JOURNAL OF APPLIED ENTOMOLOGY
J. Appl. Entomol.

ORIGINAL CONTRIBUTION

Development of a standardized protein immunomarking protocol for insect mark–capture dispersal research

J. R. Hagler, S. E. Naranjo, S. A. Machtley & F. Blackmer

USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ, USA

Keywords
enzyme-linked immunosorbent assays, *Hippodamia convergens*, marking efficiency, mark–release–recapture, protein marking

Correspondence
James R. Hagler (corresponding author), Arid-Land Agricultural Research Center, USDA-ARS, Maricopa, AZ 85138, USA. E-mail: james.hagler@ars.usda.gov

Received: February 24, 2014; accepted: April 2, 2014

doi: 10.1111/jen.12135

Abstract

A field study was conducted to test the marking efficiency of broadcast spray applications of protein marks on stationary (represented by cadavers) and free-roaming lady beetles *Hippodamia convergens* Guérin-Méneville that were strategically placed in blooming alfalfa plots. The marks tested included three different concentrations of egg albumin from chicken egg white, casein from bovine milk and trypsin inhibitor from soy milk. The cadaver and free-roaming beetle treatments served to measure the acquisition and retention of each protein treatment regime by direct contact with the spray solution and by residual contact with protein-marked residue on alfalfa, respectively. In addition, the vertical distribution of marking efficacy was determined by sampling alfalfa plant tissue and beetle cadavers that were located on the upper and lower portion of the plant canopy. The data indicated that the backpack spray apparatus was very effective at uniformly administering the various protein marks, regardless of the concentration, throughout the entire plant canopy. Also, the free-roaming beetles readily self-marked by contact exposure to protein-treated plants. We also identified concentrations of each protein type that will mark about 90% of the resident beetle population. Moreover, if a mark–capture-type study only requires two unique protein marks, we determined that concentrations of 25% for egg white and 100% for bovine milk could be used to mark 98% of the population. Our results provide a significant step towards standardizing protein immunomarking protocols for insect mark–capture dispersal research. In addition, we identify several areas of research that are needed to further standardize the protein mark–capture procedure.

Introduction

A thorough understanding of insect pest and natural enemy dispersal patterns is an important component for making informative pest management decisions. Often, the key tool needed to track insect movement is a reliable method to tag insects directly in their natural habitat. Unfortunately, marking insects is generally more difficult than marking vertebrates due to their relatively small size and cryptic behaviour. As such, there are very few materials available that are effective at tagging insects. The type of insect mark chosen for a study usually depends on whether the researcher is employing a mark–release–recapture- or mark–capture-type experiment. For mark–release–recapture experiments, the insects are usually marked *en masse* in the confines of the laboratory, released into the field at a centralized location, and recaptured using predetermined temporal and spatial sampling schemes. Historically, several types of paints, dyes, powders or rare elements have been used for mark–release–recapture experiments (Hagler and Jackson 2001). For mark–capture studies, the mark is usually applied to insects directly in the field. As such, the most useful markers for mark–capture research are those materials that can be easily sprayed onto insects or the plants they inhabit directly in the field. Dyes and dusts are rarely used for mark–capture research

because they are difficult to uniformly apply in the field (but see Byrne et al. 1996). Rare elements such as rubidium, caesium and strontium are easy to apply, but they have been shown to vary in efficacy and, in many cases, to have lethal or sublethal effects on certain species (reviewed by Hagler and Jackson 2001). Also, detection of trace element markers on insects is difficult, expensive, and time-consuming. In short, most of the materials available for tagging insects are not well suited for large-scale mark–capture research. This is especially true if two or more distinct marks are needed for a particular study.

Over two decades ago, a protein marking (immunomarking) procedure was described that consisted of applying vertebrate-derived immunoglobulin G (IgG) proteins onto insects (Hagler et al. 1992). The IgGs could then be detected on insects using very precise anti-IgG sandwich enzyme-linked immunosorbent assays (ELISA). As these marks have been used individually or in tandem for internally and externally labelling a wide variety of insects for mark–release–recapture research (Hagler 1997; Hagler and Jackson 1998; Hagler et al. 2002; Blackmer et al. 2004; Hagler and Naranjo 2004; Peck and McQuate 2004; Buczkowski and Bennett 2006; Jasrotia and Ben-Yakir 2006; Janke et al. 2009; Baker et al. 2010; Kelly et al. 2012). However, the major limitation with the IgG markers is that they are too expensive for mark–capture research, which requires the application of the marks over relatively large areas. Recently, this drawback was overcome with the development of a succession of second-generation protein detection ELISAs that detect proteins found in relatively inexpensive food products such as egg albumin in chicken egg white, casein in bovine milk and trypsin inhibitor in soy milk (Jones et al. 2006).

Over the past few years these three protein markers have been used alone or in combination to study the dispersal patterns of a wide variety of insects (Jones et al. 2006; Boina et al. 2009; Horton et al. 2009; Basoalto et al. 2010; Hagler et al. 2011a; Krugner et al. 2012; Sivakoff et al. 2012; Swezey et al. 2013). However, research has shown that these three marks vary in efficacy. Specifically, data published to date indicate that the detectability and retention of egg white > bovine milk > soy trypsin inhibitor (Jones et al. 2006; Hagler and Jones 2010; Klick et al. 2014). Hence, the goal of this study was to standardize the various protein marking protocols so that the same fraction of the targeted insect populations are marked regardless of which protein is used. To this end, we field tested the marking efficiency of these various proteins when applied at various concentrations using a backpack spray device. Our marking targets included alfalfa leaf tissue, stationary *Hippodamia convergens* Guérin-Méneville, represented by cadavers that were strategically placed on the alfalfa canopy, and free-roaming *H. convergens* that were caged on protein-marked alfalfa.

Materials and Methods

Test insects

Stationary insect cadavers

Cadaver beetles were strategically placed in the field to measure the acquisition and retention of each protein treatment by direct contact with the topical spray solutions (see below). Adult *H. convergens* were purchased from ARBICO Organics (Oro Valley, AZ). Upon arrival, the beetles were killed by freezing at −80°C. Fifteen beetle cadavers were then glued (Elmer's Glue-All™; Elmer's Products Inc., Columbus, OH) to 9.0 cm long × 2.0 cm wide push-on plant labels (similar to plastic twist-lock 'bread' tags) (#49-7032; Hummert International, Earth City, MO). The beetles were oriented with their dorsal surface facing up. The 15 cadavers were attached so that there were three rows of five individuals on each plant label.

Free-roaming insects

Free-roaming beetles were placed in the field to measure the temporal acquisition (by self-marking) and retention of each protein treatment after exposure to protein-marked alfalfa plants. Adult *H. convergens* were purchased from ARBICO Organics and were provided *Pectinophora gossypiella* (Saunders) eggs for a food source and a wetted sponge for a water source. The day before the beetles were released into the field, they were immobilized by chilling at 4°C. After chilling, groups of 10 beetles were placed into 30-ml snap vials and left in the refrigerator until release into the field cages the next day (see below).

Study site

The study was conducted in a 0.76-ha blooming alfalfa field located at the University of Arizona's Maricopa Agricultural Center, Maricopa, AZ. The alfalfa plants were 68.7 ± 12.6 cm (n = 240 plants) tall at the time of the study. Weather data (e.g. temperature, solar radiation, humidity, etc.) were recorded every hour by a weather station located 0.2 km from the study site (The University of Arizona, Arizona Meteorological Network (AZMET), Maricopa Station, http://ag.arizona.edu/azmet/).

The experiment was arranged in a randomized complete block design consisting of four linear blocks, each of which contained 12, 6.3 × 5.9 m plots. The middle 2.0 m × 6.3 m swath of each 5.9-m-wide plot was designated as the spray zone. On 15 June 2011, six randomly selected alfalfa plants in each spray zone were 'tagged' with the plant labels containing 15 cadavers as described above. Each of the six plants had two cadaver labels placed on it; one on the bottom half (Approximately 20 cm from the ground) and one on the top half (Approximately 20 cm from the top of the plant canopy). The bottom and top cadaver treatments served to measure the proportion of beetle cadavers marked directly by each spray treatment as a function of beetle vertical distribution within the vegetation.

On 16 June 2011, the middle 2.0 m × 6.3 m swath in each plot was broadcast sprayed over the plant's canopy at a volume of 2.0-l/0.5 min with one of three 2.0-l protein marking solutions using a commercial backpack mister (#MD155DX Mist Duster gas-powered backpack sprayer; Maruyama, Denton, TX). The three protein marks were as follows: (i) chicken egg white (AllWhite® 100% egg white; Papetti Foods, Elizabeth, NJ), (ii) bovine skim milk (Shamrock Farms, Phoenix, AZ) and (iii) unsweetened soy milk (Westsoy, The Hain Celestial Group Inc., Melville, NY). Each protein was tested at four different concentrations including a water (0%) control. The concentrations tested for each protein type were selected based on previous research and experience (J.R. Hagler, unpublished data). The bovine milk and soy milk marks were tested at 0% (water control) and low, medium and high concentrations of 33, 66 and 100% (v/v), respectively. The chicken egg white marker was tested at 0% and at low, medium and high concentrations of 10, 25 and 50%, respectively. Each of the 12 possible protein treatments (three protein types × four concentrations) was applied to a randomly selected plot within each of the four replicate blocks. All of the 0% treatments (the water controls) were sprayed first. Then, the soy milk treatments were applied starting with the lowest concentration (33%) and ending with the highest concentration (100%), followed by the bovine milk and then chicken egg white at their lowest through highest concentrations. The backpack sprayer was thoroughly washed three times between protein treatment applications (three wash treatments adequately cleans the spray device; James. R. Hagler personal observation). The protein marks were allowed to dry on the alfalfa for 1 h at an ambient temperature of ca. 30°C. Then, six randomly selected alfalfa plants from each plot were prepared for caging with whole plant sleeve cages. The sleeve cages (1 m long × 0.5 m dia.) were constructed from nylon tulle (mesh size = 1 mm^2; Tempe Sales, Tempe, AZ) and had an opening on each end. The bottom of each cage was tied at ground level around the base of an individual plant with a zip tie. The top of the cage was then rolled down and placed at ground level and left in this position until the next day (see below).

Insect and plant sampling procedures

Stationary insect cadavers

One day after the topical sprays were applied, the first cadaver samples were collected by cutting the lower third of each plant label (five cadavers) from the bottom and top of each of the six tagged plants in each plot (30 cadavers per plot at each height). Each tag was labelled, placed in an envelope and frozen at −80°C. Subsequently, the middle third and upper third of each plant label were collected four and 7 days after marking, respectively. The cadavers were removed from the freezer, and individuals were excised from the plant labels with a clean razor blade. Each individual cadaver was then placed into a 1.5-ml centrifuge tube and frozen at −80°C. The samples were thawed, and 1.0 ml of tris-buffered saline (TBS, pH 7.4) was added to each tube. The samples were then soaked on an orbital shaker (100 rpm) at 27°C for 1 h. Each individual insect sample was assayed for the presence of the mark by the protein-specific ELISA described below. Many of the cadavers were scavenged, primarily by *Collops vittatus* (Say) (Zilnik and Hagler 2013). We recorded whether a cadaver was intact, had been partly scavenged or devoured (missing).

Free-roaming insects

The day after each plot was sprayed with protein, the top of the sleeve cages described above was pulled up over the top of each plant. Then, 10 living *H. convergens* were released into each sleeve cage, and the top of the cages was sealed with a zip tie. The insects were allowed to roam freely within each cage for 1, 4 or 7 days. At each post-marking interval, two of the six caged plants from each of plot (10 beetles per cage, 20 beetles per plot) were cut at their base just below the bottom zip tie, placed in a labelled plastic trash bag and frozen within 1 h at −80°C. Note that in many instances, we did not find all 10 beetles in the cage and in a few instances, we found more than 10. For those caged plants

containing <10 beetles, we presumed that the beetle (s) escaped from the cage. For those plants containing more than 10 beetles, we presumed that there were native beetles on the plant when it was caged. Frozen insects were processed by removing the caged plants from the freezer and carefully searching the entire contents for *H. convergens*. Each individual insect was then placed into a 1.5-ml centrifuge tube and frozen at −80°C. The samples were thawed, and 1.0 ml of TBS was added to each tube. The samples were then soaked on an orbital shaker (100 rpm) at 27°C for 1 h. Each individual insect was assayed for the presence of the targeted mark by the protein-specific ELISA described below.

Alfalfa leaf tissue

Alfalfa leaf disc samples were also collected from each caged plant to determine the vertical distribution and temporal persistence of the marks on the plant. Five leaf disc samples were randomly collected from the top half and five from the bottom half of each protein-marked plant 1, 4 or 7 days after treatment application. Individual leaf disc samples were taken with a clean 6.0-mm-diameter soda straw (Kroger Foods, Cincinnati, OH). Each leaf disc sample was then placed into a 1.5-ml centrifuge tube and frozen at −80°C. The leaf samples were thawed, and 1.0 ml of TBS was added to each tube. The samples were then soaked on an orbital shaker (100 rpm) at 27°C for 1 h. Each individual leaf disc was assayed for the presence of the targeted mark by the protein-specific ELISA described below.

Protein-specific ELISAs

Anticasein ELISA for bovine milk

An indirect anticasein ELISA (Jones et al. 2006) was performed on each plant and insect sample collected from those plants marked with bovine milk. A 100 μl aliquot sample was placed into a well of an ELISA plate (#442404 MaxiSorp; Nalgene-Nunc International, Rochester, NY) and incubated for 1 h at 27°C. Each well was then washed by emptying and refilling the well five times with 300 μl phosphate-buffered saline–tween 20 (PBST, 0.5% tween, pH 7.4). Then, 300 μl of a 25% chicken egg white solution (diluted with DI water) was added to each well to block nonspecific binding. Each plate was incubated for 30 min at 27°C. The blocking solution was removed, and the ELISA plate was washed twice with PBST. Next, 50 μl of sheep anticasein, (#K200258; Meridian Life Sciences, Saco, ME) diluted 1 : 2000 in a buffer solution consisting of 25% chicken egg white in TBS, was added to each well and incubated for 1 h at 27°C. Plates were washed again five times with PBST, and 50 μl of mouse antigoat/sheep IgG (#A9452; Sigma Chemical, St Louis, MO) conjugated to horseradish peroxidase diluted 1 : 4000 in a 25% chicken egg white solution (diluted in TBS buffer) was added to each well and incubated at 27°C for 1 h. Finally, plates were again washed five times with PBST and 50 μl of TMB substrate (#TMBW-1000: TMB Microwell One Component Peroxidase Substrate, BioFX Laboratory Inc, Owings Mills, MD) was added to each well, incubated 10 min at 27°C and the ELISA optical density (OD) measured with a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA) set at 650 nm.

Antiegg albumin ELISA chicken egg white

An indirect antichicken egg albumin ELISA (Jones et al. 2006) was performed on each plant and insect sample collected from those plants marked with chicken egg white. A 100 μl aliquot of each sample was placed into a well of an ELISA plate (#351172: Falcon Pro-Bind™, Becton Dickinson Labware, Franklin Lakes, NJ) and incubated for 1 h at 27°C. Wells were washed five times with PBST, and then, the wells were blocked with 300 μl of PBS-BSA (1.0% BSA, w/v, pH 7.4, #P3688; Sigma Chemical). Following a 30-min incubation at 27°C, the ELISA plates were emptied and washed twice with PBST. A 50 μl aliquot of rabbit antichicken egg albumin (ovalbumin) (#C6534; Sigma Chemical) diluted 1 : 8000 in a buffer solution consisting of PBS-BSA (1%) and Silwet L-77 (1.3 μl/ml; Setre Chemical Company, Memphis, TN) was added to each well of the plate. Plates were incubated for 1 h at 27°C, emptied and washed five times with PBST before adding 50 μl of goat antirabbit IgG (#A6154; Sigma Chemical) conjugated to horseradish peroxidase diluted 1 : 1000 in the PBS-BSA-Silwet buffer described above to each well. Plates were incubated again for 1 h at 27°C, emptied and washed five times with PBST before applying 50 μl of TMB substrate to each well. Plates were incubated for 10 min at 27°C before being measured as described above.

Antisoy trypsin inhibitor ELISA soy milk

An indirect antisoy trypsin inhibitor ELISA (Jones et al. 2006) was performed on each plant disc and insect sample collected from those plants marked with soy milk. Each sample was soaked in 1.0 ml of TBS for 1 h at 27°C prior to plating the samples, and then, a 100 μl aliquot of sample was placed into a well of the ELISA plate (MaxiSorp) and incubated

for 1 h at 27°C. Wells were washed five times with PBST and then blocked with 300 μl of PBS-BSA. Following a 30-min incubation at 27°C, the ELISA plates were emptied and then washed twice with PBST. A 50 μl aliquot of rabbit antisoy trypsin inhibitor (#K59971R; Meridian Life Sciences) diluted 1 : 6000 in a buffer solution consisting of PBS-BSA (1%) and Silwet L-77 (1.3 μl/ml) was added to each well. Plates were incubated for 1 h at 27°C, emptied and washed five times with PBST before adding to each well 50 μl of goat antirabbit IgG (#A6154; Sigma, St. Louis, MO) conjugated to horseradish peroxidase diluted 1 : 1000 in the PBS-BSA-Silwet buffer described above. Plates were incubated again for 1 h at 27°C, emptied and washed five times with PBST before applying 50 μl of TMB substrate to each well. Plates were incubated for 10 min at 27°C before being measured as described above.

Data analysis

All alfalfa leaf discs and *H. convergens* (cadaver and free-roaming) samples serving as negative ELISA controls were collected from the plots sprayed with only water and assayed by the ELISAs described above. The beetle and plant samples that were sprayed with the various protein treatment regimes were scored positive for the presence of the targeted mark if the ELISA optical density reading exceeded the mean negative control reading of the water-treated samples by three standard deviations (Hagler 1997).

The proportion of leaf disc and insect samples positive for the presence of each targeted mark was determined for each protein treatment combination. The *a priori* experimental design for this study was constructed to run a factorial ANOVA model based on mark type (egg white, bovine milk, soy milk), mark concentration (control, low, medium, and high), days after marking (1, 4, 7) and the location of the sample on the canopy (upper and lower for the stationary beetle cadavers and plant samples). However, there was little to no variability among treatments and thus no reliable method to fit statistical models to these data (B. Mackey, personal communications). Therefore, we evaluated differences by estimating binomial confidence intervals for the proportion of marked samples and compared treatments by assessing overlap between the 95% confidence limits using the exact method for confidence interval estimation (Zar 1984). We focused on the comparisons among protein mark types and concentration, days or plant strata (this latter factor for alfalfa foliage and beetle cadavers only).

Results

Alfalfa leaf tissue

The leaf disc samples collected from the plants treated with water (controls) consistently yielded very low ELISA readings. Of these (n = 720), 11 (1.5%) yielded a false-positive ELISA reaction (table 1). Conversely, >95% of all the plant discs collected from the various protein-treated plots – regardless of the protein type (egg white, bovine milk or soy milk), protein concentration (low, medium and high), time after application (1, 4 and 7 days) and canopy location (upper and lower) – yielded positive ELISA reactions for the presence of the targeted marks (table 1). However, there was a slight trend in marking efficiency with respect to where the leaf discs were collected from the plant. Specifically, a higher proportion (about 5% more for the bovine milk and soy milk markers) of positive ELISA reactions were observed in samples collected from the lower portion of the plant canopy (table 1).

Stationary insect cadavers

Only 25 (1.2%) of the 2114 stationary beetle cadaver samples collected from the plots that were treated with water yielded a false-positive ELISA reaction for the presence of a targeted protein mark (table 2). In general, all three proteins at the concentrations tested were effective at directly marking the stationary beetles. The medium and high concentrations of egg white and the high concentration of bovine milk marked approximately 98% of the stationary beetles. The low-protein concentration treatment of soy milk marked a higher proportion of cadavers (0.88) than the medium (0.84)- and high (0.82)-concentration treatments. The proportion of marked beetles declined with time for cadavers marked with bovine milk (7 day < 1 or 4 day after application). Again, there was a trend in marking efficiency with respect to where the cadavers were collected from the plant. A higher proportion (about five and 10% for the bovine milk and soy milk, respectively) of positive ELISA reactions were observed in beetles collected from the lower portion of the plant canopy (table 2).

Free-roaming insects

The free-roaming beetles collected from the plants that were treated with water yielded low ELISA readings. Of these (n = 684), 22 (3.2%) yielded a false-positive ELISA response (table 3). All of the protein types at all the concentrations tested marked >90% of

Table 1 Proportion of alfalfa leaf disc samples scoring positive by ELISA for the presence of chicken egg white, bovine milk and soy milk. The alfalfa plants were sprayed with one of the four protein concentration treatments (control, low, medium and high), and then, leaf disc samples were collected 1, 4 and 7 days after marking from the bottom half and top half of the plant canopy

Mark	Mark X Treatment	N	No. of positive	Lower 95% CI	Proportion[1] Positive	Upper 95% CI	Mark[2] X Conc	Mark[2, 3] X Day	Mark[2, 3] X Strata
Egg white	Concentration								
	Control (0%)	240	4	0.005	0.017	0.042	B		
	Low (10%)	240	233	0.941	0.971	0.988	A		
	Medium (25%)	240	232	0.935	0.967	0.986	A		
	High (50%)	240	240	0.985	1.000	1.000	A		
	Days after marking								
	1	240	230	0.925	0.958	0.980		B	
	4	240	235	0.952	0.979	0.993		AB	
	7	240	240	0.985	1.000	1.000		A	
	Plant strata								
	Bottom	360	354	0.964	0.983	0.994			A
	Top	360	351	0.953	0.975	0.989			A
Bovine milk	Concentration								
	Control (0%)	240	3	0.003	0.013	0.036	C		
	Low (33%)	240	234	0.946	0.975	0.991	AB		
	Medium (66%)	240	229	0.919	0.954	0.977	B		
	High (100%)	240	239	0.977	0.996	1.000	A		
	Days after marking								
	1	240	233	0.941	0.971	0.988		A	
	4	240	237	0.964	0.988	0.997		A	
	7	240	232	0.935	0.967	0.986		A	
	Plant strata								
	Bottom	360	359	0.985	0.997	1.000			A
	Top	360	343	0.925	0.953	0.972			B
Soy milk	Concentration								
	Control (0%)	240	4	0.005	0.017	0.042	B		
	Low (33%)	240	233	0.941	0.971	0.988	A		
	Medium (66%)	240	232	0.935	0.967	0.986	A		
	High (100%)	240	236	0.958	0.983	0.994	A		
	Days after marking								
	1	240	235	0.952	0.979	0.993		A	
	4	240	232	0.935	0.967	0.986		A	
	7	240	234	0.946	0.975	0.991		A	
	Plant strata								
	Bottom	360	360	0.99	1	1			A
	Top	360	341	0.919	0.947	0.968			B

[1]Proportion of the samples testing positive for the presence of the targeted protein.
[2]Treatments within factors (protein concentration, days after marking, plant strata) followed by a different letter were deemed different based on the lack of overlap of the 95% binomial confidence intervals (CI).
[3]Mark × day and mark × strata differences do not include the 0% control.

the free-roaming beetles. In addition, >90% of the free-roaming beetles acquired each protein mark within the first 24 h of exposure to marked foliage (table 3).

Discussion

The second generation of protein markers has enormous potential for tagging insects directly in the field for mark–capture research (Jones et al. 2006; Horton et al. 2009; Swezey et al. 2013). The proteins are readily available, inexpensive and can be rapidly and uniformly dispersed over large areas using conventional spray equipment. Moreover, the detection of the marks by protein-specific ELISAs is simple, precise, relatively inexpensive and highly adaptable for high throughput (Hagler and Jones 2010).

Table 2 Proportion of stationary *Hippodamia convergens* cadaver samples scoring positive by ELISA for the presence of chicken egg white, bovine milk and soy milk. The beetle cadavers were sprayed with one of the four protein concentration treatments (control, low, medium and high) and then collected 1, 4 and 7 days after marking from the bottom half and top half of the plant canopy

Mark	Mark X Treatment	N	No. of positive	Lower 95% CI	Proportion[1] positive	Upper 95% CI	Mark[2] X Concentration	Mark[2, 3] X Day	Mark[2, 3] X Strata
Egg white	Concentration								
	Control (0%)	708	14	0.011	0.020	0.033	C		
	Low (10%)	715	666	0.910	0.931	0.949	B		
	Medium (25%)	718	702	0.964	0.978	0.987	A		
	High (50%)	710	698	0.971	0.983	0.991	A		
	Days after marking								
	1	719	683	0.931	0.950	0.965		A	
	4	719	700	0.959	0.974	0.984		A	
	7	705	683	0.953	0.969	0.980		A	
	Plant strata								
	Bottom	1075	1040	0.956	0.967	0.977			A
	Top	1068	1026	0.949	0.961	0.973			A
Bovine milk	Concentration								
	Control (0%)	714	2	0.000	0.003	0.010	C		
	Low (33%)	718	629	0.876	0.876	0.899	B		
	Medium (66%)	713	647	0.884	0.907	0.928	B		
	High (100%)	715	700	0.966	0.979	0.988	A		
	Days after marking								
	1	717	680	0.930	0.948	0.963		A	
	4	719	683	0.931	0.950	0.965		A	
	7	710	613	0.836	0.863	0.888		B	
	Plant strata								
	Bottom	1068	1009	0.929	0.945	0.958			A
	Top	1078	967	0.877	0.897	0.915			B
Soy milk	Concentration								
	Control (0%)	692	9	0.006	0.013	0.025	C		
	Low (33%)	714	630	0.856	0.882	0.905	A		
	Medium (66%)	710	594	0.807	0.837	0.863	AB		
	High (100%)	706	578	0.786	0.819	0.844	B		
	Days after marking								
	1	719	681	0.928	0.947	0.962		A	
	4	719	602	0.808	0.837	0.864		B	
	7	692	519	0.716	0.752	0.782		C	
	Plant strata								
	Bottom	1059	952	0.879	0.899	0.916			A
	Top	1071	850	0.768	0.794	0.818			B

[1]Proportion of the samples testing positive for the presence of the targeted protein.
[2]Treatments within factors (protein concentration, days after marking, plant strata) followed by a different letter were deemed different based on the lack of overlap of the 95% binomial confidence intervals (CI).
[3]Mark × day and mark × strata differences do not include the 0% control.

To date, researchers have used a wide variety of devices to apply both liquid and dry formulations of these proteins for mark–release–recapture-, mark–capture- and self-marking-type studies (Jones et al. 2006; Boina et al. 2009; Horton et al. 2009; Basoalto et al. 2010; Hagler et al. 2011a,b; Krugner et al. 2012; Sivakoff et al. 2012). However, both the amount and concentration of protein(s) used in these studies were arbitrarily selected and not determined by testing in the field. While the data generated thus far indicate that the second-generation protein detection ELISAs are sensitive, it is also apparent that the acquisition and retention of these three proteins differ with regard to abiotic and biotic conditions. As such, our goal was to determine the optimal concentration of each protein type needed to tag an equally high proportion of insects inhabiting a pre-defined area of crop, in this case an alfalfa field. Based on previous results, we assumed that we would need to apply a higher concentration of bovine milk and soy milk

Table 3 Proportion of free-roaming *Hippodamia convergens* samples scoring positive by ELISA for the presence of chicken egg white, bovine milk and soy milk. The beetles were placed on alfalfa plants that had been sprayed with one of the four protein concentration treatments (control, low, medium and high) and then collected 1, 4 and 7 days after marking

Mark	Mark × Treatment	N	No. of positive	Lower 95% CI	Proportion[1] positive	Upper 95% CI	Mark[2] × Concentration	Mark[2, 3] × Day
Egg white	Concentration							
	Control (0%)	226	9	0.018	0.040	0.074	B	
	Low (10%)	241	237	0.958	0.983	0.996	A	
	Medium (25%)	227	227	0.984	1.000	1.000	A	
	High (50%)	228	228	0.984	1.000	1.000	A	
	Days after marking							
	1	226	225	0.976	0.996	1.000		A
	4	230	228	0.969	0.991	0.999		A
	7	240	239	0.977	0.996	1.000		A
Bovine milk	Concentration							
	Control (0%)	224	8	0.016	0.036	0.069	C	
	Low (33%)	218	217	0.975	0.995	1.000	A	
	Medium (66%)	218	197	0.857	0.904	0.939	B	
	High (100%)	236	228	0.934	0.966	0.985	A	
	Days after marking							
	1	232	213	0.875	0.918	0.950		B
	4	217	213	0.953	0.982	0.995		A
	7	223	216	0.936	0.969	0.987		AB
Soy milk	Concentration							
	Control (0%)	234	5	0.007	0.021	0.049	B	
	Low (33%)	215	197	0.871	0.916	0.950	A	
	Medium (66%)	240	226	0.904	0.942	0.968	A	
	High (100%)	203	183	0.860	0.901	0.934	A	
	Days after marking							
	1	231	209	0.859	0.905	0.939		A
	4	210	194	0.879	0.924	0.956		A
	7	217	203	0.894	0.935	0.964		A

[1]Proportion of the samples testing positive for the presence of the targeted protein.
[2]Treatments within factors (protein concentration, days after marking) followed by a different letter were deemed different based on the lack of overlap of the 95% binomial confidence intervals (CI).
[3]Mark × day differences do not include the 0% control.

(e.g. 33, 66 or 100%) to mark an equivalent proportion of insects as the egg white mark (e.g. 10, 25 or 50%) (Hagler and Jones 2010; Slosky et al. 2012; Swezey et al. 2013).

Our experiments to test the acquisition and retention of each protein treatment by direct spray (beetle cadavers) showed that all three proteins, regardless of the tested concentration, were effective markers. Our data also support the findings from previous studies, albeit to a lesser extent, that the acquisition and retention of egg albumin > milk casein > soy trypsin inhibitor (Jones et al. 2006; Slosky et al. 2012; Klick et al. 2014). However, if three marks are needed for a particular study, our data suggest that we could mark about 90% of the resident target population with a 10, 66 and 33% concentration of egg white, bovine milk and soy milk, respectively. Likewise, if a study required only two marks, our data indicate that almost all (98%) of the beetles could be marked with a medium (25%) and high (100%) concentration of egg white and bovine milk, respectively.

Results from the study to test the acquisition and retention of each protein treatment by beetle contact with protein-covered plants showed that the vast majority of beetles readily acquired the marks (e.g. self-marked) by the first day of exposure and that they remained marked throughout the 7-d duration of the study. Our data also showed that lower protein concentrations were generally as effective as higher concentrations. The minimum exposure period tested for this study was 24 h. However, Hagler and Jones (2010) showed that *H. convergens* and *Lygus hesperus* Knight acquired egg albumen within 5 min of contact exposure to egg white-treated cotton leaf tissue and

Trichoplusia ni (Hübner) acquired the mark within 4 h. Jones et al. (2006) showed that the residual contact acquisition and subsequent retention of these three proteins by pear psylla, *Cacopsylla pyricola* Foerster, were significantly different. Respectively, egg white, bovine milk and soy milk were detectable on 79, 19 and 2% of the psyllids that were exposed to protein-treated apple leaves for 19 days. Klick et al. (2014) also showed high variability in protein acquisition by spotted wing drosophila, *Drosophila suzukii* Matsumura, exposed to protein-treated blackberry leaf tissue. That study reported that flies rapidly acquired and retained egg white, but not bovine milk and soy milk markers. Clearly, studies are needed to compare the self-marking capability of different insect species on different host plants prior to embarking on protein mark–capture research.

The type of spray equipment used to apply a protein will likely play a key role in marking efficiency. The backpack sprayer used here and by Swezey et al. (2014) proved to be very effective at evenly distributing the protein treatments throughout the alfalfa canopy. Others have reported satisfactory results in protein marking efficiency in other types of crops using tractor-mounted boom and nozzles (Horton et al. 2009), airplane- and helicopter-mounted boom and nozzles (Sivakoff et al. 2012), and air blast spray rigs (Jones et al. 2006; Krugner et al. 2012; Klick et al. 2014). Whether large-scale commercial spray rigs are as effective at uniformly distributing protein throughout the landscape as the portable backpack sprayer used here is another area for future research.

The persistence of each protein mark will likely be influenced by the climatic conditions encountered at any given study site. This study was conducted under what we considered to be ideal weather conditions (e.g. hot and dry). The average air temperature during the study (±SE) was 31.8 ± 6.5°C (ranging from 19.1 to 42.8°C), the relative humidity was 16.1 ± 8.0%, the solar radiation (daylight hours only) was 87.5 ± 4.3 kJ/m^2, and no rainfall was recorded. Finally, the average wind speed was relatively low at 4.0 ± 0.1 kph. Obvious abiotic factors that could interfere with protein acquisition and retention include extreme temperature, precipitation, humidity and high wind. Jones et al. (2006) examined the resilience of these proteins on apple leaves that were subjected to several simulated rain treatments. That study revealed that egg white and soy milk proteins might be retained on the plant canopy after a light rainfall, but would wash off after a heavy rainfall. However, bovine milk showed greater resilience than egg white and soy milk after a heavy rainfall. A recent field study confirmed that a light but steady rainfall did not adversely affect egg protein retention on blackberry leaves. In fact, the residual acquisition of egg white protein by *D. suzukii* exposed to protein-treated leaves improved over the course of the study (Klick et al. 2014). However, we recently determined that egg white and bovine milk residues were completely washed off cotton plants after single monsoon rain events of 4.2 and 2.8 cm, respectively (J.R. Hagler, unpublished data).

Our study revealed a very slight trend in marking efficiency with regard to location of the targeted specimens on the plant canopy. Specifically, the proportions of protein-marked plant and beetle cadaver specimens were almost always as high or higher on the lower portion of the canopy. The most plausible explanation for this trend is a slight degradation of protein due to more exposure to direct sunlight in the upper portion of the plant canopy. A fruitful area of research would be to compare the protein marking efficacy of proteins used alone with proteins mixed in various sun-blocking agents such as those used to protect photosensitive insect pathogens (Behle et al. 2009).

Protein retention and acquisition could also be influenced by the insect species. Important factors might include insect body type (e.g. large, small, smooth, hairy and scaly), life stage and basic behaviour (active, inactive, solitary and social). For the most part, the first- and second-generation proteins have proven to be very effective at tagging a wide variety of insect species (Jones et al. 2006; Boina et al. 2009; Horton et al. 2009; Hagler and Jones 2010; Hagler et al. 2011b; Krugner et al. 2012; Klick et al. 2014; Swezey et al. 2014). However, marking efficiency data published to date also suggest that certain insects acquire and retain externally applied proteins more readily than others (Jones et al. 2006; Hagler and Jones 2010). Further research is needed to determine whether various biological solvents [e.g. dimethyl sulphoxide (DMSO), Sylgard®, Dow Chemical Co., Midland, MI] could be added to the protein mixtures to increase protein mark acquisition and retention on various insect species (Jones et al. 2011; Williams et al. 2013).

In conclusion, this study showed that the backpack spray apparatus was very effective at administering the various protein treatment combinations directly on the target and that the free-roaming insects readily self-marked with protein via exposure to protein-treated alfalfa. We identified a concentration of each protein type that could be used to mark about 90% of the resident beetle population. Moreover, for studies only requiring two protein types, we determined that egg

white and bovine milk could be used to mark almost all the targeted insects. Our data also suggest that even lower concentrations of protein (especially egg white) might be sufficient to mark the resident arthropod population. Our results provide baseline information for researchers to conduct more precise mark–capture research. We also identify several areas of research that are needed to further standardize the protein mark–capture procedure.

Acknowledgements

We thank Lucy Li, Priyanka Mohanty, Juan Sandoval, Anna Sonoqui, Leticia Sonoqui, Melissa Stefanek and Gabe Zilnik for their technical assistance. A special thanks to Dr. Bruce Mackey (Statistician General; USDA-ARS, Albany, CA) for providing expert statistical advice. This project was supported, in part, by Agriculture and Food Research Initiative Competitive Grant no. 2011-67009-30141 from the USDA National Institute of Food and Agriculture. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Baker PB, Hagler JR, Marchosky R, Machtley SA, Brown JM, Riehle MA, Bellamy DE, 2010. Utilizing rabbit immunoglobulin G protein for mark-capture studies on the desert subterranean termite, *Heterotermes aureus* (Snyder). Insectes Soc. 57, 147–155.

Basoalto E, Miranda M, Knight AL, Fuentes-Contreras E, 2010. Landscape analysis of adult codling moth (Lepidoptera: Tortricidae) distribution and dispersal within typical agroecosystems dominated by apple production in central Chile. Environ. Entomol. 39, 1399–1408.

Behle RW, Compton DL, Laszlo JA, Shapiro-Ilan DI, 2009. Evaluation of soyscreen in an oil-based formulation for UV protection of *Beauveria bassiana* conidia. J. Econ. Entomol. 102, 1759–1766.

Blackmer JL, Hagler JR, Simmons GS, Cañas LA, 2004. Comparative dispersal of *Homalodisca coagulata* and *Homalodisca liturata* (Homoptera: Cicadellidae). Environ. Entomol. 22, 88–99.

Boina DR, Meyer WL, Onagbola EO, Stelinski LL, 2009. Quantifying dispersal of *Diaphorina citri* (Hemiptera: Psyllidae) by immunomarking and potential impact of unmanaged groves on commercial citrus management. Environ. Entomol. 38, 1250–1258.

Buczkowski G, Bennett GW, 2006. Dispersed central-place foraging in the polydomous odorous house ant, *Tapinoma sessile* as revealed by a protein marker. Insectes Soc. 53, 282–290.

Byrne DN, Rathman RJ, Orum TV, Palumbo JC, 1996. Localized migration and dispersal by the sweet potato whitefly, *Bemisia tabaci*. Oecologia 105, 320–328.

Hagler JR, 1997. Field retention of a novel mark-release-recapture method. Environ. Entomol. 26, 1079–1086.

Hagler JR, Jackson CG, 1998. An immunomarking technique for labeling minute parasitoids. Environ. Entomol. 27, 1010–1016.

Hagler JR, Jackson CG, 2001. Methods for marking insects: current techniques and future prospects. Annu. Rev. Entomol. 46, 511–543.

Hagler JR, Jones VP, 2010. An approach to mark arthropods for mark-capture research. Entomol. Exp. Appl. 135, 177–192.

Hagler JR, Naranjo SE, 2004. A multiple ELISA system for simultaneously monitoring intercrop movement and feeding activity of mass-released predators. Int. J. Pest Mange. 50, 199–207.

Hagler JR, Cohen AC, Bradley-Dunlop D, Enriquez FJ, 1992. New approach to mark insects for feeding and dispersal studies. Environ. Entomol. 21, 20–25.

Hagler JR, Jackson CG, Henneberry TJ, Gould JR, 2002. Parasitoid mark-release-recapture techniques – II. Development and application of a protein marking technique for *Eretmocerus* spp., parasitoids of *Bemisia argentifolii*. Biocontrol Sci. Tech. 12, 661–675.

Hagler J, Mueller S, Teuber LR, Machtley SA, Van Deynze A, 2011a. Foraging range of honey bees, *Apis mellifera*, in alfalfa seed production fields. J. Insect Sci. 11, 144.

Hagler J, Mueller S, Teuber LR, Van Deynze A, Martin J, 2011b. A method for distinctly marking honey bees, *Apis mellifera*, originating from multiple apiary locations. J. Insect Sci. 11, 143.

Horton DR, Jones VP, Unruh TR, 2009. Use of a new immunomarking method to assess movement by generalist predators between a cover crop and tree canopy in a pear orchard. Am. Entomologist. 55, 49–56.

Janke J, Bandte M, Ulrichs C, Grabenweger G, Jäckel B, Balder H, Büttner C, 2009. Serological marking of *Pnigalio agraules* (Hymenoptera: Eulophidae) for field dispersal studies. J. Pest. Sci. 82, 47–53.

Jasrotia P, Ben-Yakir D, 2006. An immuno-marking technique for thrips. Entomol. Exp. Appl. 120, 155–160.

Jones VP, Hagler JR, Brunner JF, Baker CC, Wilburn TD, 2006. An inexpensive immunomarking technique for studying movement patterns of naturally occurring insect populations. Environ. Entomol. 35, 827–836.

Jones VP, Melton TD, Baker CC, 2011. Optimizing immunomarking systems and development of a new marking system based on wheat. J. Insect Sci. 11, 87.

Kelly JL, Hagler JR, Kaplan I, 2012. Employing immunomarkers to track dispersal and trophic relationships of a piercing-sucking predator, *Podisus maculiventris* (Hemiptera: Pentatomidae). Environ. Entomol. 41, 1527–1533.

Klick J, Lee JC, Hagler JR, Bruck DJ, Yang WQ, 2014. Evaluating *Drosophila suzukii* (Diptera: Drosophilidae) immunomarking for mark-capture research. Environ. Entomol. (in press).

Krugner R, Hagler JR, Groves RL, Sisterson MS, Morse JG, Johnson MW, 2012. Plant water stress effects on the net dispersal rate of the insect vector *Homalodisca vitripennis* (Hemiptera: Cicadellidae) and movement of its egg parasitoid, *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae). Environ. Entomol. 41, 1279–1289.

Peck SL, McQuate GT, 2004. Ecological aspects of *Bactrocera latifrons* (Diptera: Tephritidae) on Maui, Hawaii: Movement and host preference. Environ. Entomol. 33, 1722–1731.

Sivakoff FS, Rosenheim JA, Hagler JR, 2012. Relative dispersal ability of a key agricultural pest and its predators in an annual agroecosystem. Biol. Control 63, 296–303.

Slosky LM, Hoffmann EJ, Hagler JR, 2012. A comparative study of the retention and lethality of the first and second generation arthropod protein markers. Entomol. Exp. Appl. 144, 165–171.

Swezey SL, Nieto DJ, Hagler JR, Pickett CH, Bryer JA, Machtley SA, 2013. Dispersion, distribution and movement of *Lygus* spp. (Hemiptera: Miridae) in trap-cropped organic strawberries. Environ. Entomol. 42, 770–778.

Swezey SL, Nieto DJ, Pickett CH, Hagler JR, Bryer JA, Machtley SA, 2014. Spatial density and movement of the *Lygus* spp. parasitoid *Peristenus relictus* (Hymenoptera: Brachonidae) in organic strawberries with alfalfa trap crops. Environ. Entomol. 43, 363–369.

Williams L III, Hagler JR, Tonkel KC, 2013. Does dimethyl sulfoxide increase protein immunomarking efficiency for dispersal and predation studies? Entomol. Exp. Appl. 148, 275–286.

Zar JH, 1984. Biostatistical analysis, 2nd edn. Prentice-Hall, Englewood Cliffs, New Jersey.

Zilnik G, Hagler JR, 2013. An immunological approach to distinguish arthropod viviphagy from necrophagy. Biocontrol 58, 807–814.