie dyes could be used as internal markers of insects, mainly staining the gut. These dyes are not commonly used for insect marking, but they have been used mainly in mammals (Tanaka, 2006). Therefore, our study brings insight into possible alternatives for marking insects. Despite their binding affinity to proteins (Tal et al., 1985), spermatophores were not stained and the high protein content in certain insects such as Lepidoptera (Foo et al., 2006) must have contributed to the reduction of dye availability.

The marking of internal insect structures, such as the gut, can be observed without the need of dissection. This becomes evident when the food load passes through the digestive tract (Vail et al., 1966). The change in diet color, obtained from the lowest concentrations of dye added, has the potential to change the internal coloration of insects. Therefore, it can be an important tool for studies of larval movement, as well as an indication for feeding biological control agents.

The use of a water-soluble-dye-containing diet for marking immature insects via feeding could be used as a method for the evaluation of the larval movement, making ease the distinction between marked and wild insects. However, the duration of marking after ceasing the supply of artificial diet-containing dyes needs further evaluation. Moreover, adult longevity was not evaluated in our study, but several studies have already indicated this biological parameter is not affected by water-soluble dyes (Vail et al., 1966; Sparks and Cheatham, 1973).

Marking insect spermatophores is crucial for evaluating insect movement, temporal mating status, and gene flow, but it remains a challenge in entomological research. The inefficiency of larval feeding as a marking method can be attributed to dye excretion by insects (Nijhout, 1975) and the reduction in feeding as well as clearing of the insect gut towards the end of the last larval instar before entering the pupal stage (Spranghers et al., 2018). Consequently, for the dye to persist into adulthood, it must be absorbed into the internal structures of the insect, such as the hemocoel and imaginal discs. Given that spermatophores consist of proteins (Heller et al., 1998), it is crucial for dyes to bind with protein compounds during the larval phase. Attempts to label spermatophores by providing liposoluble diets to the larvae have proven ineffective as these dyes bind with the fat body rather than proteins (Qureshi et al., 2006; Vilarinho et al., 2011). Noteworthy exceptions such as the studies of Hendricks and Graham (1970) and Cantelo (1973) exist in which successful marking of lepidopteran spermatophores using liposoluble dyes was observed. This phenomenon might be explained by the high concentration of dye bound to the fat body of insects during the metamorphosis phase, causing some dye molecules to bind to the male's reproductive system.

Our study demonstrated that marking *S. frugiperda* larvae, especially the gut, hemolymph, and imaginal discs by incorporating water-soluble dyes into the artificial diet was feasible. However, as spermatophores were not able to be marked using these dyes suggests that further studies or new techniques need to be developed. Although Eosin-Nigrosin, Ponceau, and Coomassie are efficient protein markers, they were not potential candidates for *in vivo* marking the internal structures of adult insects via larval feeding.

Acknowledgments

This work was carried out with the support of the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Financing Code 001.

Conflicts of interest

The authors declare no conflicts of interest.

Author contribution statement

OAF and GFR designed the experiment. GFR carried out the assays, performed the statistical analysis, created the graphs, and wrote the first draft of the manuscript; OAF coordinated the study development, provided funds, discussed the results, and reviewed the manuscript. All authors reviewed the manuscript prior to submission.

References

Aviles, E.I., Rotenberry, R.D., Collins, C.M., Dotson, E.M., Benedict, M.Q., 2020. Fluorescent markers rhodamine B and uranine for *Anopheles gambiae* adults and matings. Malar. J. 19, 1–19. https://doi.org/10.1186/s12936-020-03306-5.

Barbosa, P., Peters, T. M., 1971. The effects of vital dyes on living organisms with special reference to methylene blue and neutral red. Histochem. J. 3, 71-93. https://doi.org/10.1007/BF01686508.

Bentivenha, J.P., Rodrigues, J.G., Lima, M.F., Marcon, P., Popham, H.J., Omoto, C., 2019. Baseline susceptibility of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to SfMNPV and evaluation of cross-resistance to major insecticides and *Bt* proteins. J. Econ. Entomol. 112 (1), 91–98. https://doi.org/10.1093/jee/toy342.

Blanco, C.A., Sumerford, D., López, J.D., Hernández, G., 2006. Mating incidence of feral *Heliothis virescens* (Lepidoptera: Noctuidae) males confined with laboratory-reared females. J. Cotton Sci. 10, 105–113. https://doi.org/10:105–113.

Cantelo, W.W., 1973. Dye markers for moths of the Tobacco Hornworm. Environ. Entomol. 2, 393–396. https://doi.org/10.1093/ee/2.3.393.

Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Fresia, P., Santos, A.C., Omoto, C., 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. Crop Prot. 64, 150–158. https://doi.org/10.1016/j.cropro.2014.06.019.

Fatoretto, J.C., Michel, A.P., Silva Filho, M.C., Silva, N.. 2017. Adaptive potential of fall armyworm (Lepidoptera: Noctuidae) limits Bt trait durability in Brazil. J. Integr. Pest Manag., 8 (1), 17. https://doi.org/10.1093/jipm/pmx011

Foo, C. W. P., Bini, E., Hensman, J., Knight, D. P., Lewis, R. V., Kaplan, D. L., 2006. Role of pH and charge on silk protein assembly in insects and spiders. Appl. Phys., A Mater. Sci. Process. 82, 223-233. https://doi.org/10.1007/s00339-005-3426-7.

Fondren Junior, J. E., Heitz, J. R., 1979. Dye-sensitized house fly toxicity produced as a function of variable light sources. Environ. Entomol. 8 (3), 432-436. https://doi.org/10.1093/ee/8.3.432.

Gangwere, S. K., Chavin, W., Evans, F. C., 1964. Methods of marking insects, with special reference to Orthoptera (Sens. Lat.). Ann. Entomol. Soc. Am. 57, 662-669. https://doi.org/10.1093/aesa/57.6.662.

Ge, S., Sun, X., He, W., Wyckhuys, K. A., He, L., Zhao, S., Zhang, H., Wu, K., 2021. Potential trade-offs between reproduction and migratory flight in *Spodoptera frugiperda*. J. Insect Physiol. 132, 104248. https://doi.org/10.1016/j.jinsphys.2021.104248.

Gilreath, R. T., Kerns, D. L., Huang, F., Yang, F., 2021. No positive cross-resistance to Cry1 and Cry2 proteins favors pyramiding strategy for management of Vip3Aa resistance in Spodoptera frugiperda. Pest Manag. Sci. 77, 1963-1970. https://doi.org/10.1002/ps.6224.

Hagler, J.R., Jackson, C.G., 2001. Methods for marking insects: current techniques and future prospects. Annu. Rev. Entomol. 46, 511–543. https://doi.org/10.1146/annurev.ento.46.1.511.

Heller, K.G., Faltin, S., Fleischmann, P., Helversen, O. V., 1998. The chemical composition of the spermatophore in some species of phaneropterid bushcrickets (Orthoptera: tettigonioidea). J. Insect Physiol. 44, 1001–1008. https://doi.org/10.1016/S0022-1910(97)00171-6.

Hendricks, D.E., Graham, H.M., 1970. Oil-soluble dye in larval diet for tagging moths, eggs, and spermatophores of Tobacco Budworms. J. Econ. Entomol. 63, 1019–1020. https://doi.org/10.1093/jee/63.3.1019.

ISAAA, 2019. Global Status of Commercialized Biotech/GM Crops: 2019. Biotech Crops Drive Social Economic Development and Sustainable

Environment in the New Frontier. ISAAA: Ithaca, NY, USA. (ISAAA Brief no. 55)

Johnson, B.J., Mitchell, S.N., Paton, C.J., Stevenson, J., Staunton, K.M., Snoad, N., Beebe, N., White, B.J., Ritchie, S.A., 2017. Use of rhodamine B to mark the body and seminal fluid of male *Aedes aegypti* for mark-release-recapture experiments and estimating efficacy of sterile male releases. PLoS Negl. Trop. Dis. 11, e0005902. https://doi.org/10.1371/journal.pntd.0005902.

Kottarathil, H.A., Kappalli, S., 2019. Reproductive system in the male phase of a parasitic isopod (Crustacea) – Morphological, histological and ultrastructural evidence for sequential protandrous hermaphroditic changes. Zool. Stud. 58, 4. https://doi.org/10.6620/ZS.2019.58-04.

Mazaheri, F., Aliabad, K.K., Kalantar, S.M., Ziya, N., Khdmehr, A., Anvari, M., 2020. Effects of phosalone plant pesticide on sperm parameters and sexual hormone levels in Wistar rats: an experimental study. IJRM 18, 785–794. https://doi.org/10.18502/ijrm.v13i9.7683.

Monnerat, R., Martins, E., Macedo, C., Queiroz, P., Praca, L., Soares, C. M., Moreira, H., Grisi, I., Silva, J., Soberon, M., Bravo, A., 2015. Evidence of field-evolved resistance of *Spodoptera frugiperda* to Bt corn expressing Cry1F in Brazil that is still sensitive to modified Bt toxins. PLoS One 10 (4), e0119544. https://doi.org/10.1371/journal.pone.0119544.

Nijhout, H.F., 1975. Excretory role of the midgut in larvae of the tobacco hornworm, *Manduca sexta* (L.). J. Exp. Biol. 62, 221–230. https://doi.org/10.1242/jeb.62.1.221.

Noaman, N., Coorssen, J.R., 2018. Coomassie does it (better): A Robin Hood approach to total protein quantification. Anal. Biochem. 556, 53–56. https://doi.org/10.1016/j.ab.2018.05.012.

Qureshi, J.A., Buschman, L.L., Throne, J.E., Ramaswamy, S.B., 2006. Dispersal of adult *Diatraea grandiosella* (Lepidoptera: Crambidae) and Its Implications for Corn Borer Resistance Management in *Bacillus thuringiensis* maize. Ann. Entomol. Soc. Am. 99, 279–291. https://doi.org/10.1603/0013-8746(2006)099[0279:DOADGL]2.0.CO;2.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Robinson, J.R., Photodynamic insecticides: a review of studies on photosensitizing dyes as insect control agents, their practical application, hazards and residues. In: Heitz, J.R. (Ed.), Residue Reviews. Vol. 88. Springer Verlag, New York, pp. 69–100, 1983.

Showers, W.B., Hellmich, R.L., Derrick-Robinson, M.E., Hendrix, W.H., 2001. Aggregation and dispersal behavior of marked and released European Corn Borer (Lepidoptera: Crambidae) Adults. Environ. Entomol. 30, 700–710. https://doi.org/10.1603/0046-225X-30.4.700.

Sousa, P.C., Santos, E.A.A., Souza, A.L.P., Lima, G.L., Barros, F.F.P.C., Oliveira, M.F., Silva, A.R., 2013. Sperm morphological and morphometric evaluation in captive collared peccaries (*Peccary tajacu*). Pesq. Vet. Bras. 33, 924–930. https://doi.org/10.1590/S0100-736X2013000700014.

South, A., Sota, T., Abe, N., Yuma, M., Lewis, S.M., 2008. The production and transfer of spermatophores in three Asian species of *Luciola* fireflies. J. Insect Physiol. 54, 861–866. https://doi.org/10.1016/j.jinsphys.2008.03.008.

Southwood, T.R.E., 1978. Ecological Methods with Particular Reference to the Study of Insect Populations. 2nd ed. London, UK, Springer Science.

Sparks, M.R., Cheatham, J.S., 1973. Tobacco hornworm: marking the spermatophore with water-soluble stains. J. Econ. Entomol. 66, 719–721. https://doi.org/10.1093/jee/66.3.719.

Spranghers, T., Michiels, J., Vrancx, J., Ovyn, A., Eeckhout, M., De Clercq, P., De Smet, S., 2018. Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia illucens* L.) prepupae for weaned piglets. Anim. Feed Sci. Technol. 235, 33-42. https://doi.org/10.1016/j.anifeedsci.2017.08.012.

Tabashnik, B. E., Carrière, Y., 2017. Surge in insect resistance to transgenic crops and prospects for sustainability. Nat. Biotechnol. 35, 926-935. https://doi.org/10.1038/nbt.3974.

Tal, M., Silberstein, A., Nusser, E., 1985. Why does Coomassie Brilliant Blue R interact differently with different proteins? A partial answer. JBC 260 (18), 9976-9980. https://doi.org/10.1016/S0021-9258(17)39198-6.

Tanaka, T., 2006. Reproductive and neurobehavioural toxicity study of Ponceau 4R administered to mice in the diet. FCT 44 (10), 1651-1658. https://doi.org/10.1016/j.fct.2006.05.001.

Terra, W. R., 2001. The origin and functions of the insect peritrophic membrane and peritrophic gel. Arch. Insect Biochem. Physiol. 47 (2), 47-61. https://doi.org/10.1002/arch.1036.

Vail, P. V., Howland, A. F., Henneberry, T. J., 1966. Fluorescent dyes for mating and recovery studies with Cabbage Looper Moths. J. Econ. Entomol. 59, 1093-1097. https://doi.org/10.1093/jee/59.5.1093.

Van Der Reijden, E.D., Monchamp, J.D., Lewis, S.M., 1997. The formation, transfer, and fate of spermatophores in *Photinus* fireflies (Coleoptera: lampyridae). Can. J. Zool. 75, 1202–1207. https://doi.org/10.1139/z97-143.

Venkatesha, T.G., Nayaka, Y.A., Chethana, B.K., 2013. Adsorption of Ponceau S from aqueous solution by MgO nanoparticles. Appl. Surf. Sci. 276, 620–627. https://doi.org/10.1016/j.apsusc.2013.03.143.

Vilarinho, E.C., Fernandes, O.A., Omoto, C., Hunt, T.E., 2006. Oil-soluble dyes for marking *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J. Econ. Entomol. 99, 2110–2115. https://doi.org/10.1093/jee/99.6.2110.

Vilarinho, E.C., Fernandes, O.A., Hunt, T.E., Caixeta, D.F., 2011. Movement of *Spodoptera frugiperda* adults (Lepidoptera: Noctuidae) in maize in Brazil. Fla. Entomol. 94, 480–488. Available in: https://doi.org/10.1653/024.094.0312 (accessed 20 July 2023).

Vilela, M., Mendes, S.M., Valicente, F.H., Carvalho, S.S.S., Santos, A.E., Santos, C.A., Araújo, O.G., Barbosa, T.A.N., Carvalho, E.A.R., Costa, V.H.D., 2014. Methodology for rearing and maintaining *Helicoverpa armigera* in the Laboratory. Embrapa Corn and Sorghum, Sete Lagoas (Technical Circular, 203).

Wang, Z., Huang, Y., Pierce, N. E., 2019. Radio telemetry helps record the dispersal patterns of birdwing butterflies in mountainous habitats: Golden Birdwing (Troides aeacus) as an example. J. Insect Conserv. 23, 729-738. https://doi.org/10.1007/s10841-019-00167-5.

Yoho, T. P., Weaver, J. E., Butler, L., 1973. Photodynamic action in insects. 1. Levels of mortality in dye-fed light-exposed house flies. Environ. Entomol. 2 (6), 1092-1096. https://doi.org/10.1093/ee/2.6.1092.