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A potential contamination error associated with insect protein mark-capture data

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Abstract

Various types of protein-spray solutions have proven effective for externally tagging arthropods for mark-release-recapture and mark-capture type dispersal research. However, there is concern that certain standardized arthropod collection methods, such as sweep netting, might lead to high incidences of protein transfer from field-marked to unmarked arthropods during sample collection and sample handling. Native arthropods were collected in sweep nets from a field of alfalfa, *Medicago sativa* L. (Fabaceae). The nets also contained 10 egg white-, 10 bovine milk-, 10 soy milk-, and 10 water (control)-marked *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) that were visually distinguishable by a yellow, white, green, and blue dot, respectively. The plant debris and arthropods from each sweep net collection were then placed into either a paper or a plastic bag and frozen for storage. The contents of each sweep net sample were thawed and the color-coded *H. convergens* and field-collected arthropods were examined for the presence of each protein by an egg white (albumin), bovine milk (casein), and soy milk (soy trypsin) enzyme-linked immunosorbent assay (ELISA). Data revealed that only 0.67, 0.81, and 0% of the field-collected unmarked arthropods acquired an egg white, bovine milk, and soy milk mark, respectively. ELISA results also showed that all the egg white-marked *H. convergens* retained their mark, but 22.1% of the bovine milk-marked and 5.1% of the soy milk-marked *H. convergens* (color-coded beetles) lost their mark during the collection and sample handling processes.

Introduction

Spot or broadcast applications of various protein marks for mark-release-recapture (MRR)- and mark-capture-type dispersal research have become an increasingly popular means for marking arthropods (Jones et al., 2006; Boina et al., 2009; Hagler et al., 2011; Krugner et al., 2012; Sivakoff et al., 2012; Swezey et al., 2013, 2014). The proteins are inexpensive, easy to apply, and detectable by protein-specific enzyme-linked immunosorbent assays (ELISA). However, the sensitivity of the protein detection ELISAs is such that there is concern that an unmarked arthropod could acquire a false-positive mark if it contacts a protein-marked specimen. If so, this would lead to erroneous estimates of the dispersal capability of any captured arthropod species.

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Perhaps the greatest potential sources of error for studies employing protein marking methodology is the contamination of unmarked specimens during the arthropod collection and sample handling processes. That is, conventional methods used to mass collect insects might be unsuitable for protein MRR- or mark-capture-type research. Common methods for collecting arthropods include physical methods such as hand picking, sweep netting, suction (vacuum) netting, and shaking or beating plants (beat cloth), and passive methods such as pitfall trapping, adhesive trapping, and pheromone trapping (Ellington et al., 1984; McEwen, 1997). A review by King et al. (2008) on 'best practices' for prey-specific ELISA and polymerase chain reaction (PCR) predator gut content analyses cautioned that so-called 'harsh' (e.g., sweep netting, vacuum netting, shaking, or beating) collection methods could lead to high incidences of false-positive predator gut content assays due to direct contact of the prey with a predator in the nets or beat cloths during the collection process. Subsequently, four studies tested this

hypothesis. Two of the studies determined that there were no significant differences in the frequency of spiders scoring positive by a prey-specific ELISA when collected by sweep net or hand picking (Harwood, 2008) or by a prey-specific PCR assay when collected by vacuuming or hand picking (Chapman et al., 2010). However, Greenstone et al. (2011) reported that there was a higher frequency of false-positive gut content reactions yielded by predators collected by a beating method than a hand picking method. Most recently, it was reported that there was a higher frequency of PCR gut assay reactions yielded by the cotton predator assemblage on three of four prey species that were collected by sweep netting vs. whole plant collecting (Hagler & Blackmer, 2013).

Here, we examined the possibility of obtaining false-positive-marked arthropods due to the transfer of protein marks from marked convergent lady beetles, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), to unmarked arthropods during the sweep net collection and sample handling processes. Such studies are necessary to ensure that the data collected for protein MRR and mark-capture studies are reliable.

Materials and methods

Insect marking procedures

Adult *H. convergens* were purchased from Nature's Control (Medford, Oregon, USA). The beetles were separated into four groups of 250 individuals and chilled at 4 °C for 15 min. All the individuals in each group were first tagged with a visual mark by placing either a yellow (DecoColor™ Opaque Paint Marker, 300-S Yellow; Uchida of America, Torrance, CA, USA), white (Sharpie® Paint, Oil-based Opaque Paint Marker; Newell Rubbermaid, Freeport, IL, USA), green (Sharpie® Paint, Oil-based Opaque Paint Marker; Newell Rubbermaid), or blue (DecoColor™, 300-S blue; Uchida of America) dot on their pronotum. The colored marks served as a visual confirmation of the invisible protein mark that was applied to each cohort of beetles as described below.

The following day the yellow-, white-, and green-marked beetles were placed in separate 1-l Erlenmeyer flasks and sprayed with 2.5 ml of 100% egg white (All Whites™; Papetti Foods, Elizabeth, NJ, USA), bovine milk (Shamrock Farms, Phoenix, AZ, USA), or soy milk (Westsoy, Hain Celestial Group, Melville, NY, USA), respectively. The blue-marked beetles, which served as the negative control protein-marked treatment, were sprayed with 2.5 ml of dH_2O. Each mark was applied with a hand-held spray device (Uline Shipping Supply Specialists, Chicago, IL, USA). Each cohort of beetles was allowed to roam freely in the flask for 15 min. After that, the beetles were dried for 2 h at 27 °C in a 1-l Tupperware® dish that contained facial tissue paper. The beetles were then immobilized by chilling at 4 °C for 15 min and groups of 10 beetles, all containing the same colored dot and invisible protein mark, were placed into 30-ml snap cap vials. The vials were placed in an ice chest containing three 0.95-l packs of blue ice (Uline Shipping Supply Specialists) and transported to the field.

Sweep net sampling procedure

The sweep net samples were taken from a 0.8-ha blooming alfalfa, *Medicago sativa* L. (Fabaceae), field located at the University of Arizona's Maricopa Agricultural Center (Maricopa, AZ, USA). In total 20 sweep sample units were taken. Each sample was taken with a clean 38-cm-diameter heavy-duty canvas sweep net (BioQuip Products, Rancho Dominguez, CA, USA). A sweep sample unit consisted of: (1) performing 25 sweeps of the top half of the alfalfa canopy while walking at a pace of ca. 4.0 km h^{-1}, (2) stopping and placing 40 marked *H. convergens* into the net (10 of each of the three color-coded and protein-marked treatments and 10 of the control beetles), (3) performing an additional 25 sweeps of the alfalfa canopy, and (4) placing the contents of each sweep sample into a sample bag for storage. Ten of the sweep samples were each placed into a 20.0 × 15.6 × 40.3-cm paper bag (Shorty Kraft Paper Grocery Bag, Uline Shipping Supply Specialists) and 10 were each placed into a 30.5 × 30.5-cm plastic bag (Envision® Seal Closure Bags; Skillcraft, Wichita, KS, USA). The samples were then placed back into the ice chest, returned to the laboratory within 30 min after collection, and frozen at −80 °C.

Insect sample preparation

Frozen arthropods were processed by removing the sample bags from the freezer and carefully emptying the contents onto a clean piece of butcher paper. The samples (plant debris and arthropods) were dried at 27 °C for at least 1 h. Individual arthropods in each sample were picked from the plant debris with a clean toothpick, placed into a 1.5-ml microcentrifuge tube, and frozen at −80 °C for later examination to detect the presence of the various protein marks by the protein-specific ELISAs described below.

Detection of the protein marks

The samples were removed from the freezer and 1.0 ml of tris-buffered saline (pH 7.4) was added to each microtube. The samples were mixed at 100 r.p.m. for 1 h (27 °C) using an orbital shaker. Every arthropod sample was then screened for the presence of egg albumin in egg white, casein in cow milk, and soy trypsin inhibitor in soy milk using the assays described by (Hagler et al., 2014) with

only one slight modification. The modification consisted of adding 100 μl of hydrogen peroxide to each ELISA sample well for 30 min between the first step of the ELISA (i.e., after the arthropod sample incubated for 1 h in the well of the plate) and the blocking step. The addition of hydrogen peroxide has been proven useful for reducing ELISA background noise for unmarked insects (JR Hagler, unpubl.)

ELISA critical (positive) threshold values

Unmarked arthropod samples serving as negative controls were collected from the same alfalfa field and assayed by each ELISA for the presence of protein. Eight negative controls per arthropod species were included on each 96-well ELISA plate. The color-coded *H. convergens* and field-collected arthropods were scored positive by ELISA for the presence of a protein mark using the standard normal variate transformation threshold criteria described by Sivakoff et al. (2011). That method consists of calculating the standard deviation rate from the pooled sample of all the negative controls and adding 3× that value to the mean ELISA optical density reading yielded by the negative controls from each individual plate. Any sweep net sample that yielded an optical density above this value was then scored as a protein-marked insect.

Statistical analysis

The proportions z-test statistic (SigmaPlot, version 11.0; SPSS, Chicago, IL, USA) was used to determine whether differences occurred in the frequencies of protein acquisition (contamination) by each arthropod species as a function of the type of bag (e.g., paper or plastic) they were stored in. The Yate's correction for continuity was applied to each z-statistic calculation (Glantz, 1997). There were no significant differences in the rate of protein acquisition by any of the species examined based on bag type; therefore all 20 of the sweep net samples were pooled by species and the frequencies of positive ELISA reactions for each arthropod taxon were tallied. Only data for those species represented by >20 individuals are presented.

Results

There were no significant differences in protein acquisition by any of the arthropod species regardless of whether they were stored in paper or plastic bags. The P-value yielded for each species*bag type comparison was always ≥0.49 (data not shown). Overall (all field-collected arthropods pooled by bag type), there were only 11 contaminated (false-positive ELISA reactions) arthropod samples of the 2 232 assays conducted (n = 744 field-collected arthropods ×3 assays each). Of these, only 0.58% (n = 1 203) of the paper-bagged specimens and 0.39% (n = 1 029) of the plastic-bagged specimens acquired a false protein mark ($z = 0.37$, $P = 0.74$).

The majority of color-coded and protein-marked *H. convergens* yielded strong ELISA reactions for the presence of their targeted protein. As expected, all of the yellow and egg white-marked *H. convergens* yielded very strong reactions for the presence of the egg white mark. However, 22.1% of the white and bovine milk-marked and 5.1% of the green and soy milk-marked beetles failed to react to their targeted ELISA (false-negative reactions) (Table 1). None of the blue-marked (water controls) *H. convergens* reacted to the various ELISAs. Only one of the yellow and egg white-marked *H. convergens* responded to the bovine milk ELISA and none reacted to the soy milk ELISA. Overall, only 0.22% (four of 1 785 assays) of the color-marked *H. convergens* yielded a false-positive immunoreaction when examined by the non-target ELISAs (Table 1). The optical density readings yielded by four color-marked *H. convergens* that cross-reacted to the various protein-specific ELISAs are given in Table 2. Of these, one (e.g., a green and soy milk-marked *H. convergens*) yielded a relatively weak ELISA absorbance value of 0.083 for the presence of bovine milk, which was marginally above the critical threshold value of 0.061. However, the other three color-marked *H. convergens* that were contaminated yielded strong ELISA reactions of 0.195 and 0.244 to bovine milk, and 0.418 for the presence of soy milk (Table 2).

Overall, 0.67, 0.81, and 0% of the field-collected arthropods yielded a positive ELISA response for the presence of egg white, bovine milk, and soy milk, respectively (Table 1). All six of the arthropods that scored positive for the presence of bovine milk yielded reactions that were equal to or only slightly greater than the calculated positive threshold value (i.e., they yielded a marginally positive reaction for the presence of bovine milk; Table 2). However, four of the five arthropods, all of which were *Orius tristicolor* (White), yielded very strong ELISA reactions for the presence of egg albumin protein.

Discussion

We have been collecting arthropods in dispersal studies with sweep and vacuum nets for more than 2 decades (Hagler & Naranjo, 1994; Hagler, 2002; Hagler et al., 2002; Blackmer et al., 2004; Swezey et al., 2014). Over this span, we have indiscriminately stored the contents of these samples in variously sized paper or plastic zipper bags. The moisture yielded by the plant debris collected in the nets tends to soak into the paper bags, whereas it accumulates in the plastic bags (JR Hagler, pers. obs.). As such, we

Table 1 ELISA results yielded by the protein-marked and field-captured arthropods collected in sweep nets and assayed for the presence of the egg white, bovine milk, and soy milk marks

					Egg white ELISA			Bovine milk ELISA			Soy milk ELISA		
	Taxon	Visible mark	Protein mark	No. assayed	No. expected	No. observed	% contaminated samples[1]	No. expected	No. observed	% contaminated samples[1]	No. expected	No. observed	% contaminated samples[1]
Color-coded and protein-marked *Hippodamia convergens*	*H. convergens*	Blue	Water control	199	0	0	0.00	0	0	0.00	0	0	0.00
		Green	Soy milk	196	0	0	0.00	0	2	0.01	196	186	5.10
		White	Bovine milk	199	0	0	0.00	199	155	22.11	0	1	0.01
		Yellow	Egg white	199	199	199	0.00	0	1	0.01	0	0	0.00
Field-collected arthropods	*H. convergens*			119	0	0	0.00	0	1	0.01	0	0	0.00
	Coccinella septempunctata			31	0	0	0.00	0	0	0.00	0	0	0.00
	Collops vittatus			21	0	0	0.00	0	0	0.00	0	0	0.00
	Lygus spp.			70	0	0	0.00	0	0	0.00	0	0	0.00
	Orius tristicolor			472	0	5	1.06	0	1	0.00	0	0	0.00
	Misumenops celer			31	0	0	0.00	0	4	0.13	0	0	0.00
Field-collected arthropod totals				744	0	5	0.67	0	6	0.81	0	0	0.00

[1]Percentage of contaminated samples (false-positive reactions) = (number observed/number assayed)*100; percentage of falsely-scored negative *H. convergens* (red-colored values) = 100 − [(number observed/number assayed)*100)].

Table 2 ELISA readings and critical threshold values for the 15 contaminated arthropods

Origin[1]	Species	ELISA reading[2]	Critical value[3]	Assay yielding contaminated sample[4]	Visible color mark[5]	Protein mark[5]
Color-coded	*H. convergens*	0.244	0.061	Bovine milk	Yellow	Egg white
and protein-marked		0.195			Green	Soy milk
Hippodamia		0.083				
convergens		0.418	0.070	Soy milk	White	Bovine milk
Field-collected	*Orius tristicolor*	0.662	0.073	Egg white		
arthropods		0.212				
		0.197				
		0.108				
		0.081				
		0.057	0.057	Bovine milk		
	Misumenops	0.066	0.054	Bovine milk		
	celer	0.063				
		0.063				
		0.055				
	H. convergens	0.061	0.061	Bovine milk		

[1]Purchased *H. convergens* were color and protein marked and added to the contents of the sweep net samples. Field-collected arthropods were collected in the alfalfa.
[2]Quantitative absorbance value yielded by the 15 cross-contaminated arthropods.
[3]Calculated critical ELISA threshold value yielded by the negative control specimens for each arthropod species for the various protein-specific ELISAs. Any arthropod collected in a sweep net yielding a value greater than the critical threshold values was deemed as cross contaminated (false-positive) for the non-target protein.
[4]Protein-specific ELISA yielding the false-positive absorbance value.
[5]Visible color and protein mark placed on the protein-marked *H. convergens* treatments that yielded the false-positive ELISA reaction.

hypothesized that there would be differences in protein contamination rates between the arthropods placed into the two types of bags. Data revealed that only 0.49% (11 of 2 232 assays) of the field-collected arthropods acquired a mark during the sampling (placement of sweep net contents in the bags and then subsequent transport of the samples to the laboratory freezer) and post-sampling (thawing and sorting of the specimens in the laboratory for ELISA analyses) handling procedures, regardless of the type of bag that they were stored in. Moreover, seven of the 11 field-collected arthropods that yielded a positive ELISA response for the presence of a protein mark yielded a weak positive response. These weak reactions might be attributed to day-to-day variations (e.g., assay error) that are inherent to the ELISA procedure (Crowther, 1995; Hagler & Naranjo, 1997; Hagler et al., 1997; Hagler, 1998). Those samples that are marginally positive for a protein mark could be re-assayed in triplicate on different plates and/or days to verify that the weak reaction is due to the presence of a minute trace of protein (if all are positive) and not to assay error.

Further results of this study revealed that none of the egg white-marked *H. convergens* lost their mark. However, 22.1 and 5.1% of the bovine milk- and soy milk-marked *H. convergens*, respectively, did not retain their mark. These results support previous studies that showed that egg white is a more steadfast mark than bovine and soy milk (Jones et al., 2006; Hagler & Jones, 2010; Slosky et al., 2012; Hagler et al., 2014).

This study demonstrated that transfer of protein marks from *H. convergens* to various unmarked arthropods during the sweep net sampling and sample handling process is rare (i.e., <1%). Moreover, we believe that even lower rates of contamination will likely occur in mark-capture studies because the research protocol used maximized exposure to the protein markers. First, we thoroughly covered the beetles with a topical application of undiluted (100%) protein solutions. It is unlikely that such a large volume of protein would be administered to an arthropod in its natural habitat using conventional spray equipment. Second, no mark-capture research protocol used to date has applied pure proteins to the arthropods. Protein mark-capture studies published to date have applied <20% solutions of the various protein marks (Jones et al., 2006; Boina et al., 2009; Horton et al., 2009; Krugner et al., 2012; Sivakoff et al., 2012; Swezey et al., 2013, 2014; Peck et al., 2014). Finally, the number of protein-marked specimens (n = 30 per sample unit) placed into each sweep net sample was much higher than one would expect to encounter in mark-capture type studies. As such, a lower density of

protein-marked specimens per sample unit would decrease the likelihood of obtaining falsely marked specimens.

Although the mechanical (horizontal) transfer of a protein from a marked *H. convergens* to unmarked arthropods was rare, we caution that higher contamination rates could occur under different study conditions. For instance, local weather conditions could negatively affect the chances of obtaining falsely marked samples. The weather was hot (ca. 30 °C) and dry (<12% r.h.) during this study. Obvious abiotic factors that could lead to increased protein contamination include rainfall, dew, and high humidity. In addition, the method used to collect arthropods could play a key factor in protein mark contamination. Perhaps other 'harsh' (e.g., vacuum, beat cloth, etc.) or passive (e.g., sticky trap, pitfall trap, pheromone trap, etc.) sampling methods would yield higher incidences of contaminated specimens. Protein contamination could also be influenced by the arthropods collected in the samples. Important factors might include insect body type (e.g., soft bodied aphids or whiteflies), life stage, and arthropod behavior (active, inactive). Finally, arthropod predators might yield false-positive reactions if they feed on protein-marked prey (i.e., vertical transfer of the protein through the food chain) prior to collection or while confined in the sample net (Horton et al., 2009). Specifically, marking prey with protein has proven a viable method for studying various aspects of predation via molecular gut content analysis (Hagler, 2006, 2011; Zilnik & Hagler, 2013; Kelly et al., 2014).

In conclusion, this study showed that collecting arthropods by sweep nets and storing the contents of the samples in paper or plastic bags are reliable research protocols for MRR or mark-capture type research under the abiotic and biotic conditions encountered during this study. Further studies may be warranted if a different sample collection procedure is used or if the weather conditions are different than the ones encountered in this study.

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