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Source: Environmental Entomology, 48(6) : 1260-1269

Published By: Entomological Society of America

URL: <https://doi.org/10.1093/ee/nvz118>

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Egg Albumin as a Protein Marker to Study Dispersal of Noctuidae in the Agroecosystem

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Subject Editor: Matthew Ginzl

Received 6 May 2019; Editorial decision 4 September 2019

Abstract

Knowledge of dispersal and spatial dynamics of pest populations is fundamental for implementation of integrated pest management and integrated resistance management. This study evaluated 1) the effectiveness of egg white albumin protein to mark larvae and adults of two polyphagous and highly mobile pests, *Spodoptera frugiperda* (J.E. Smith) (fall armyworm) and *Helicoverpa zea* (Boddie) (corn earworm) (Lepidoptera: Noctuidae), and 2) the sensitivity of polyvinylidene difluoride membrane (dot blot) in detecting albumin on marked insects. Laboratory and field experiments tested egg albumin as a protein marker, which was detected using two enzyme-linked immunosorbent assay (ELISA), microplate, and dot blot. In the laboratory, 100% of the moths sprayed with 20% egg white solution acquired the albumin marker, which was detected through the last time point tested (5 d) after application. Egg albumin was not effective at long-term marking of larvae, detected only prior the molting to the next instar. Albumin application in field cages resulted in a high percentage of moths detected as marked at 24 h and 5 d for both species. Egg albumin applied in the open field resulted in 15% of the recaptured corn earworm moths marked with most of them collected 150 m from the application area, although some were captured as far as 1,600 m within approximately 6 d after adult emergence. The results indicated egg albumin is a suitable marker to study the dispersion of fall armyworm and corn earworm in the agroecosystem and dot blot was as effective to detect egg albumin as was indirect ELISA.

Key words: enzyme-linked immunosorbent assay, dot blot, *Spodoptera frugiperda*, *Helicoverpa*, mark–capture

Dispersal is defined as any movement of organisms away from their parent source (Nathan 2001). This behavior is a fundamental biological process that has important ecological and evolutionary consequences (Kokko and López-Sepulcre 2006, Ronce 2007). By dispersing, insects find food, mates, and favorable environment conditions affecting their survival, growth, and reproduction. As a consequence, insect dispersal has enormous implications, both negative and positive, including crop loss, the spread of diseases, the provision of essential ecosystem services such as crop pollination (Holland et al. 2006), and gene flow (Kokko and López-Sepulcre 2006, Ronce 2007). Understanding and measuring the patterns of insect dispersal is valuable for managing pest populations because dispersal affects pest population dynamics as well as the distribution of genetic diversity throughout space (Ronce 2007).

Spodoptera frugiperda (J.E. Smith) (fall armyworm) and *Helicoverpa zea* (Boddie) (corn earworm) are highly mobile and

economically important plant pests throughout the western hemisphere (Sparks 1979, Capinera 2008, Luttrell and Jackson 2012, Olmstead et al. 2016). Recently, fall armyworm was introduced to and has spread over 30 countries in Africa and Asia (Goergen et al. 2016, Wild 2017, Chormule et al. 2019). These pests are known for their ability to migrate long distances annually, resulting in infestation and significant economic losses. The dispersal behavior associated with the wide range of crops utilized by these species constitutes a challenge for effective local management. Although long-distance movement of fall armyworm and corn earworm is well documented (Westbrook and López 2010, Nagoshi et al. 2012, Westbrook et al. 2016), little is known about the dispersal behavior of these insects throughout the agricultural landscape. Developing a method to assess local movement patterns could provide important information about the range of cultivated and wild hosts utilized by these pests. Thus, understanding the dispersal pattern as well as the sequence

and distribution of hosts around the agricultural landscapes can contribute to their effective management in the agricultural landscape (Luttrell and Jackson 2012).

Tracking insect movement is a major challenge due to their relatively small size and cryptic behavior (Hagler and Jackson 2001). According to these authors, an effective marker should be non-toxic to the insect and environment, easy to apply, durable, inexpensive, and clearly identifiable. Also, the marker should not cause any injury to the insect or affect its normal dispersal behavior, growth, reproduction, and life span (Hagler and Jackson 2001). Although several methodologies have been used to mark insects, some of them fail in one or more of these characteristics. An immunoassay was initially used to detect protein on insects, and was proved to be effective, easy, safe, and stable (Hagler et al. 1992, Hagler 1997a). Initially, specific vertebrate protein (rabbit or chicken IgG) was applied externally as a spray or incorporated into the insect diet (Hagler 1997a, b; Hagler and Jackson 1998). The IgG could be easily detected by applying an anti-IgG sandwich enzyme-linked immunosorbent assay (ELISA). This assay is a very sensitive technique (Hagler 1997a, Hagler and Miller 2001), but its major limitation as a marker is the high cost of the purified protein, which makes it impractical to apply directly in the field for mark-capture type studies (Jones et al. 2006, Hagler 2019).

In the face of this limitation, Jones et al. (2006) developed an inexpensive immunomarking alternative to mark insects in the field using easily available food proteins, such as chicken egg albumin (as egg white), bovine casein (as cow's milk), and soy protein (as soy milk). Over the past years, several studies have been using this technique to study insect dispersal patterns (Jones et al. 2006, Boina et al. 2009, Hagler and Jones 2010, Hagler et al. 2014, Klick et al. 2016, Boyle et al. 2018a, Irvin et al. 2018, Hagler 2019). Although using protein to study insect dispersal has proven effective, the protein detection method currently performed using microplates could be run by other commonly immunoassay methods. An alternative to microplate ELISA is applying the protein (antigen) directly onto a membrane for antibody detection (Hawkes et al. 1982). This method is known as dot blot, a protein-based detection method that has shown to be at least as sensitive as microplate ELISA (Stuart and Greenstone 1990, Hagler et al. 1995, Hagler 1998).

In this study, the effectiveness and persistence of albumin (egg white) as a foreign protein to mark fall armyworm and corn earworm larvae and adults was tested to determine the technique efficacy and document the movement of adults in the agricultural landscape. Laboratory tests were performed to demonstrate the efficacy for mark-release-recapture studies, and the acquisition of egg albumin by moths when applied under field conditions was assessed for mark-capture studies.

Materials and Methods

Lepidoptera Stock Colony

Eggs and pupae of fall armyworm and corn earworm were obtained from Benzon Research Inc. (Carlisle, NE). Eggs were placed in a plastic bag until hatching. Neonates (<24 h old) of both species were sprayed with chicken egg albumin to measure its acquisition and retention. Pupae were placed in containers containing vermiculite and stored in small cages (25 × 25 × 25 cm) until the emergence of the adults. Adults were sprayed with chicken egg albumin under laboratory and field. Larvae, pupae, and adults were kept at a controlled temperature of 27 ± 2°C, relative humidity of 70 ± 10%, and a 12L: 12D photoperiod.

Protein Marker

Albumin from chicken white egg was tested as a potential marker for fall armyworm and corn earworm larvae and adults. The source of the albumin was from Egg Beaters Original Real Egg® (ConAgra Foods, Omaha, NE). The insects were marked by spraying with 20% (vol/vol) egg white/water solution. The presence of egg albumin on the insect body was tested using indirect ELISA and dot blot (described below).

Effectiveness and Persistence of Egg Albumin on Larvae and Moths in the Laboratory

The experiments were conducted in the insect toxicology laboratory located at the University of Nebraska-Lincoln Plant Science Hall, Lincoln, NE. Larvae and adults of both species were kept in a chamber at 27 ± 2°C, relative humidity of 70 ± 10%, and a 12L: 12D photoperiod.

Fall armyworm and corn earworm neonates (<24 h old) were separated into four groups ($n = 60$ per group) and placed inside a petri dish (90 mm diameter and 15 mm height) containing filter paper (one group per petri dish). Three groups were sprayed with 1 ml of a 20% egg albumin solution and one group was not sprayed (negative control). All treatments were applied using a 24-oz trigger spray bottle (Skilcraft applicator spray, from Taiwan purchased Grainger.com). One hour after application, larvae were placed in a small container (8.5 cm diameter and 3.5 cm height) with artificial diet and held in a growth chamber. Four days after treatment, each group of sprayed and non-sprayed larvae were placed individually into small cups (30 ml) containing artificial diet. For each instar, three larvae from each sprayed group ($n = 9$) and eight larvae from the negative control group were sampled. Individual samples were placed into a clean 1.5 ml microtube and frozen at -10°C for subsequent testing for the presence of egg albumin. Larval manipulation was carried out using gloves and tweezers to avoid contamination and transference of egg albumin between marked and unmarked larvae.

Pupae of fall armyworm and corn earworm were separated by sex before emergence of adults (Capinera 2008). Male and female pupae were placed separately inside cages (25 × 25 × 25 cm) until moth emergence. Three days after moth emergence, three small cages for each species were prepared and 12 females and 12 males were placed in each cage. Immediately after the placement of the moths in each cage, 10 ml of 20% egg albumin solution was sprayed using a trigger spray bottle. For a negative control, a fourth cage was not sprayed. Cages were held in a growth chamber at 27 ± 2°C, relative humidity of 70 ± 10%, and a 12L: 12D photoperiod. Two couples were sampled from each cage (sprayed and non-sprayed cages) every other day starting on the day of the egg albumin application. Moths were sampled throughout 5 d after the albumin application. To avoid contamination and occurrence of false positives, each moth was placed individually into a clean 2 ml microtube and frozen at -10°C for subsequent testing for the presence of egg albumin by indirect ELISA and dot blot.

Acquisition and Persistence of Egg Albumin on Adults in the Field

The experiments were conducted during 2016 in a corn field located at the University of Nebraska Haskell Agricultural Laboratory, Concord, NE (42°22'50.4"N and 96°57'17"W). The corn was cultivated following the recommended agronomic practices for the region.

A study site in V9 stage corn was inspected for natural infestation of noctuid eggs and larvae (none observed). Six large mesh cages

(1.6 × 3.2 × 1.8 m) and six small mesh cages (1.6 × 1.6 × 1.8 m) were installed at the site, spaced 3 m from each other. Fall armyworm and corn earworm pupae were placed in small containers (13 × 13 × 5 cm) with vermiculite. One day after initial moth emergence, at least 200 pupae of each species were placed in each large cage (three large cages received fall armyworm and three large cages received corn earworm). The emergence of the moths was observed and after 90% of the adults emerged (3 d after), the containers containing the unemerged pupae were removed. Immediately after, the plants inside each cage were sprayed with 2.5 liters of a 20% egg albumin solution using a backpack sprayer (R&D Sprayers, Opelousas, LA). As a negative control (no marker application), at least 100 pupae of each species were placed in two small cages. The first sampling was performed on the day after the albumin marker application. Approximately 50 adults of each species from the sprayed large cages were transferred to the other three small cages 24 h after spray. This transference was done to ensure that the moths did not pick up the marker by contact after the marker dried on the corn leaf surface. A second sample was collected 5 d after the egg albumin application. The moths were placed into a clean 2 ml microtube and frozen at -10°C for subsequent testing for the presence of egg albumin by indirect ELISA and dot blot.

Another field study was performed without cages. Approximately 1,000 corn earworm pupae were placed in six containers (26 cm diameter and 8 cm height) with vermiculite and held in a growth chamber until the initiation of moth emergence. The day after initial moth emergence, the containers were placed 3 m apart in the middle of pre-selected albumin application zone (8 × 21 m). When 80% of the adults had emerged (3 d after placement in field), the containers with unemerged pupae were removed. Immediately after, 17.5 liters of a 20% egg albumin solution was sprayed on the albumin application zone (plants at V9) using a backpack sprayer. Ten light traps and four pheromone traps were placed throughout the area for recapture of corn earworm. Light traps were attached to small live capture cages (Paula-Moraes et al. 2013) and kept illuminated from sunset to sunrise during the recapture period. Light traps were positioned from 16 to 880 m from the application zone. Pheromone traps were positioned 1,260 to 1,620 m from the application zone. The live capture cages were checked for corn earworm moths each day for 6 d after the egg albumin application in the corn field. Pheromone traps were used to recapture corn earworm males. Pheromone traps were also checked daily during 6 d after marker application. Moths captured in the light and pheromone traps were transported to the laboratory. Corn earworm moths were placed into a clean 2 ml microtube and frozen at -10°C for subsequent testing for the presence of egg albumin. The date and trap location of each specimen was recorded.

Transference of Egg Albumin Between Marked to Unmarked Moths

An experiment was performed to examine the possibility of egg albumin being transferred from marked to unmarked moths. The marker transference could result in the incidence of false positive, which would lead to erroneous estimates of the dispersal capability of insects (Hagler 2019). There is a possibility that egg albumin could be transferred between moths, such as in the small recapture cages placed under light traps. The transference could depend on how long prior to recapture the moth was sprayed, as well as the presence of individuals of the same or different gender. To test these hypotheses, we placed marked and unmarked corn earworm moths together inside small cages that were also used to catch moths from

light traps. Pupae of corn earworm were separated by sex and placed in small containers. After the emergence of adults, a group of moths was sprayed with egg albumin (20% vol/vol), and another group was kept unmarked. A total of eight marked and eight unmarked moths from the same (male × male and female × female) or different sex (female × male and male × female) was placed into a cage 1 and 24 h after spray. A total of eight cages was used in this experiment, four to hold moths transferred at 1 h and four to hold moths transferred at 24 h post egg albumin application. Another cage was prepared to keep unmarked moths, used as negative control. All moths from each cage were collected 24 h after being placed together. Moths were placed into a clean 2 ml microtube and frozen at -10°C to be later tested for the presence of egg albumin by indirect ELISA and dot blot.

Indirect ELISA

Microtubes containing collected insect samples were removed from the freezer and 1 ml of Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl) was added in each one. The samples were soaked at room temperature for 1 h on a laboratory shaker (Reliable Scientific, Inc.) at 50 rocking motions per minute. After that, the moths were immediately discarded, and the remaining solution was centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was used for ELISA and dot blot.

The detection limit of egg albumin by indirect ELISA assay was performed by preparing a triple serial dilution from a chicken egg white (Cat. #A5503, Millipore Sigma, St. Louis, MO) solution starting at 3.33 ppm and ending at 1.52 ppb. TBS solution and samples from non-sprayed adults were used as negative controls. Each serial dilution and blank samples were evaluated in four replicates following the indirect ELISA procedure as described below. The assay sensitivity was determined as the concentration of protein giving an optical density (OD) mean value higher than the mean plus three times the SD value of the TBS buffer and of the insect negative control blank.

Egg albumin was quantified in all samples by an indirect ELISA assay following the protocol described by Hagler and Jones (2010) with some modifications. Briefly, 96-well microplates containing 80 µl of each supernatant were incubated for 1.5 h at 37°C and then blocked for 2 h using 300 µl of blocking solution. After adding TMB substrate, 50 µl of 2 N sulfuric acid solution were used to stop the enzymatic reaction before reading. Color development was measured using a 96-well microplate reader (BioTek Instruments, Inc, Highland Park, Winooski, VT) at 490 nm of wavelength. The standard curve ($R^2 = 0.97$) established using serial dilutions was saved and used in quantification of egg albumin. Negative controls were also used in indirect ELISA. Each sample was evaluated by triplicate.

Dot Blot

A triple serial dilution was prepared using albumin from chicken egg white (Cat. #A5503, Millipore Sigma, St. Louis, MO) in order to evaluate the sensitivity to detect egg albumin using the dot blot approach. The serial dilution was performed with eight chicken egg white concentrations diluted in TBS (pH 7.4) starting at 10 ppm and ending at 4.5 ppb. The limit of detection for each concentration was tested using seven different aliquot volumes (1, 3, 5, 7, 10, 15, and 20 µl). Non-spray and sprayed samples from the laboratory experiment were used as negative and positive controls, respectively. Aliquots from each concentration were manually transferred to a polyvinylidene difluoride (PVDF) membrane (Cat. #162-0175, Bio Rad, Hercules, CA) as described below.

The PVDF membrane was cut with clean scissors in rectangular portions according to the number of samples assayed. The membrane was then wetted in 100% methanol for 5 s, until the entire membrane became translucent and then placed in a container with pure water for 3 min for equilibration. To prevent the membrane from drying out before applying protein samples, the membrane was placed on the top of three sheets of filter paper soaked in pure water. An aliquot of 5 μ l from each individual protein sample was manually spotted on the PDVF membrane. After the samples dried, the membrane was blocked for 2 h at room temperature using 300 μ l of a non-fat milk solution (3%) per each cm^2 of membrane diluted in phosphate-buffered saline (PBS) (Cat. #1610780, Bio Rad). The primary antibody, rabbit anti-chicken egg albumin (Cat. #C6534, Millipore Sigma, St. Louis, MO), was diluted 1:5,000 in 3% non-fat milk/PBS, as previously described. After washing the membrane three times, 10 min each, with PBS solution (0.5% Tween, pH 7.4) (Cat. #P3563, Millipore Sigma), the secondary antibody, goat anti-rabbit IgG (Cat. #6154, Millipore Sigma), conjugated to horseradish peroxidase and diluted 1:5,000 in the 3% non-fat milk/PBS, was added and kept at room temperature for 1 h. After washing steps, the horseradish peroxidase substrate (Cat. #170-8235, Bio Rad, Hercules, CA) was added following the manufacturer recommendations. After 30 min of incubation time at room temperature, the reaction was then stopped by washing the membrane with pure water, dried, and a picture was taken for further record.

Data Analysis

Unmarked samples were used to calculate the indirect ELISA critical threshold values. For those experiments performed in laboratory conditions, the threshold values were determined by the mean of the indirect ELISA OD from negative control greater three times the SD (Crowther 2001). For the experiments performed in field conditions, the presence of false positive could lead to inflated estimates of long-distance dispersal (Sivakoff et al. 2011). To provide more protection against the incidence of false positive, the threshold for those experiments was calculated by the method described by Sivakoff et al. (2011). Thus, samples were scored positive for egg albumin marker if the indirect ELISA OD were above the threshold previously calculated.

Each PVDF membrane assayed in dot blot study included a negative control (unmarked), positive control (known marked samples),

and samples in which the presence of egg albumin would be confirmed. The reaction between the enzyme peroxidase conjugated with the secondary antibody and the Opti-4CN substrate results in the production of a black/gray solid product in the blotting where the sample aliquot was placed. Thus, a sample was considered positive when a visual and well-defined black/gray spot was observed on the PVDF membrane. To test if the egg albumin detection depends on the methods adopted in this study, ELISA and dot blot, data of each experiment were grouped into chi-squared contingency tables (PROC FREQ, SAS Institute 2012). The chi-squared results were statistically significant at $\alpha = 0.05$.

Results

ELISA and Dot Blot Sensitivity

The indirect ELISA detected egg albumin up to 4.5 ppb with a positive threshold value of 0.039 from TBS buffer blank and 41.1 ppb with a positive threshold value of 0.109 from negative control blank (non-spray adults) (Fig. 1a). These results indicate that indirect ELISA sensitivity is lower when calculated with the indirect ELISA OD from non-spray adults. The limit of detection for egg albumin by dot blot was 41.0 ppb (Fig. 1b). The sensitivity was not influenced by the aliquot volume and the spot color intensity was basically dependent on the antigen (egg albumin) concentration.

Acquisition and Persistence of Egg Albumin on Larvae

Egg albumin was detected on fall armyworm and corn earworm larvae at the first instar by using both indirect ELISA and dot blot (Fig. 2). However, egg albumin does not persist throughout the larval stages. Eight corn earworm and fall armyworm larvae in each instar were assayed (Fig. 2a). The samples from negative control yielded a mean (\pm SEM) ELISA OD of 0.047 ± 0.002 ($n = 46$) for corn earworm and 0.046 ± 0.003 ($n = 43$) for fall armyworm. That resulted in ELISA threshold values of 0.0985 and 0.116, respectively. Like the ELISA, dot blot was able to detect egg albumin marker of larvae sprayed with egg white solution (Fig. 2b). Dot blot was assayed only for those samples of the first instar since egg albumin does not persist in older instars.

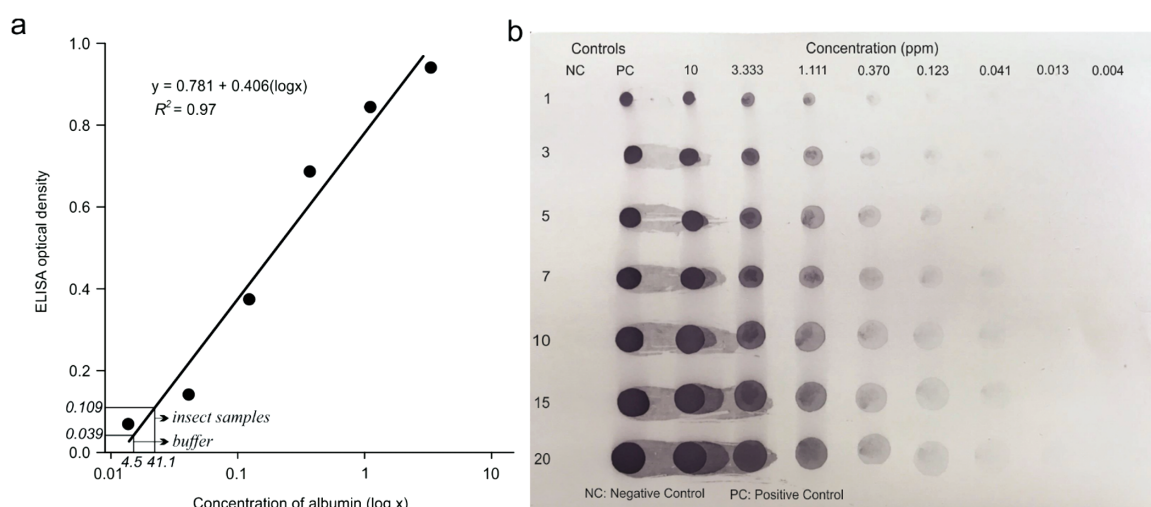


Fig. 1. Sensitivity of ELISA and dot blot to detect egg albumin. (a) ELISA standard curve with the concentration and its respective threshold value for negative samples performed with buffer and unmarked moths. (b) Dot blot standard tested with a serial dilution applied at different aliquot volume onto a PVDF membrane. Black/gray spots mean positive scored samples for albumin.

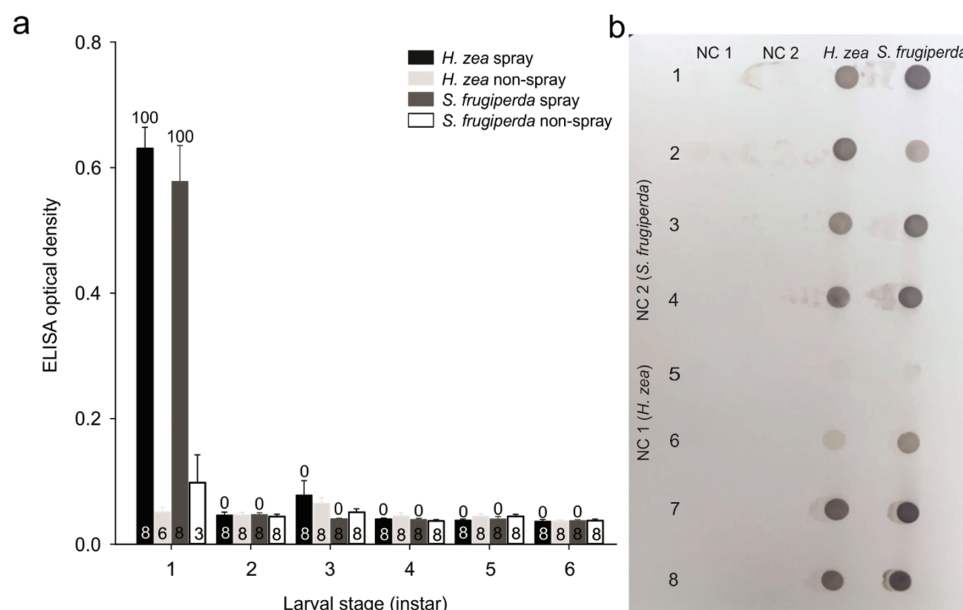


Fig. 2. Immunoassay results for the presence of egg albumin in larvae of corn earworm and fall armyworm sprayed with a 20% egg white solution. (a) ELISA OD values (mean \pm SEM) and the percentage of those samples that scored positive for egg albumin. Indicated above the SE bars is the % positive samples and inside the columns is the number of samples tested. (b) Dot blot for the detection of egg albumin in first-instar larvae. The numbers before the first column indicate the dot lines. The first and second columns (NC 1, NC 2) show the negative control for corn earworm and fall armyworm, respectively. The next two columns represent the results from samples sprayed with egg white solution. Black/gray spots are the samples that scored positive for albumin.

Acquisition and Persistence of Egg Albumin on Adults in Laboratory

The presence of egg albumin was confirmed by ELISA and dot blot (Fig. 3). All the samples assayed in each species and throughout the collection period were scored positive (Fig. 3a). Samples from negative controls yielded a mean (\pm SEM) ELISA OD of 0.052 ± 0.006 for corn earworm and 0.060 ± 0.010 for fall armyworm. That resulted in ELISA threshold values of 0.104 and 0.144 and for corn earworm and fall armyworm, respectively. The number of samples assayed for the presence of egg albumin by dot blot ($n = 8$) was less than those assayed by ELISA. Importantly, dot blot detected egg albumin in all samples scored positive in the regular microplate ELISA (Fig. 3b and c).

Acquisition and Persistence of Egg Albumin on Adults in Field Cages

Adults of fall armyworm and corn earworm sprayed with a 20% egg white solution in field cages were marked and egg albumin was detectable until the last time point tested (5 d) after spray (Fig. 4). The negative samples tested by ELISA (Fig. 4a) yielded a mean (\pm SEM) ELISA OD of 0.058 ± 0.005 and 0.061 ± 0.006 for corn earworm and ELISA OD of 0.219 ± 0.013 and 0.052 ± 0.002 for fall armyworm 1 and 5 d after egg white spraying, respectively. The calculated threshold values from those mean was 0.122 and 0.113 for corn earworm and 0.308 and 0.078 for fall armyworm 1 and 5 d after spray with an egg white solution, respectively. Likewise, dot blot detected egg albumin in moths from samples sprayed in field cages of both species throughout the collecting time (Fig. 4b–c). For CEW, more positive samples were statistically significant scored by dot blot compared to ELISA (Table 1).

Acquisition of Albumin on Adults Sprayed in the Corn Field

A total of 126 and 191 corn earworm moths were captured during 6 d in light and pheromone traps, respectively. Of those samples

from light traps, 38 (30.16%) and 46 (36.51%) were marked with albumin according to ELISA and dot blot, respectively. Of the 191 moths caught from the pheromone trap, 8 (4.19%) and 9 (4.71%) scored positive for egg albumin according to ELISA and dot blot. Overall, there was a difference in the percentage of moths collected through time, and a greater number of marked moths were caught in light traps, which were located nearest to the albumin application area (Fig. 5). No statistical difference was observed between ELISA and dot blot according to chi-squared test for the detection of egg albumin in light and pheromone traps (Table 1). Our results clearly showed that corn earworm has a great ability to move throughout the area being collected, and at least as far as 1,600 m from the albumin application site within approximately 6 d after emergence.

Marker Transference Between Marked to Unmarked Moths

Egg albumin can be transferred from marked moths to unmarked moths if they are kept in small cages tied to light traps within 24 h (Table 2). However, the percentage of marker transference was not statistically influenced by the time that the moths were placed together after sprayed with egg albumin for both ELISA and dot blot methods (Table 3). Also, no statistical difference was observed when males and females were placed single or mixed in the cages for both marker detection methods (Table 3). Overall, the ELISA and dot blot methods were not statistically differently for the marker transference experiment, despite a smaller number of positive samples had been detected by dot blot compared to ELISA (Table 3).

Discussion

The potential of chicken egg albumin to mark fall armyworm and corn earworm larvae and moths was tested for efficacy and persistence. Chicken egg albumin was found to be a suitable protein marker for tracking and monitoring the movement of moths in the field. This conclusion is supported by the high percentage of moths

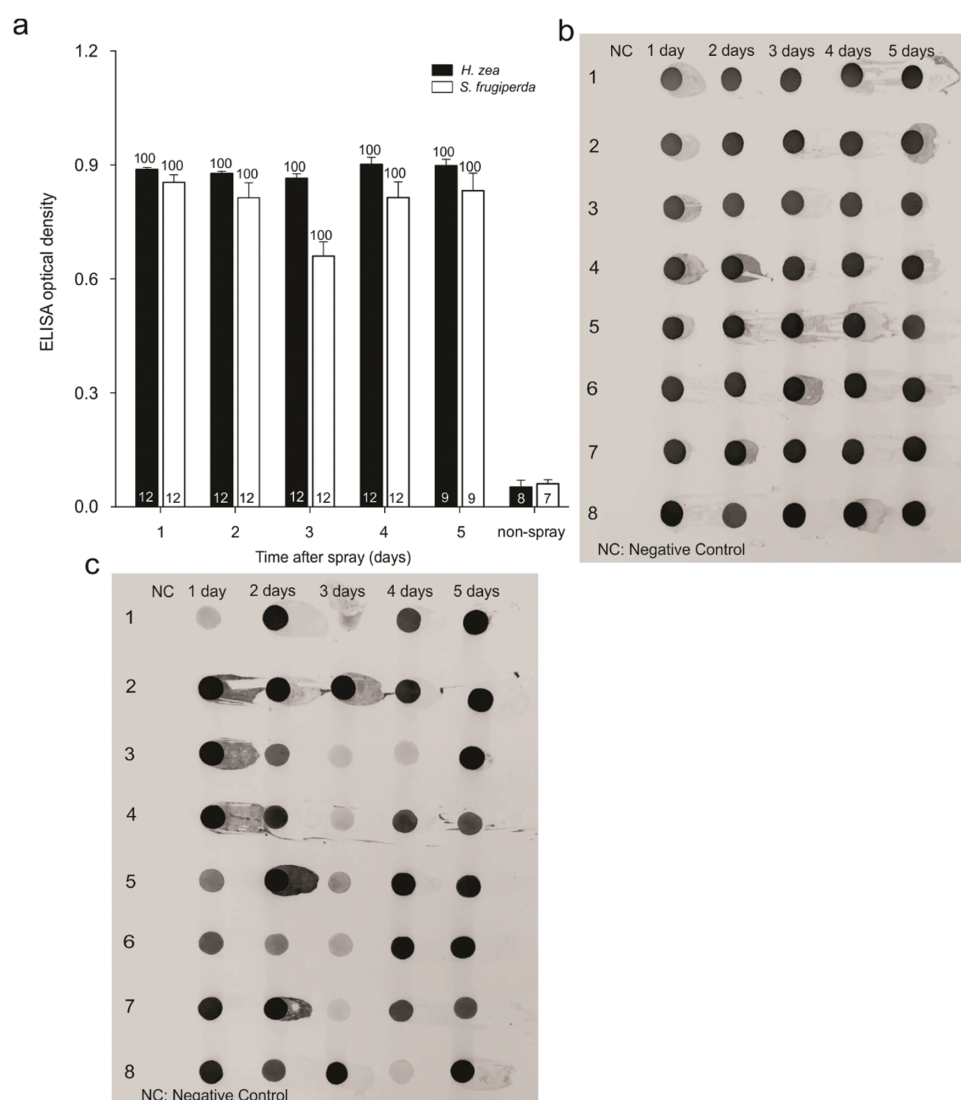


Fig. 3. Immunoassay results for the presence of egg albumin in adults of corn earworm and fall armyworm sprayed with egg whites in the laboratory. (a) ELISA OD values (mean \pm SEM) and the percentage of the samples that scored positive for the presence of egg albumin (number above the bars). Inside the bar is the number of samples tested. (b, c) Dot blot for egg albumin detection in corn earworm and fall armyworm, respectively. The first column shows the negative (non-sprayed) control; the other columns show the samples collected in different days after spraying the egg albumin. Black/gray spots were the samples that scored positive for the marker.

with a positive score when sprayed with a 20% chicken egg albumin solution under laboratory and field conditions. In addition, our findings demonstrated that egg albumin can be detected using PVDF membrane (dot blot), an alternative method to conventional microplate ELISA, which is the most often method used in previous studies (Jones et al. 2006, 2011; Boina et al. 2009; Hagler and Jones 2010; Klick et al. 2016; Blaauw et al. 2017; Hagler 2019).

The efficacy of vertebrate protein to mark insects in the field has been reported for different species of insects, contributing to the understanding of movement patterns of important agricultural pests (Boina et al. 2009, Sivakoff et al. 2012, Reisig et al. 2013, Lewis-Rosenblum et al. 2015, Klick et al. 2016, Blaauw et al. 2017, Bastola and Davis 2018, Hagler 2019), natural enemies (Horton et al. 2009, Hagler and Jones 2010, Sivakoff et al. 2012, Lefebvre et al. 2017, Irvin et al. 2018), and pollinators (Hagler et al. 2011; Biddinger et al. 2013; Boyle et al. 2018a, b). Our findings demonstrate that chicken egg albumin is also a suitable protein source to mark fall armyworm and corn earworm moths. However, this study showed that chicken

egg albumin does not persist throughout larval development, being only detectable within instar, in our case at the first instar. Because insect growth and development depend on the molting process, the residue of egg albumin absorbed on the first-instar integument is likely discarded during subsequent ecdysis. On the other hand, our results showed that chicken egg albumin has potential for mark-release-recapture and for mark-capture studies. All adults assayed in the laboratory acquired egg albumin, confirming the efficacy of this protein to be used for mark-release-recapture studies. In addition, egg albumin applied in field cages and directly in the field resulted in a high percentage of positive samples, which indicates that direct application of albumin in the field can mark natural populations of corn earworm, and therefore be used for mark-capture studies. Compared with previous studies (Jones et al. 2006, Boina et al. 2009, Hagler and Jones 2010), the percentage of corn earworm adults positive scored for egg albumin from the field experiment was significantly lower. However, some points should be considered. The percentage of positive samples was calculated based on the total of

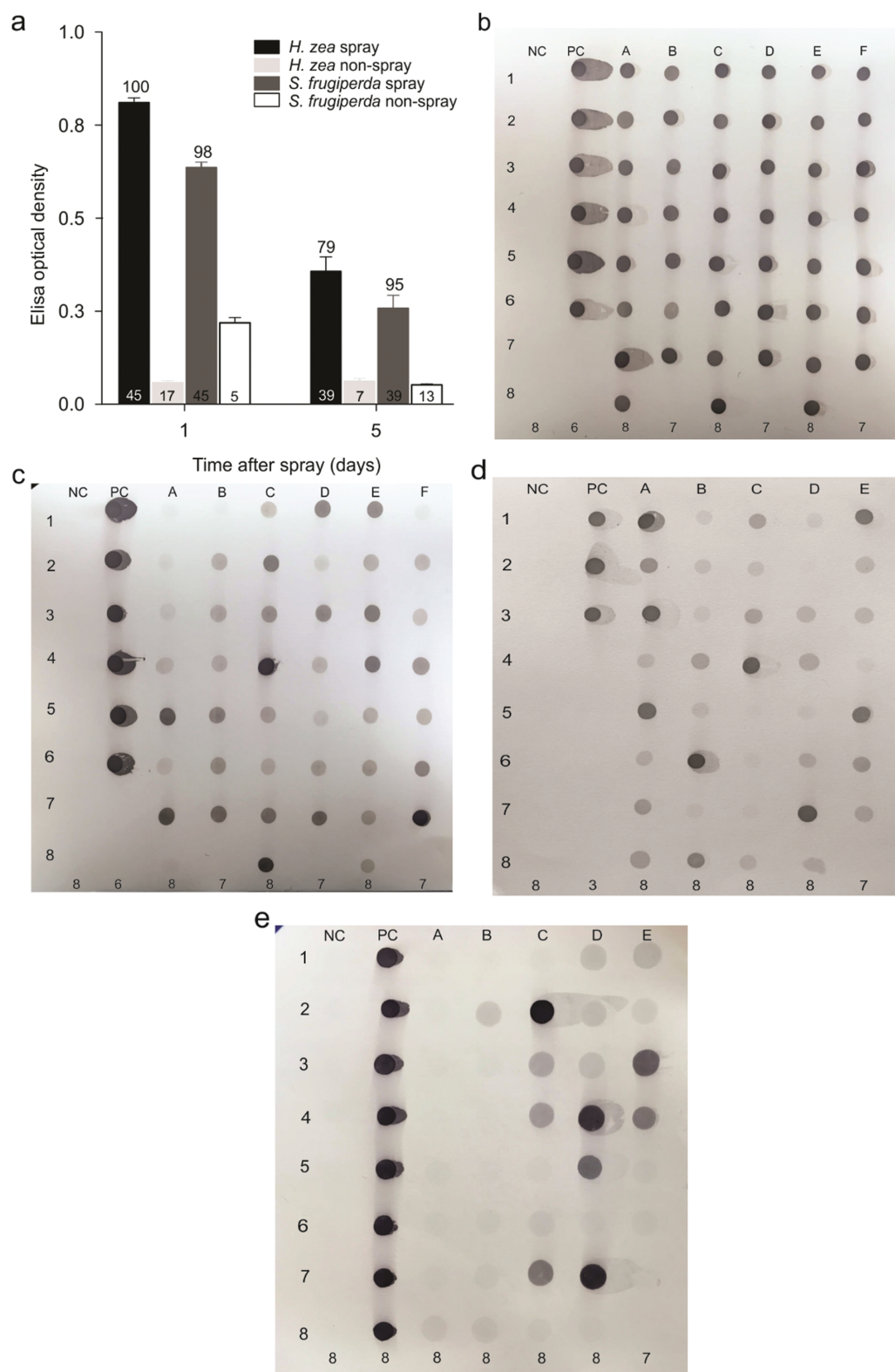


Fig. 4. Immunoassay results for the presence of egg albumin in adults of corn earworm and fall armyworm sprayed with 20% egg white solution in the field cages. (a) ELISA OD values (mean \pm SEM) and the percentage of samples scored positive for the presence of egg albumin (number above the bars) in corn earworm and fall armyworm. The number inside the bar indicates the sample size tested. (b, c) Dot blot for the detection of egg albumin in corn earworm and fall armyworm 1 d after spray, respectively. (d, e) Dot blot for the presence of egg albumin in corn earworm and fall armyworm 5 d after spray, respectively. The black/gray spots mean scored positive samples for the marker.

Table 1. Detection of egg albumin by ELISA and dot blot methods

Study	Time after spray/trap	Insect	Test	Positive	Negative	df	χ^2_{cal}	P value ^a
Adults/field cages	1 and 5 d	<i>S. frugiperda</i>	ELISA	81	3	1	0.15	0.70
			Dot blot	80	4			
Adults/open field	Light	<i>H. zea</i>	ELISA	76	8	1	8.40	0.00
			Dot blot	84	0			
		<i>H. zea</i>	ELISA	38	88	1	0.40	0.52
			Dot blot	46	90			
	Pheromone	<i>H. zea</i>	ELISA	8	183	1	0.06	0.80
			Dot blot	9	182			
Total	—	—	ELISA	203	282	1	0.45	0.50
			Dot blot	219	279			

Experiments performed during larval stage and adults in the laboratory are not presented since negative samples were not found.

^aDifferences among ELISA and dot blot methods using chi-squared test ($P < 0.05$).

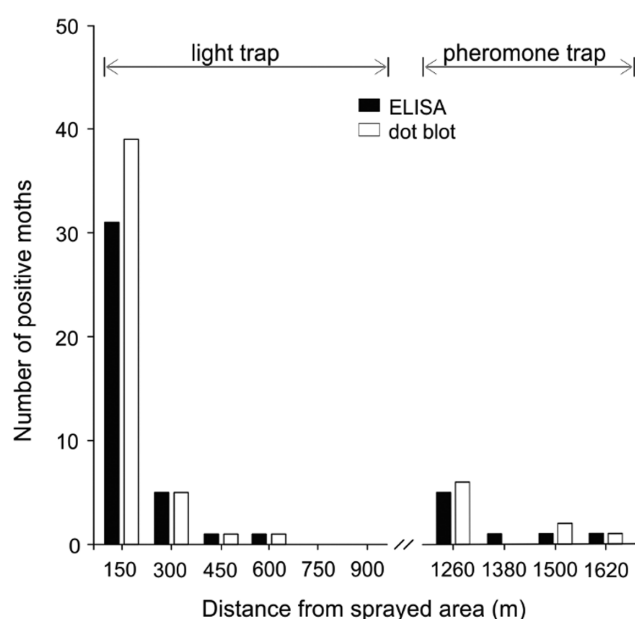


Fig. 5. Number of positive moths detected by ELISA and dot blot. Egg white albumin was sprayed in an open field and the moths were collected in light and pheromone traps placed at different distances around the application area. The distances from the sprayed area are represented in intervals.

insects collected, and some of them are likely from natural populations, since the study was performed during the northern migration of corn earworm in the United States. In addition, egg albumin was sprayed when at least 80% of adults had emerged, which suggests that some had already moved away from the spraying area.

The advantages of using protein as marker include its availability, low cost, and possibility to be applied directly in the field (Jones et al. 2006). Also, the ELISA is a simple, rapid, sensitive, and relatively inexpensive method of protein detection (Jones et al. 2006), and ELISA performed on microplates showed to be effective to detect egg albumin in this current study. In addition to ELISA, we also demonstrated that an alternative immunoassay (dot blot), in which protein detection is performed on PVDF membrane, has equal sensitivity as microplate ELISA. According to our results, the limit of detection of egg albumin by both ELISA and dot blot ELISA was 41 ppb, which indicates that both methods are equally sensitive. Despite the high sensitivity, dot blot is a qualitative method which could lead to a subjective interpretation of the results. A quantitative analysis could be carried out using

ImageJ (an image processing software) (Abramoff et al. 2004) which can detect the blots and objectively determine if a sample is positive or negative. However, the lack of well photographed PVDF membranes makes such alternative unsuitable for this study. Regarding its subjectivity, dot blot could be a useful protein detection method when no equipment is available to read a microplate ELISA. This is especially applicable to remote locations and/or places with lower resources and equipment, such as in Africa, South America, or Asia.

A major benefit of having an effective and easily detectable marker is to study the dispersion pattern and ecology of agricultural pests (Hagler et al. 1995). The laboratory and field studies performed clearly demonstrated that egg albumin is a suitable marker and can be used to measure the movement of fall armyworm and corn earworm. With a protein marker, it would be possible to evaluate the range of cultivated and wild host plants colonized by these pests as sink and source sites. The movement of *Diaphorina citri*, vector of the citrus greening disease, has been documented using egg albumin and milk proteins. Boina et al. (2009) found that this pest moved between managed and unmanaged areas suggesting that abandoned groves may function as a pest source for cultivated areas. Klick et al. (2016), using egg albumin, demonstrated that *Drosophila suzukii* utilize a wild alternative host, which may play a role as a source of infestation of *D. suzukii* in cultivated fruiting crops. In addition, egg albumin could be used to investigate some assumptions involved in the adoption of refuge to slow the evolution of resistance to Bt crops targeting fall armyworm and corn earworm. An assumption of the refuge is that non-Bt plants must be cultivated concomitantly with Bt plants to provide susceptible individuals to mate with eventual resistant individuals coming from the Bt area (Gould 1998). However, the effectiveness of this strategy is conditional on random adult movement and the distance between non-Bt and Bt fields (Gould 1998). Despite that many studies have reported that refuge can delay the evolution of resistance (Tabashnik 2008), most are based on mathematical models which do not consider the pest movement patterns in the field. Therefore, egg albumin could be an effective marker for tracking and monitoring movement of adults of fall armyworm and corn earworm between Bt and non-Bt crops, and consequently, better inform research to improve resistance management. These results could also be considered a template to be used in the study of the dispersion of other noctuids.

Although the use of protein markers have overcome several disadvantages associated with methodologies previously adopted to study the movement pattern of insects (Hagler and Jackson 2001, Jones et al. 2006), some points about its application deserve attention. Protein persistence can be affected by abiotic factors, such as rain, humidity, and wind (Jones et al. 2006, Boina et al. 2009, Hagler 2019), and this should be taken into consideration during the experimental design.

Table 2. Egg albumin transference between marked and unmarked moths at different time after sprayed and between individuals of the same or different gender

Time (hour)	Sex	No. sprayed	No. non-sprayed	No. positive scored		Positive increasing (%)	
				ELISA	Dot blot	ELISA	Dot blot
1	M or F	15	16	20	15	16.13	0
	M and F	16	16	21	20	15.62	12.5
24	M or F	16	16	19	17	9.37	3.12
	M and F	16	16	19	17	9.37	3.12

M, male; F, female.

Table 3. Chi-squared summary results for the marker transference between marked and unmarked moths evaluated by ELISA and dot blot

Method	Test	Positive	Negative	df	χ^2_{cal}	P value ^a
ELISA	1 h	41	22	1	0.44	0.50
	24 h	38	26			
Dot blot	1 h	35	28	1	0.07	0.78
	24 h	34	30			
ELISA	M or F	30	24	1	0.58	0.44
	M and F	40	24			
Dot blot	M or F	32	31	1	0.63	0.42
	M and F	37	27			
ELISA	Total	79	48	1	1.62	0.20
Dot blot	Total	69	58			

M, male; F, female.

^aDifferences among ELISA and dot blot methods using chi-squared test ($P < 0.05$).

Concerns have arisen about the risk of underestimating false positives, particularly in migration studies, which can alter dispersal estimates (Sivakoff et al. 2011). In addition, externally marked individuals may pass the marker to unmarked individuals during the capture (Hagler et al. 2015). This concern is relevant because in this study moths were kept together in a small cage until being collected. As consequence, physical contact between them may pass the protein marker from marked to unmarked individuals, which can impact the understanding of the insect dispersal pattern in the landscape. By simulating marker transference between marked and unmarked moths into a small cage (the same tied to the light trap used in the field experiment), we demonstrated that the incidence of false positive may be a disadvantage of this technique when documenting insect dispersion. As ELISA and dot blot are very sensitive methods, the transference of even a small amount of protein can be detected. The marker transference can occur between moths inside the cage as we already shown or during the sample collection. However, the marker protein is still a viable method to track the dispersion of noctuids. To decrease the risk of marker transference, small cages tied to light traps could be coated with adhesive material which would immobilize the moths avoiding physical contact. In addition, sticky liners could be used in pheromone and light traps to immobilize the trapped insects without compromising the effectiveness of the marker (Blackmer et al. 2006, Hagler 2019). Therefore, improvement of collection methods and protein detection could decrease the risk of false positive due to marker transference.

The results of the present work demonstrate that fall armyworm and corn earworm acquired egg albumin applied in the laboratory and under field conditions, the protein persisted in moths of both species, and egg albumin is suitable as a marker for the study of dispersion of these pests in the landscape. In addition, dot blot was found to be a feasible protein detection method that could be considered as an alternative to microplate ELISA, especially in those cases that ELISA could not be performed. Despite the incidence of false positive due to

marker transference, insect collection and protein detection improvements could avoid the incidence of false positive.

The dispersal of noctuids in the agricultural landscape is a key component to when designing integrated pest management and integrated resistance management programs. Tracking and monitoring the movement of these species with protein marker (e.g., egg albumin) would provide useful information about their dispersion in the landscape as well as the size that should be considered when recommending IPM management tactics (e.g., considering the dynamics of pest sink and source), and to validate the IRM recommendation for the design of refuge areas for Bt and other transgenic crops.

Acknowledgments

We would like to thank Terence Spencer and Bruce Kohler from the University of Nebraska-Lincoln Toxicology Lab for the support during the performance of the experiments in the laboratory. C.S.T., S.V.P.-M., T.E.H., and E.J.G.P. designed the experiments; C.S.T., T.E.H., A.V.-J., and A.M.V. performed the experiments; C.S.T., S.V.P.-M., T.E.H., and A.V.-J. performed the immunoassays to detect the marker. All the authors contributed and approved the content of this research.

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