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Research

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Refinement of the Protein Immunomarking Technique for Mark-Capture Research

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Abstract

A follow-up study was conducted to further evaluate the marking efficiency of broadcast spray applications of egg albumin (from chicken egg whites) on *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) in alfalfa. A previous study recorded exceptional marking efficiency (e.g., >95% of the population) on *H. convergens* when using relatively high concentrations (10 to 50%) of chicken egg whites. The present study examines marking efficiency of egg whites using lower concentrations of 2.5, 5.0, and 10.0%. We used cadaver and free-roaming beetles to measure protein mark acquisition (and retention) of each protein concentration by direct contact with the spray application and incidental contact with protein residue on the plant tissue, respectively. The vertical distribution of the protein mark was also determined by sampling the upper and lower portions of the alfalfa canopy. The data indicate, regardless of the egg white treatment, that the backpack sprayer provided uniform coverage of egg albumin on the alfalfa plants and cadaver beetles. Also, almost every free-roaming beetle acquired a mark within 24 h after contact exposure to protein marked plants. This study shows that a very low concentration of egg albumin is sufficient for marking arthropods directly in the field.

Key words: ELISA, dispersal, *Hippodamia convergens*

Mark-capture research requires marking arthropods directly in their natural habitat (Hagler and Jackson 2001). Over 25 yr ago, the protein immunomarking technique (PIT) was described for mark-release-recapture (MRR) research (Hagler et al. 1992). The procedure consisted of applying a foreign protein via ingestion of foodstuffs or by topical application to arthropods in the laboratory and then releasing them at a central point release site in the field. In turn, field-collected arthropods were identified for the presence of a protein mark by a protein-specific enzyme-linked immunosorbent assay (ELISA) (Hagler et al. 2002, Hagler and Naranjo 2004).

Since its inception, the PIT has been adapted for mark-capture research (Jones et al. 2006). The main difference between using the PIT for MRR and mark-capture research is, for the latter, that the arthropods are marked directly in their natural habitat, usually by a broadcast application of protein solution. The PIT mark-capture method has proven to be a valuable tool for studying arthropod dispersal patterns (see Hagler 2019 for a review). The most popular protein marker used to date for mark-capture type research is egg albumin. Egg albumin is found in commercially available chicken egg whites (Jones et al. 2006, Hagler and Jones 2010). For the most part, the concentration of egg whites applied for any given study has been by a best-guess estimate. To date, the concentration of egg whites used has ranged from 10 to 20% (Jones et al. 2006, Boina et al. 2009, Horton et al. 2009, Irvin et al. 2012, Sivakoff et al. 2012, Swezey et al. 2013, Bastola et al. 2016, Klick et al. 2016, Leach et al. 2019). Only one study was designed specifically to determine the optimal concentration of egg whites needed to effectively mark arthropods in alfalfa. For that study, egg white concentrations tested were arbitrarily chosen to be 10, 25, and 50% whole chicken egg white mixtures. The data obtained showed that all three egg white treatments were equally and highly (95%) effective at marking *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) over a seven day period (Hagler et al. 2014). The present study was designed to determine whether even lower concentrations of egg whites could be effective (i.e., >90% of the population) for marking arthropods for mark-capture research. If so, a more diluted protein solution would be easier to apply directly into the field while, at the same time, reducing the cost of conducting a large-scale mark-capture experiment.

Materials and Methods

Test Beetles

Adult *H. convergens* were purchased from Arbico (Oracle, Arizona, United States). Half of these beetles were euthanized by freezing at –80°C. These beetles were designated as the cadaver beetle treatment. They were used to test for the presence of the protein mark via a direct topical application. The cadavers served to measure the

frequencies of stationary beetles being marked directly in the field by the topical application of the protein sprays as a function of their location on the plant. Fifteen cadavers were attached, dorsal side up, to plastic twist-lock 'bread' tags (Hummert Int., Topeka, Kansas) as described by Hagler et al. (2014). The cadavers were arranged on each tag so that five individuals occupied roughly every third of a tag. The remainder of the beetles were kept alive and reserved for the part of the experiment designed to measure the acquisition of the protein mark by contact exposure with residue left on alfalfa plant tissue. These beetles were designated as the free-roaming beetle treatment. These beetles were immobilized by chilling at 4°C in a walk-in refrigerator. After chilling, 10 beetles were placed into 30-ml snap vials and left in the refrigerator until just before their release into the field cages.

Study Site

The study was conducted within a 1.0 ha blooming alfalfa field at the University of Arizona, Maricopa Agricultural Research Center, Maricopa, Arizona. The alfalfa plants were in full bloom and stood approximately 0.7-m tall at the time of the study. There was no precipitation recorded throughout the study, and the average daytime and nighttime temperatures were 33.9 ± 1.5°C and 15.0 ± 3.2°C, respectively.

Experimental Setup

The alfalfa plots were arranged in a four-block randomized design. Each block contained four, 6.3- × 5.9-m alfalfa plots. The middle 2.0- × 6.3-m swath of each 5.9-m wide plot was designated as the protein mark spray zone. Before spraying, six alfalfa plants were randomly chosen within the spray zone of each plot. Two bread tags (15 cadavers each) were attached to each plant. A cadaver bread tag was placed on the lower (≈15 to 20 cm from the ground) and the upper (≈15 to 20 cm from the top) portion of each plant.

Once the cadavers were placed on the plants, the 2.0-m wide swath in each plot was sprayed with 2.0 liters of one of the four designated egg albumin marking treatments with a backpack sprayer (MD155DX Mist Duster gas-powered backpack sprayer; Maruyama, Denton, TX). The protein marker was crude chicken egg whites (All Whites, Papetti Foods, Elizabeth, NJ) diluted in tap water. The four concentrations tested consisted of a 0 (no mark water control), 2.5, 5.0, or 10.0% egg whites solution. The treatments were applied to a randomly selected plot within each of the four blocks. The 0% control treatment was sprayed first, followed by the 2.5, 5.0, and 10.0% treatments, respectively. After the marks had dried for 2 h, six randomly selected alfalfa plants from each plot were caged as described by Hagler et al. (2014). Briefly, the bottoms of the nylon tulle (mesh size 1 mm^2; Tempe Sales, Tempe, AZ) sleeve cages (1 m long × 0.5 m diameter) were tied at ground level around the base of an individual plant with a permanent zip-tie. The cages were left in this position until the next day.

Beetle and Plant Sampling Procedures

Cadaver Beetles

One day after marking the first cadaver, samples were collected by cutting the outer third (5 of the 15 cadavers) of each bread tag on the bottom and top of each tagged plant and frozen at −80°C. Then, the cadaver beetles located on the middle third and bottom third of each bread tag were sampled 4 and 7 d after marking, respectively. The bread tags were removed from the freezer, and each cadaver was excised from the bread tag with a clean razor blade and placed in a 1.5-ml microcentrifuge tube containing 1.0 ml of tris-buffered saline (TBS). The samples were soaked for 1 h on an orbital shaker at 100 rpm at room temperature. Each sample was assayed for the presence of egg albumin protein by the ELISA described by Hagler et al. (2014).

Free-Roaming Beetles

On the day after protein application, the top of each sleeve cage was grabbed and pulled up over the top of the plant. Then, ten living *H. convergens* described above were placed in each cage (note that these beetles were marked with a green ink dot to distinguish them from their native counterparts). The top of the cage was then immediately tied with a zip-tie. This study was designed to determine the frequencies of free-roaming beetles acquiring a mark after residual contact exposure to the protein-marked alfalfa plants. The beetles then allowed to roam freely within each cage for 1, 4, or 7 d. After each time interval, two of the six caged plants from each of plot were cut at their base, immediately transported back to the laboratory, and frozen at −80°C. The free-roaming beetles were processed by removing the caged plants from the freezer and carefully collecting them from the samples. Each beetle was placed into a 1.5 ml centrifuge tube containing 1.0 ml of TBS and prepared for ELISA as described above.

Plant Samples

Alfalfa leaf disc samples were also taken from the upper and lower portion of each caged plant to determine the homogeneity and persistence of the egg albumin mark on the plant tissue. Ten alfalfa leaf disc samples (5 from the top and 5 from the bottom), each obtained from a separate leaf, were collected from each plant. Each leaf disc was obtained with a clean 6.0-mm diameter soda straw (Kroger, Cincinnati, OH), as described by Hagler et al. (2014). Each leaf disc was placed into a 1.5 ml centrifuge tube containing 1.0 ml of TBS and processed for ELISA analysis as described above.

Data Analysis

All beetle and alfalfa leaf samples serving as negative ELISA controls were collected from the plots marked with tap water. The mean and SD of the negative control samples were calculated. Individual leaf and beetle samples collected from all the protein mark treatments were scored positive for the presence of egg albumin protein if its ELISA reading exceeded the mean negative control reading by three SDs (Hagler 1997). Box-whisker plots were constructed to depict the mean, median, and range of each sample for each protein mark treatment. The marking efficiency of the protein application was determined by simply calculating the proportion of positively marked specimens obtained from each protein concentration treatment.

Results

Distribution of Protein Mark Throughout the Alfalfa Canopy

The leaf disc samples collected from the alfalfa plants sprayed with water (negative controls) consistently yielded low ELISA readings throughout the study. Overall, only four (1.6%; n = 240) leaf disc samples yielded a false-positive ELISA reaction (Fig. 1). Conversely, every alfalfa leaf disc sample examined from the egg albumin-marked plots, regardless of the protein concentration treatment and time expired since marking, yielded a positive ELISA reaction for the presence of the mark.

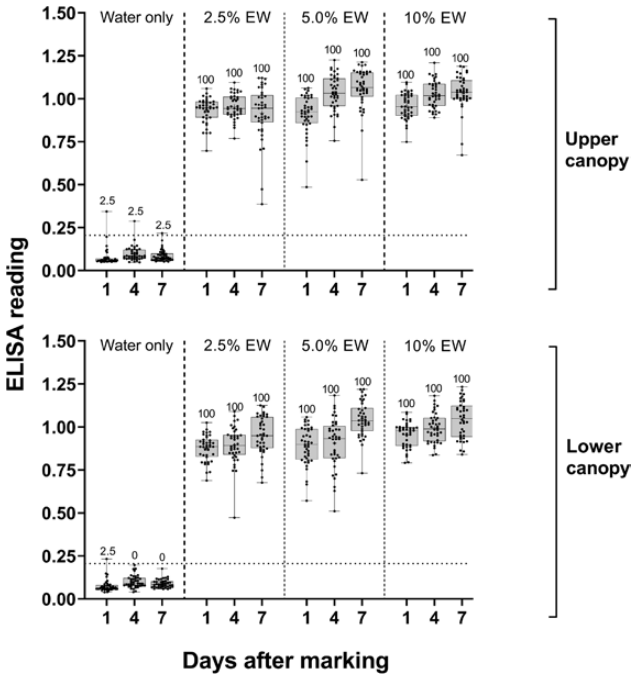

Fig. 1. The ELISA values yielded for each leaf disc sample (black dots, $n = 40$ per treatment) examined for the presence of the egg albumin protein mark. Samples were collected from the upper and lower portion of the plant canopy 1, 4, and 7 d after marking. The percentage of leaf disc samples scoring positive for the presence of the mark for each treatment is given above each boxplot. The upper and lower whiskers depict the extreme ELISA values yielded for each treatment. The median ELISA reading is depicted by the solid horizontal line in each box plot, respectively. The dotted horizontal line is the critical ELISA threshold value used to score the leaf disc samples for the presence of the egg albumin mark.

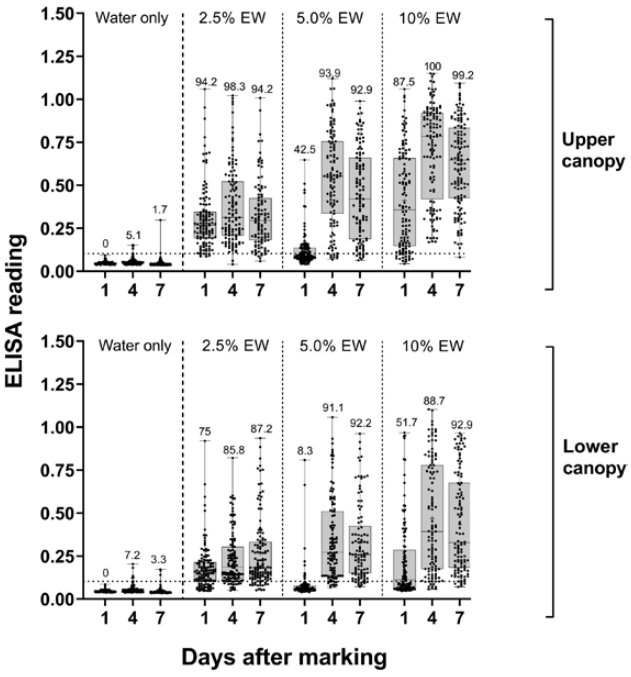

Fig. 2. The ELISA values yielded for each stationary cadaver beetle (black dots, $n = 102$ to 131 per treatment) examined for the presence of the egg albumin protein mark. Samples were collected from the upper and lower portion of the plant canopy 1, 4, and 7 d after marking. The percentage of beetle samples scoring positive for the presence of the mark for each treatment is given above each boxplot. The upper and lower whiskers depict the extreme ELISA values yielded for each treatment. The median ELISA reading is depicted by the solid horizontal line in each box plot, respectively. The dotted horizontal line is the critical ELISA threshold value used to score the cadaver beetle samples for the presence of the egg albumin mark.

Distribution of Protein Mark on Cadaver Beetles

The cadaver beetles collected from the alfalfa plants sprayed with water (negative controls) yielded low ELISA readings throughout the study. Overall, 2.8% (n = 703) of the beetles collected from the water control plots yielded a false-positive ELISA reaction for the presence of egg albumin (Fig. 2). Generally, 85 to 100% of the cadavers, regardless of the concentration of egg whites administered, their location within the plant canopy, and time-lapsed after marking, yielded positive ELISA reactions for the presence of the mark. However, the samples collected one day after marking from the 5% egg white treatment yielded relatively poor results. Specifically, only 42.5 and 8.3% of the cadavers located on the upper and lower canopy, respectively; yielded a positive ELISA reaction. In all likelihood, this was due to poor spray coverage on the stationary (sentinel) beetles that were sampled on those particular plants on that day.

Acquisition of the Protein Mark by Free-Roaming Beetles

The free-roaming beetles collected from the water-marked alfalfa plants yielded low ELISA readings. However, 4.1% (n = 240) of the beetles examined yielded false-positive ELISA reactions for the presence of the protein mark (Fig. 3). The vast majority (98.5%, n = 714) of the free-roaming beetles exposed to the various egg albumin plant residues readily acquired the mark within the first day of exposure and retained it throughout the entire study.

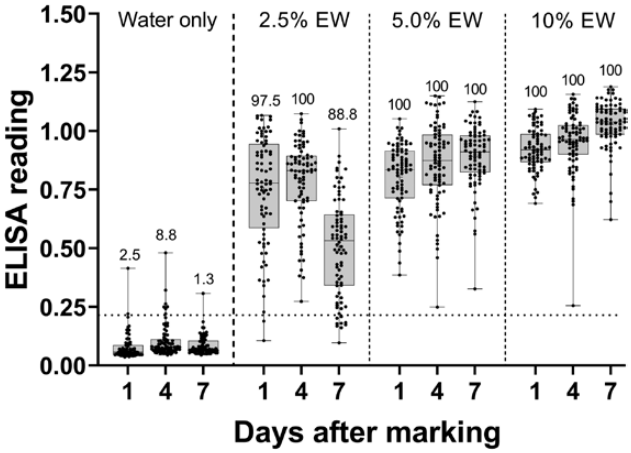

Fig. 3. The ELISA values yielded for each free-roaming beetle sample (black dots, n = 74 to 81 per treatment) examined for the presence of the egg albumin protein mark. Samples were collected from plants 1, 4, and 7 d after marking. The percentage of beetle samples scoring positive for the presence of the mark for each treatment is given above each boxplot. The upper and lower whiskers depict the extreme ELISA values yielded for each treatment. The median ELISA reading is depicted by the solid horizontal line in each box plot, respectively. The dotted horizontal line is the critical ELISA threshold value used to score the free-roaming beetle samples for the presence of the egg albumin mark.

Discussion

The PIT is a valuable tool for marking arthropods for mark-capture type research (Hagler 2019). The main attributes of the procedure are that the protein immunomarkers are affordable, effective, and can be administered over large areas with conventional spray equipment. However, research is still needed to refine the PIT for mark-capture research. For instance, in previous studies, the volume and concentration of protein applied, have been arbitrarily chosen (i.e., best-guess estimates; see the citations contained in Hagler 2019). More rigorous testing under actual field conditions is needed.

Our previous research in alfalfa using the same spray apparatus showed that 50, 25, and 10% egg white concentrations effectively mark >95% of the cadaver and free-roaming *H. convergens* (Hagler et al. 2014). The high marking efficiency in that study, even at the lowest concentration tested (10%), exceeded expectations. Other studies have shown that egg white concentrations ranging from 10 to 20% were also effective at marking indigenous arthropods in a wide variety of cropping systems (see Hagler 2019 for a review). Moreover, many methods have been used to apply protein marks. The backpack spray device used in this study has been used to mark native arthropods in trap crop and cover crop dispersal experiments (Swezey et al. 2013, 2014; Irvin et al. 2018). Air-blast, boom-and-nozzle tractor, and aerial (helicopter and airplane) applicators have also proven effective for marking arthropods in various habitats (Jones et al. 2006, Horton et al. 2009, Krugner et al. 2012, Sivakoff et al. 2012, Blaauw et al. 2016, Klick et al. 2016, Bastola and Davis 2018). The current study suggests that even lower egg white concentrations (e.g., 2.5 and 5%), regardless of the method of application, should be effective on different arthropod taxa.

There are several key advantages to using less protein mark for large-scale mark-capture research. First, it would reduce the cost of the marking procedure. Second, it would likely decrease the chance of altering the arthropod's behavior (Jones et al. 2011). Finally, lower concentrations of egg whites would be easier to apply because it would be less likely to clog the spray nozzles. Previously, we have found that high egg white concentrations are notorious for clogging spray nozzles (personal observation).

The present study was conducted under hot and dry environmental conditions. In a previous study, a heavy rainfall (i.e., 0.56 cm in 1 h) washed off all the egg white albumin residue that was sprayed over a cotton field the day before the storm (J. R. Hagler, unpublished data). As such, the protein mark had to be re-applied to the field site. The impact that abiotic factors, such as rain, humidity, dew, and radiation have on marking efficiency merits further investigation.

In summary, the acquisition and retention of low concentrations of egg albumin protein by direct spray and residual contact were examined on *H. convergens*. Results showed that low egg white concentrations were as effective as the higher concentrations used in previous studies. Lower concentrations of egg whites will improve the cost and application efficiency of the PIT for future mark-capture type research.

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