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Special Collection: Tools of the Behavior and Biocontrol Trade
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Super Mark It! A Review of the Protein Immunomarking Technique

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Abstract

Having an effective method to track movement of arthropods in nature is essential for any mark-release-recapture (MRR) or mark-capture (MC) type experiment. A simple protein immunomarking technique (PIT) was described over a quarter of a century ago that has since been proven to be a highly useful and versatile tool for tracking arthropod dispersal patterns. The PIT consists of tagging arthropods with a specific protein. In turn, recaptured arthropods are examined for the presence of the protein tag by a highly sensitive and specific enzyme-linked immunosorbent assay. In this article, I review the progression of the PIT procedure, provide guidelines for conducting a successful PIT (MRR or MC) dispersal study, and highlight some of the ways this procedure has been adapted to study the dispersal patterns of a wide variety of arthropod species. My goal is that this information will provide researchers with the motivation to develop even more creative uses for the PIT.

Key words: dispersal, mark-capture, mark-release-recapture, ELISA

Entomologists often need an effective method to track arthropod dispersal patterns. To date, a variety of physical (e.g., tags, dusts, dyes) and chemical (e.g., rare elements, radioisotopes, nitrogen-15) markers have been used to mark arthropods. Unfortunately, no single type of marker has been shown to be effective for all arthropods or in all circumstances. The kind of marking material and technique used is highly dependent on the species and habitat under investigation, as well as the type of dispersal study being conducted.

Arthropod dispersal studies can be broadly classified as mark-release-recapture (MRR) or mark-capture (MC)-type research (Hagler and Jackson 2001). MRR research, sometimes referred to as the central point release approach, usually consists of marking arthropods in the laboratory and then releasing them at a strategic location in the field (e.g., the central point of the area under investigation). Sometimes MRR-type studies can be conducted on arthropods that 'self-mark' themselves by contacting markers, such as dusts, at discrete sites that are strategically placed in the field (e.g., feeding stations, nest entrances, pheromone lures, etc.).

For MC-type research, arthropods are directly marked in the field through a broader application of marker. Typically, the marker is in liquid form and is administered with a spray apparatus. Unfortunately, most of the current conventional marking materials do not have wide-scale appeal for MC research because they are either ineffective, expensive, difficult to apply, or difficult to detect (Hagler and Jackson 2001).

This review focuses on the 'protein immunomarking technique' (PIT) for use with MRR- and MC-type research. Thorough reviews of other methods used to mark arthropods are provided by Hagler and Jackson (2001), Henderson and Southwood (2016), and Lavandero et al. (2004a,b). The PIT consists of marking arthropods with one or more foreign protein(s) that, in turn, can be detected by a sensitive protein-specific enzyme-linked immunosorbent assay (ELISA). Protein markers can be applied to arthropods, which facilitates the use of the PIT for both MRR and MC approaches. Here, I will discuss the milestones of PIT research, explain the fundamental steps for conducting a PIT study, provide examples of how the PIT has been used for MRR and MC research, and describe a new approach for conducting PIT research.

Milestones

The PIT was described over a quarter of a century ago. The techniques described in the original study by Hagler et al. (1992) and those that followed over the next 14 years were used solely for MRR research. Henceforth, I refer to that era as the first generation of PIT research. The next milestone was established by Jones et al. (2006),

which described PIT methods that were well suited for MC research. I refer to this study and subsequent MC studies as the second generation of PIT research.

First-Generation PIT Research

The proof-of-concept PIT study was a simple laboratory experiment that consisted of spraying a topical solution of rabbit immunoglobulin G (IgG) onto *Lygus hesperus* Knight (Hemiptera: Miridae) adults using a hand-held spray bottle (Hagler et al. 1992). The IgG-marked *L. hesperus* were temporally sampled and examined for the presence of the mark using an anti-rabbit IgG sandwich ELISA. The data showed that the ELISA was 100% effective at detecting this external mark over a period of a week.

The next PIT study did not occur until 5 years later. In that study, another sandwich ELISA was developed to detect chicken IgG (Hagler 1997a). The persistence of external rabbit IgG and chicken IgG markers was also compared using fluorescent dust, which was placed on *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae). Data revealed that the IgGs were detectable on the beetles over a much longer time perioid using ELISA, when compared with visual inspection for the presence of the dust. It was also determined that the ELISA was less tedious, labor intensive, and prone to human error than the visual inspection of hundreds of individuals for the presence of dust particles.

A series of studies soon followed that described methods for administering external and internal protein marker to various parasitoid species (Hagler 1997b, Hagler and Jackson 1998). A medical nebulizer, which produces a fog-like mist, was shown to be an ideal tool for delivering an external IgG mark onto the tiny parasitoids. A significant feature of the nebulizer applicator was that it only required a small volume of IgG solution (1.0 ml at 10 mg/ml) to mark thousands of parasitoids simultaneously. For internally marking, it was shown that parasitoids could be induced to self-mark by feeding on honey or sucrose solutions laced with IgG. In turn, the internal mark was detected by gut analysis of the parasitoids. These studies showed that the external and internal markers were retained throughout the seven to 10-d adult lifespan of the various parasitoid species tested.

The first-generation PIT is still regularly used for MRR and self-marking type experiments. The anti-IgG sandwich ELISAs are simple to perform, inexpensive, protein-specific, and effective at detecting small amounts of IgG in simple (homogenous) and complex (heterogenous) sample mixtures. The major drawbacks are that IgGs are very expensive and typically available only in small quantities. As such, IgG markers are not practical for MC research (Hagler and Jackson 2001).

Second-Generation PIT Research

In my previous review of marking methods (Hagler and Jackson 2001), I emphasized a need for protein-specific ELISAs to detect inexpensive protein markers that could be acquired in large quantities. Subsequently, Jones et al. al. (2006) described a suite of ELISAs designed to detect albumin in chicken egg whites, casein in cow's milk, and soy trypsin inhibitor in soy milk. We also conducted an MC study that showed codling moths, *Cydia pomonella* L. (Lepidoptera: Tortricidae) could be marked directly in an apple orchard with commercial spray equipment. Since that study, the second-generation PIT has been used on a regular basis to study the dispersal patterns of many arthropods.

Basic Steps for Conducting PIT Research

The basic steps for conducting an MRR or MC study using the PIT consists of the following: (1) administering the mark to the arthropods, (2) temporally and spatially sampling the targeted habitat for marked arthropods, and (3) detecting the mark on field-collected specimens. One of the main benefits of the PIT is that it is adaptable; the specifics for each of the three steps can be widely modified based on the study parameters. As such, there is opportunity for researchers to develop creative methods for administering the markers, sampling the habitat, and analyzing the specimens for the presence of the tags (as shown below).

Administering the Protein Mark

The PIT is unique because the markers can be applied externally, internally, or both internally and externally (as a double mark) to most arthropod species. The choice of application method is dependent on the type of study conducted, the arthropod species and life stage of interest, and the terrain of the study site.

Internal Marking Procedures

Internal markers are administered to arthropods by providing them a known protein-laced food. The ingested protein is subsequently detected in arthropods by gut content analysis using the protein-specific ELISA. Food items used, thus, far to deliver the markers include protein-laced honey or sugar solutions (Hagler and Jackson 1998, DeGrandi-Hoffman and Hagler 2000, Buczkowski and Bennett 2006, Hogg et al. 2018), cellulose baits (Buczkowski et al. 2007, Hagler et al. 2009, Baker et al. 2010), artificial diets (Hagler and Miller 2002), prey tissues (Hagler and Durand 1994), plant tissues (Lundgren et al. 2013, Blubaugh et al. 2016), and vertebrate blood meals (Sivakoff et al. 2016).

An advantage of an internal mark is that it is unlikely to be affected by harsh environmental conditions, such as heavy rainfall, heat, dew, etc. A minor inconvenience of this approach is that internally marked specimens must be homogenized to examine the gut contents for the presence of the protein marker. Specifically, tissue grinding requires extra time and labor during the sample preparation process (see below). Another potential disadvantage of internal marking is that the marker might degrade rapidly due to the arthropod digesting the protein-marked food item. However, several studies have shown that internal markers are well retained (e.g., for many days) in most of the arthropods tested to date (Hagler 1997b, Hagler and Jackson 1998, Hagler and Miller 2002, Hagler et al. 2009). It is, therefore, essential to determine the typical internal mark retention rate for your target arthropod before the study is conducted.

External Marking Procedures

Both first- and second-generation protein markers have proven to be excellent external markers. External marks are usually administered by a topical application of a protein solution. The choice of the spray device used to apply a topical mark will depend on the type of study being conducted, as well as the size and fragility of the arthropod species under examination. For MRR research, hand-held spray bottles, air paint brushes, perfume atomizers, and nebulizers have all proven effective for a wide variety of arthropod species (Hagler 1997b, Hagler and Jackson 1998, Hagler and Naranjo 2004, Blackmer et al. 2006, Slosky et al. 2012).

Second-generation protein markers are typically applied to arthropods directly in their natural habitat for MC research. Again, a wide variety of spray devices have been used to administer the protein markers. For instance, arthropods have been marked in various crops with industrial air-blast, and boom- and nozzle-type tractor sprayers (Jones et al. 2006, Krugner et al. 2012, Klick et al. 2016,

Bastola and Davis 2018). In some instances, electric spray devices mounted on an all-terrain vehicle (Horton et al. 2009) and gas-powered backpack sprayers (Swezey et al. 2013, 2014; Irvin et al. 2018) have been used to apply pinpoint applications of protein markers to specific sites within a field. These methods have been useful for marking arthropods inhabiting trap crops and cover crops, respectively. The most 'extreme' method for administering a broadcast mark has been with aerial crop dusters (Sivakoff et al. 2012).

Protein markers can also be administered externally to arthropods in powdered form. For example, Hagler et al. (2011a,b) placed a device at the entrance of 112 honey bee hives that sprinkled either powdered chicken eggs, cow's milk, or variously colored fluorescent dusts onto foraging honey bees as they exited their hives. Most recently, Boyle et al. (2018a) described a method to mark adult blue orchard bees, *Osmia lignaria* Say (Hymenoptera: Megachillidae), as they emerge from protein-dusted cocoons.

Collecting and Preserving Arthropod Specimens

The next step of a PIT experiment consists of collecting and preserving field-collected samples for the ELISA analysis. A proper sampling technique should trap as many arthropods as possible within a reasonable amount of time and space. Most importantly, the method should not compromise the integrity of marked and unmarked specimens that are trapped in the sampling devices. Like most conventional marking methods, the PIT is vulnerable to yielding false-negative and false-positive assay reactions, if the field-collected specimens are not handled properly. False-negative sampling errors can occur when a protein marked specimen prematurely loses its mark during the sampling and handling processes. A false-positive error can occur when an unmarked specimen obtains the mark during these processes.

Arthropod sampling procedures can be classified into two categories: 1) physical and 2) passive sampling (McEwen 1997). To date, almost every available sampling method (i.e., various types of trapping and netting devices) has been used for PIT research (Hagler et al. 2002a,b, 2011b; Jones et al. 2006; Boina et al. 2009; Krugner et al. 2012; Swezey 2013; Klick et al. 2016).

Abiotic and biotic conditions in the field must be considered when choosing a reliable collection technique. Key abiotic factors include temperature, rainfall, and dewdrops. Ideally, arthropods should be collected under the driest conditions possible to prevent sample contamination or loss of mark. Biotic factors that may affect the reliability of a given mark include the body type (e.g., hard vs soft-bodied, smooth vs hairy, large vs small), life stage, and feeding characteristics (e.g., herbivore, carnivore) of the target arthropod species. The size of the research area, type of host plant (e.g., row crop, grass, orchard), and primary habitat of the arthropod (e.g., arboreal, ground-dwelling) must also be considered.

In all cases, collected arthropods must be handled carefully and with the goal of avoiding contamination (i.e., transfer of marker proteins). For example, insects stored *en masse* in a single specimen container, or handled sequentially with protein-contaminated forceps, may result in the unintended transfer of the mark. Use clean tools (e.g., toothpicks, grinders) for each specimen to minimize the risk of obtaining false positives. If it is necessary to reuse tools, thoroughly wash between specimens. Lay down fresh paper towels or waxed paper as a handling surface between specimens or sample units, and wash hard surfaces thoroughly before and after handling specimens.

Physical Sampling Methods

Physical sampling methods include handpicking, sweep netting, vacuum netting, and shaking or beating plants until arthropods fall onto a ground cloth or into a bucket (Henderson and Southwood 2016). Collecting individuals by hand or with tweezers is probably the method that is least prone to yielding sample contamination errors but is not an efficient mass trapping technique.

Sweep and vacuum netting are probably the most commonly used physical sampling techniques used for PIT research (Sivakoff et al. 2012; Swezey et al. 2013, 2014; Hagler et al. 2014; Basola and Davis 2018). The fidelity of a sweep net sampling and sample unit storage technique was examined for chicken egg white-marked *H. convergens* (note that the beetles were heavily coated with egg albumin) that were exposed to unmarked arthropods during the collection process and then preserved by freezing in plastic and paper storage containers (Hagler et al. 2015). The ELISA results showed that the directly marked *H. convergens* retained their protein mark (no false negatives) and that <1% of the unmarked collected arthropods yielded a false-positive ELISA response for the egg white mark.

There are a few procedures to follow when using physical sampling methods for PIT research. The arthropod specimens in the sample unit should be immobilized as soon as possible to minimize contact between individuals inside the net (or beat bucket), as insects interacting or colliding with one another may cause unintended transfer of the mark. Immobilization can be achieved by immediately transferring each sample unit into a large (3.8 liter) plastic zip-top bag. The bag should then be 'burped' to expel all the air out of the container. The burping process creates a vacuum that significantly reduces the mobility of arthropods within the bag. Their movement can be further reduced by tightly rolling the bag. Last, place the sample bag on dry ice contained in an ice chest as soon as possible. Ice packs can be used if dry ice is not available. Once back at the laboratory, the sample units can be stored indefinitely by freezing in an ultra-cold (−60 to 80°C) or standard (−20°C) freezer. Do not use a 'frost-free freezer' to store the specimens, because freeze-thaw cycles can destroy the integrity of the samples.

Passive Sampling Methods

Passive sample methods have also been used for PIT research. Passive sampling methods include various trapping strategies, such as adhesive, pheromone, pitfall, and passive suction (McEwen 1997). Of these, clear and colored adhesive 'sticky' traps are probably the most commonly used (Blackmer et al. 2004, 2006; Klick et al. 2016; Irvin et al. 2018). Surprisingly, the unavoidable adhesive residue arthropods obtain does not seem to affect the efficiency of the ELISA at detecting a protein mark (J.R.H., personal observation).

As with the physical sampling methods, certain precautions must be taken to avoid sample contamination errors. Again, it is critical to immobilize the trapped arthropods as soon as possible to minimize contact between marked and unmarked individuals. There are a few guidelines to follow when using sticky cards for PIT experiments. First, use as little adhesive as necessary to adequately capture and immobilize the specimens. If not enough adhesive is used, the arthropods can escape or crawl around on the card and potentially contaminate the other entrapped specimens. If too much adhesive is applied, then it could entomb the arthropods; this would likely affect the protein detection efficiency of the ELISA. Second, remove each specimen from the sticky card with an individual clean toothpick and place it in an empty sample vial. For delicate insects, such as tiny parasitoids and soft-bodied arthropods, it can be difficult to dislodge individuals from a sticky toothpick into a sample vial. If

so, the toothpick can be snapped in half and left in the sample vial along with the arthropod. The presence of a toothpick in the sample will not adversely affect the efficacy of the ELISA (J.R.H., personal observation). Finally, arthropods can be removed from sticky traps directly in the field or in the comfort of the laboratory. If the latter, cover the sticky portion of each trap with wax paper and then lightly tap the paper down onto the sticky trap. The trap can then be returned to the laboratory, frozen, and processed by ELISA at a convenient time.

Pitfall traps also have potential for capturing protein-marked arthropods. However, some pitfall trapping protocols allow the specimens to roam freely at the bottom of the trap or contain a liquid (e.g., oil or soap solution) that serves to kill or preserve the specimens. Obviously, both protocols would create errors for a PIT experiment, whether through contamination of unmarked insects or washing the mark off marked insects. I suggest that a fine layer of adhesive be applied to the bottom of a trap to immobilize arthropods that blunder into the pitfall apparatus.

Arthropod Sample Preparation

Each specimen is placed into an individual microcentrifuge tube. Then, Tris-buffered saline or phosphate-buffered saline (PBS) is added to the sample tubes. The amount of buffer used usually ranges from 500 to 1,000 µl. Typically, the samples are stirred by gentle agitation on an orbital rocker for ≥30 min. If the field-collected specimens were externally marked, then soaking in the buffer to wash off the mark is sufficient—do not homogenize them! If the specimens were internally marked, they must be homogenized in the sample buffer with a clean tissue grinder before soaking and agitation.

A caveat of the PIT sample preparation process as described above is that the field-collected arthropod specimens are destructively sampled (euthanized by freezing) for analysis by ELISA. For most arthropod studies, the destructive sampling procedure is of no concern. However, killing endangered, rare, or expensive arthropods (e.g., commercially purchased bumble bees) for protein examination by ELISA would be unacceptable. Recently, a nonlethal sample preparation protocol was developed and compared with the destructive sampling method for examining protein-marked bumble, leafcutter, and blue orchard bees for the presence of a protein marker. The method consists of capturing the bees, rinsing them in buffer solution directly in the field, and safely releasing them back into their environment. The ELISA analyses showed that the rinsed bee buffer samples compared favorably with the destructively prepared samples (Boyle et al. 2018b).

Detection of a Protein Mark

The two immunoassay formats used for detecting protein markers are the sandwich and indirect ELISAs. Both ELISA procedures are inexpensive, simple to perform, sensitive, and well suited for mass throughput.

Sandwich ELISA

The first-generation IgG protein marks are detected by standardized protein-specific sandwich ELISAs. The original anti-rabbit and anti-chicken sandwich ELISAs developed over 25 yr ago (Hagler et al. 1992, Hagler 1997a) have remained the standard assays for MRR dispersal and gut content analysis research (Hagler 2019 in this volume). The sandwich ELISA format requires two protein-specific antibodies to 'sandwich' the marker protein in an ELISA sample well. In this assay, the primary antibody (also known as a capture antibody) is coated on the plate first to 'capture' any targeted protein mark in the sample mixture that is subsequently added to the well. Then, the target protein is bound by the secondary antibody, which is also specific to the target protein. The sandwich ELISA format is more effective than the indirect ELISA at detecting target markers contained in heterogenous arthropod samples (i.e., homogenized in sample buffer). As such, the sandwich ELISA format is an excellent tool for detecting internal or external IgG markers.

The sandwich ELISA procedure consists of five simple steps that can all be conducted at room temperature (Fig. 1). First, protein-specific antibody (often referred to as the primary antibody) is added to each well of a 96-well ELISA plate for ≥1 h (Fig. 1a). The incubation period gives time for the protein-specific antibody molecules to attach to the nonspecific binding sites present on the bottom of each well on an ELISA plate. The excess primary antibody is discarded, and the wells are then coated with a protein-rich solution consisting of 1% nonfat bovine milk for ≥30 min. The milk proteins serve as a 'blocking agent' that bind with any remaining nonspecific binding sites in the well that might not be occupied with the primary antibody (Fig. 1b). Third, the excess blocking agent is discarded, and an individual arthropod sample, which has either been soaked (for an external mark) or macerated (for an internal mark) in sample buffer, is added to each well for ≥1 h (Fig. 1c). This incubation period allows any protein mark present in the sample to bind to

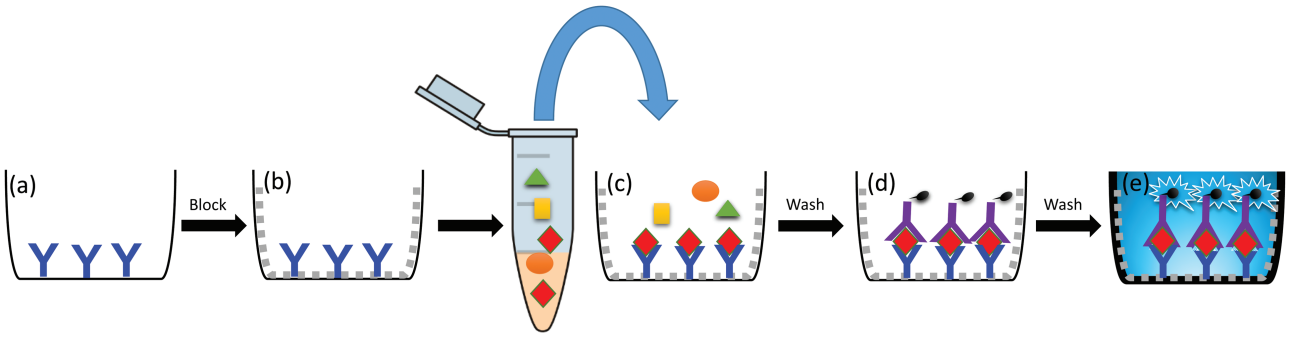

Fig. 1. Steps of the sandwich ELISA procedure: (a) ELISA well is coated with a primary (capture) antibody; (b) the well is coated with a blocking agent to bind remaining nonspecific binding sites; (c) arthropod sample is added-any marker protein present in the sample binds to the capture antibody; (d) enzyme-labeled secondary antibody is added and binds (only) to the targeted protein mark; (e) chromogenic substrate is added, which serves as a catalyst in the presence of an enzyme label; colored reaction in each well is measured with an ELISA plate reader. Note that the response turns a blue-green color if the targeted protein mark is present in the sample.

the only available binding sites in each well, which belong to the the primary antibody. Fourth, the excess sample liquid is discarded, the wells are washed with PBS–Tween, and a second protein-specific antibody (often referred to as the secondary antibody) is added to each well for ≥1 h (Fig. 1d). The difference between the primary and secondary antibody is that the latter is chemically labeled with an enzyme (usually horseradish peroxidase [HRP]). During this stage, the secondary antibody molecules bind to the only available attachment sites in each well—the targeted protein mark. Last, the excess secondary antibody is discarded, the wells are washed again, and an HRP-specific substrate (catalyst for the enzyme label) is added to each well. After a short incubation period (~10 min), the chromogenic ELISA reaction of each sample is measured with a microplate reader. Each sample will yield either a clear (negative) or blue-green (positive) response (Fig. 1e).

Indirect ELISA

The second-generation protein marks are detected by indirect ELISAs initially described by Jones et al. (2006). The indirect ELISA is a two-step binding assay that uses a primary antibody, which is specific to the target protein, as above, and an enzyme-labeled secondary antibody, which is instead usually a polyclonal antibody specific to the host animal in which the primary antibody was developed. The indirect ELISA, like the sandwich ELISA, is highly sensitive. However, it is most effective at detecting proteins contained in relatively homogenous sample mixtures, such as externally marked arthropods that just require soaking in buffer before analysis. I do not recommend the indirect ELISA procedure for detecting protein marks contained in heterogeneous sample mixtures, such as internally marked arthropods that must be homogenized before analysis (see Hagler 1998). The sheer volume of proteins released during maceration of the insect specimens tends to completely coat the binding sites on the ELISA plate. Consequently, many target protein molecules will not find their way to an open binding site, leading to a weak detection output.

The indirect assay also consists of five steps. First, the arthropod samples are added to the wells of the ELISA plate for ≥1 h (Fig. 2a). During this time, all target and nontarget proteins present in the sample competitively attach to the limited number of protein binding sites available at the bottom of each well (therefore, a homogenous sample works best). Second, the excess sample liquid is discarded, the wells are washed with PBS–Tween, and each well is blocked with a protein-rich blocking solution for ≥30 min (Fig. 2b). Third, the excess blocking agent is removed, the wells are washed, and the protein-specific primary antibody is added to each well for ≥1 h (Fig. 2c). During this incubation period, the primary antibody molecules bind to any protein marker molecules that are attached to the well. Fourth, the excess primary antibody is discarded, the wells are washed, and the HRP enzyme-conjugated secondary antibody is added to the wells for ≥1 h (Fig. 2d). During this phase, the secondary antibody molecules attach to any targeted primary antibody that is connected to the targeted protein. Finally, the excess secondary antibody is discarded, the wells are washed, HRP-specific chromogenic substrate is added to each well, and the reactivity of each sample is measured with the microplate reader (Fig. 2e).

ELISA Supplies

The minimum hardware needed to run an ELISA include a microplate reader, a single channel pipettor, and an 8- or 12-multichannel pipettor. An automated microplate washer is useful for large-scale research, but not a necessity. A microplate reader and washer are expensive (typically >US $10,000), but they are commonly found in most biological science departments. As such, it is likely that they could be loaned out for short-term use from colleagues. ELISA reagents (antibodies, substrate, etc.) and single-use supplies (pipette tips, microplates, etc.) are inexpensive. The estimated cost of reagents and single-use supplies needed to analyze one ELISA plate consisting of 96 samples is about $14.00 (J.R.H., personal observation). Two people can process approximately 10–14 plates/d (>1,000 individual specimens). The entire list of supplies and reagents required to every PIT ELISA developed to date is provided by Hagler and Machtley (2016).

Scoring Samples for the Presence of a Mark

Most marking procedures (e.g., fluorescent dusts, dyes) only yield qualitative data. That is, an observer subjectively scores arthropods visually for the presence of the mark. In most situations, it does not matter how much mark is present on a specimen, only that the observer can reliably detect it. This criteria certainly holds true for ELISA data obtained for PIT research. However, an advantage of the PIT is that the ELISA procedure generates quantitative data. These data can be used to score arthropods for the presence of a protein mark objectively. The ability to score samples by statistical analysis eliminates the human error component of subjectively (qualitatively) scoring specimens. Moreover, both the quantitative (mean ± SD values of each treatment) and qualitative (percentage of the population of each treatment) data can be depicted conveniently in tabular or graphical form for data presentation (as shown below).

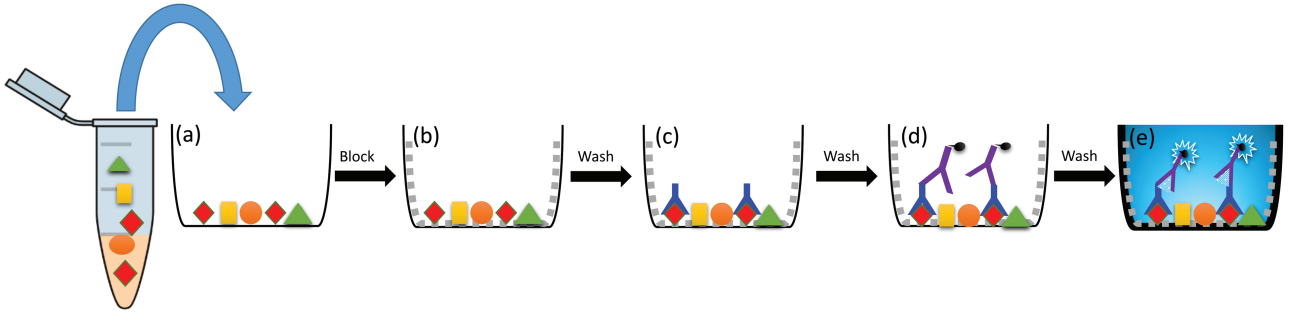

Fig. 2. Steps of the indirect ELISA procedure: (a) ELISA well is coated with the arthropod sample, (b) each well is coated with a blocking agent to bind remaining non-specific binding sites, (c) the protein-specific primary antibody is added to each well, (d) enzyme-labeled secondary antibody is added and binds (only) to the primary antibody, (e) chromogenic substrate is added, which serves as a catalyst in the presence of an enzyme label; colored reaction in each well is measured with an ELISA microplate reader. Note that the response turns a blue-green color if the targeted protein mark is present in the sample.

Negative Control Specimens

Several methods have been used to score field-collected arthropods for the presence of a protein mark. The various techniques are reliant on ELISA values obtained from unmarked arthropod specimens. The original method for scoring ELISA samples for the presence of a protein mark was adopted from the method used initially to score insects for the presence of rubidium chloride mark by atomic absorption analysis (Stimmann 1974). This original method that defined an ELISA critical threshold value (CTV) for a marked specimen was the mean ELISA reading of a group of unmarked arthropods on a single 96-well ELISA plate plus three times the SD of that mean. In some instances, researchers have intuitively selected a more conservative CTV to reduce further the risk of falsely scoring samples for the presence of a mark. In such cases, the researchers have added 4–6 SD to the mean of the negative control specimens (Horton et al. 2009, Hagler 2011b). A more sophisticated method, coined as the 'maximum negative control' threshold value was proposed by Sivakoff et al. (2011). This method further reduces the chances of obtaining false-positive assay errors by calculating the mean ELISA readings of the negative control specimens on each ELISA plate and then calculating the SD based on the pooled negative controls from all the plates assayed for a given study. The maximum negative control threshold method is best suited for large studies that require dozens of ELISA plates to analyze all the field-collected specimens of any given study.

An important caveat of the ELISA is that it yields slight plate-to-plate and day-to-day variability (Clark and Adams 1977, Crowther 2001). Therefore, no matter which CTV method is chosen, it is essential that unmarked (negative controls) arthropod samples are included on every 96-well ELISA plate. My standard operating procedure consists of dedicating the last column of eight wells on every ELISA plate to unmarked arthropod samples (negative controls). Some thought needs to go into how to obtain negative controls for any given PIT study.

In my experience, this factor is often overlooked. Ideally, negative arthropod control specimens should be either collected at the field site before the protein mark is applied (for MC research) or at a site far enough away from study area to ensure that there is no chance of collecting protein-marked specimens. Laboratory-reared specimens can also be used as negative controls. However, note that some artificial diets contain ingredients that will react with the protein-specific ELISA (e.g., they contain eggs and milk products).

Data Generated by a Typical PIT Experiment

A hypothetical set of data yielded by a PIT MC study is shown in Fig. 3. The graph depicts individual ELISA values (red dots) produced by arthropods collected from a centralized mark zone and each four adjacent sampling zones 100 and 200 m away in each direction from the marked region (n = 20 individuals per sample zone). The marked zone (0-m zone) in this scenario could represent a centralized cover crop, bait station, or trap crop as done by Horton et al. (2009), Baker et al. (2010), and Swezey et al. (2013), respectively. As emphasized above, the data depicted are both quantitative (note the mean [± SD] and individual [red dots] ELISA values obtained for the arthropods collected and the various sampling zones) and qualitative (note the percentages of each population marked at the various sampling zones).

Examples of MRR Research

The PIT has proven useful for conducting MRR research. The first open-field MRR study using PIT was conducted by DeGrandi-Hoffman and Hagler (2000). Foraging honey bees, *Apis mellifera*

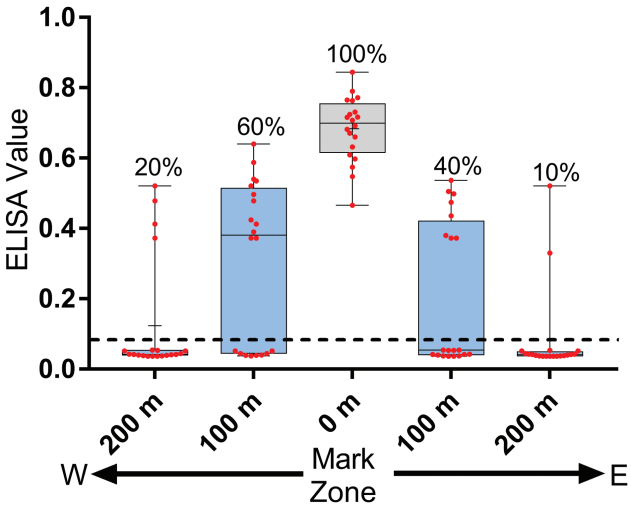

Fig. 3. A depiction of a hypothetical set of ELISA data obtained from arthropods collected in protein-marked (0-m away) and unmarked sampling zones (100- and 200-m away). The dots within the box-whisker plots show the ELISA reaction yielded by each field-collected arthropod. The whiskers show the high and low ELISA values yielded by the arthropods collected from the sampling site. The boxes and the horizontal line within each box indicate the quartiles and the median value for each spatial treatment, respectively. The percentage values represent the percentages of arthropods scoring positive for the presence of a protein mark. The dotted horizontal line is ELISA critical threshold value yielded by the negative control specimens.

L. (Hymenoptera: Apidae), were conditioned to feed on sucrose laced with rabbit IgG at a bait station that was placed near their hive. Subsequently, the IgG-marked food provisions provided by the foragers to nestmates were tracked by temporally sampling bee larva, nurse bees, and nectar within the hive and analyzing the various samples for the presence of IgG. Data revealed that the rabbit IgG-marked sucrose was transported to food storage and brood combs within 2 h after the bees were exposed to the feeding station.

The first open-field PIT study on natural enemies was performed on a cohort of over 40,000 laboratory-reared whitefly parasitoids (Hagler et al. 2002b). The parasitoids were marked internally by feeding them a honey solution laced with rabbit IgG and externally with IgG solution emitted with a nebulizer. The dual marking method proved ideal for tracking parasitoid movement from a central point release site in a complex agroecosystem.

Some MRR studies have used rabbit IgG and chicken IgG to distinctly mark different cohorts of arthropods (Blackmer et al. 2006). For example, Hagler and Naranjo (2004) released commercially purchased *H. convergens* marked with rabbit IgG or chicken IgG into adjacent cotton or cantaloupe fields, respectively. The two distinctive markers allowed the researchers to identify the intercrop dispersal patterns of the beetles between the two cropping systems.

The first-generation IgG marks have proven to be especially useful for studying various aspects of insect social behavior. Many social insect species can be easily 'self-marked' by providing protein-laced food items strategically placed at a central location in a field. For instance, various aspects of ant and termite dispersal behavior have been examined by using IgG-impregnated bait stations (Buczkowski et al. 2007, Buczkowski and Bennett 2009, Baker et al. 2010, Song et al. 2015, Hogg et al. 2018).

The second-generation marks have also been applied in powdered form for MRR research. Hagler et al. (2011a,b) installed

Table 1. A chronolgical listing of articles written that used the PIT

Reference	Target species	Method of application	Study area	Type of study
Hagler et al. (1992)	*Lygus hesperus*	Hand held sprayer	Laboratory evaluation	Proof of concept
Hagler (1997a)	*Hippodamia convergens*	Hand held sprayer	Field cage evaluation	Efficacy test
Hagler (1997b)	*Trichorammatoidea bactrae*	Nebulizer, self-mark	Laboratory evaluation	Efficacy test
Hagler and Jackson (1998)	*Anaphes iole*	Nebulizer, self-mark	Laboratory evaluation	Efficacy test
Degrandi-Hoffman and Hagler (2000)	*Apis mellifera*	Self-mark	Apiary	Self-mark, MRR
Hagler et al. (2002b)	*Eretmocerus sp.*	Nebulizer, self-mark	Cotton, surrounding fields	MRR
Hagler and Miller (2002)	*Pectinophora gossypiella*	Nebulizer, self-mark	Laboratory evaluation	Efficacy test
Blackmer et al. (2004)	*Homalodisca vitripennis*	Hand-held airbrush sprayer	Fallow field	MRR
Hagler (2004)	*H. convergens*	Hand-held airbrush sprayer	Laboratory evaluation	Efficacy test
Hagler and Naranjo (2004)	*H. convergens*	Hand-held sprayer	Cotton and cantaloupe field	MRR
Peck and McQuate (2004)	*Bactrocera latifrons*	Hand-held sprayer, self-mark	*Solanum torvum*	MRR
Blackmer et al. (2006)	*Homalodisca vitripennis*	Hand-held airbrush sprayer	Citrus grove	MRR
Buczkowski and Bennett (2006)	*Tapinoma sessile*	Self-mark	Household	Self-mark, MRR
Buczkowski et al. (2007)	*Reticulitermes flavipes*	Self mark	Laboratory evaluation	Efficacy test
Jasrotia and Ben-Yakir (2006)	*Thrips tabaci, Frankliniella occidentalis*	Self-mark	Field cage evaluation	Efficacy test
Jones et al. (2006)	*Cacopsylla pyricola*	Air-blast sprayer	Apple orchard	Proof of concept, MC
Boina et al. (2009)	*Diaphorina citri*	Hand held atomizer	Citrus grove	MC
Buczkowski and Bennett (2009)	*Tapinoma sessile*	Self-mark	Household	Self-mark, MRR
Hagler et al. (2009)	*Heterotermes aureus*	Perfume atomizer, self-mark	Laboratory evaluation	Efficacy test
Horton et al. (2009)	Various predators	Electric sprayer mounted on an all-terrarin vehicle	Cover crop embedded in a pear field	MC
Janke et al. (2009)	*Pnigalio agraules*	Perfume atomizer, self-mark	Laboratory evaluation	Efficacy test
Baker et al. (2010)	*Hetrotermes aureus*	Self-mark	Desert habitat	Self-mark, MRR
Basoalto et al. (2010)	*Cydia pomonella*	Powered skid sprayer	Apple orchard	MC
Hagler and Jones (2010)	Cotton arthropods	Self-mark, backpack sprayer	Cotton field	Efficacy test
Hagler et al. (2011a)	*Apis mellifera*	Self-mark	GMO and non-GMO alfalfa fields	Efficacy test
Hagler et al. (2011b)	*A. mellifera*	Self-mark	GMO and non-GMO alfalfa fields	Self-mark, MRR
Jones et al. (2011)	Various pests	Self-mark	NA	Efficacy test
Williams et al. (2011)	*Diorhabda carinulata*	Submersion in protein solution	Saltcedar	Efficacy test
Irvin et al. (2012)	*Cosmocomoidea ashmeadi* (formerly *Gonatocerus ashmeadi*)	Hand held sprayer	Laboratory evaluation	Efficacy test
Kelly et al. (2012)	*Podisus maculiventris*	Hand held sprayer, self-mark	Laboratory evaluation	Efficacy test
Krugner et al. (2012)	*Homalodisca vitripennis*	Tractor-mounted airblast sprayer	Citrus grove	MC
Sivakoff et al. (2012)	*Various predators, L. hesperus*	Aerial sprayer (helicopter and airplane)	Alfalfa and cotton fields	MC
Slosky et al. (2012)	*Hippodamia convergens*	Hand held sprayer	Greenhouse	Efficacy test
Reisig et al. (2013)	*Euchistus servus*	Tractor-mounted Hi-Boy sprayer	Wheat and corn fields	MC
Biddinger et al. (2013)	*Osmia conrifrons*	Self-mark	Cherry orchard	Self-mark
Swezey et al. (2013)	*Lygus hesperus*	Gas-powered backpack sprayer	Organic strawberry field with a trap crop	MC
Williams et al. (2013)	*Diorhabda carinulata*	Submersion in protein solution	Laboratory evaluation	Efficacy test
Hagler et al. (2014)	*H. convergens*	Self-mark, backpack sprayer	Alfalfa field	Efficacy test
Kelly et al. (2014)	*Manduca sexta*	Self-mark	Tomato field	MRR
Klick et al. 2014	*Drosophila suzukii*	Self-mark	Laboratory evaluation	Efficacy test
Lesso et al. (2014)	*Scaphoideus titanus*	Hand held jet sprayer	Vineyard	MC

Table 1. (Continued)

Reference	Target species	Method of application	Study area	Type of study
Sanders and Carpenter (2014)	*Culicoides obsoletus*	Self-mark	Horse stable	Self-mark, MRR
Peck et al. (2014)	*Musca autumnalis*	GunJet[R] sprayer on an all-terrain vehicle	Pastured beef cattle operation	Self-mark, MRR
Swezey et al. (2014)	*Peristenus relictus*	Gas-powered backpack sprayer	Organic strawberry field with a trap crop	MC
Hagler et al. (2015)	Various arthropods	Hand held sprayer	Alfalfa field	Efficacy test
Lewis-Rosenblum et al. (2015)	*Diaphorina citri*	Handgun sprayer mouned on an all-terrain vehicle	Citrus grove	MC
Song et al. (2015)	*Linepithema humile*	Self-mark	Natural areas	Self-mark, MRR
Bastola et al. (2016)	*H. convergens*	Tractor-driven boom and nozzle sprayer	Alfalfa and cotton fields	MC
Blaauw et al. (2016)	*Halyomorpha halys*	Pak-blast airblast sprayer	Peach orchard	MC
Blaauw et al. (2017)	*H. halys*	Hand wand electric pump sprayer	Polyculture and trap crop	MC
Hagler and Machtley (2016)	NA	Nebulizer, hand held sprayer	Laboratory demonstration	Demonstration
Klick et al. (2016)	*D. suzukii*	Cannon air blast sprayer	Raspberry field and surrounding vegetation	MC
Sivakoff et al. (2016)	*Cimex lectularius*	Self-mark	Laboratory evaluation	Proof of concept
Lefebvre et al. (2017)	Varioius predators	Backpack sprayer	Hedgerow and apple orchard	MC
Boyle et al. (2018a)	*Osmia lignaria*	Self-mark	Laboratory evaluation	Efficacy test
Boyle et al. (2018b)	Various bees	Self-mark	Laboratory evaluation	Proof of concept
Hogg et al. (2018)	*Linepithema humile*	Self-mark	Vineyard	Self-mark, MRR
Irvin et al. (2018)	Various natural enemies	Gas-powered backpack sprayer	Vineyard and cover crop	MC
Bastola and Davis (2018)	*Piezodours guildinii*	Lawn and garden sprayer	Soybean field	MC
Tait et al. (2018)	*D. suzukii*	Backpack sprayer	Forest	MC

protein dispensers at the entrances of >100 honey bee colonies (central point sites) that were designed to administer a self-mark of egg white, milk, and fluorescent powders (various colors) to foragers as they departed their hives. In turn, the foraging honey bees were spatially sampled over a 15.2 km^2 area and examined for the presence of the various types of marks. It was determined the honey bees foraged, on average, about 800 m from their nest site. The maximum foraging distance recorded was 5,984 m.

There have been several other ingenious methods used to deliver protein marks to arthropods for MRR research. Peck et al. (2014) topically doused cow dung with chicken egg whites to mark adult face flies, *Musca autumnalis* DeGeer (Diptera: Muscidae), that came into direct contact with, or emerged from, centrally marked cow dung. Biddinger et al. (2013) used a self-marking egg white disperser to mark foraging blue orchard bees. The bee's foraging range in a cherry orchard was uniquely determined by sampling and examining the cherry flowers rather than the bees, which provided indirect evidence of bee visitation to flowers based on protein residue left on the flowers. This methodological approach was significant because it offered a way for PIT studies to be conducted without destructively sampling valuable and/or rare insect populations, such as blue orchard bees. A listing of all the MRR studies using the PIT conducted to date is provided in Table 1.

Examples of MC Research

Historically, MC research has proven much more problematic than MRR research. This difficulty is due, in large part, to a lack of suitable markers for tagging arthropods in their habitat. MC studies require markers that can be easily applied in large volumes over relatively large areas. Unfortunately, the few markers used for MC type research to date (e.g., trace elements, nitrogen-15) have significant drawbacks that limit their wide-scale appeal (see Hagler and Jackson 2001, for a review).

The development of ELISAs that detect inexpensive protein markers has had a significant impact on the methodology used to conduct MC research. The effect is evidenced by the flurry of MC studies conducted over the past 12 yr using the second-generation PIT procedure (Table 1). The PIT has been used to study the dispersal characteristics of indigenous and invasive crop pests, urban pests, natural enemies, and pollinators. It has been deployed in row crops, cover crops, orchards, and deserts. A listing of all the MC studies using the PIT conducted to date is provided in Table 1.

Future Directions—A Third Generation PIT?

The best attributes of the first-generation PIT are that the sandwich ELISA format is very well established and it is an outstanding tool for detecting internally and externally marked arthropods. However, the IgG marks are prohibitively expensive and unavailable in quantities sufficient to conduct a large-scale MC type study. In contrast, the best attributes of the second-generation PIT are that the marks are inexpensive and readily available for bulk purchase. However, the indirect ELISA format is less effective than the sandwich ELISA format, especially regarding the detection of protein marks in internally marked arthropods. An ideal PIT would combine the strengths of both first- and second-generation PITs. Specifically, it would consist of an inexpensive marker that is detectable by the sandwich ELISA format.

Recently, I compared the reactivity of rabbit IgG and whole rabbit serum to the anti-rabbit IgG sandwich ELISA. The results revealed that

the rabbit serum was as reactive as IgG to the ELISA (J.R.H., in preparation). Given that whole rabbit serum is readily available for bulk purchase at a fraction of the cost of the highly purified IgGs, this finding could provide researches with an inexpesive marker that is appropriate for sandwich ELISA. Preliminary data indicate that for a sample to yield an ELISA absorbance value of 0.5 (a strong ELISA reaction), the cost per liter for the necessary concentration of rabbit IgG and rabbit serum is approximately $1.50 and $0.01, respectively. For reference, it is not uncommon for a large-scale MC study to require 1,000 to 2,000 liters of marking liquid for an experiment (Jones et al. 2006, Krugner et al. 2012, Sivakoff et al. 2012). Currently, rabbit serum and other various types of whole vertebrate sera are being examined by antisera sandwich ELISAs for marking efficacy and costeffectiveness for future MC-type research (J.R.H., in preparation). If successful, this 'hybrid' PIT could offer researchers an economical method for conducting MC research using the sandwich ELISA format.

Conclusions

Selecting the best method for marking arthropods for MRR and MC research is critical to the success of many experiments aiming at understanding dispersal (Hagler and Jackson 2001). Unfortunately, most of the current methods used to tag insects are not universally effective. The PIT has proven over the years to be useful for both MRR and MC research. Moreover, the arthropods can be marked in a variety of ways. For MRR studies, the proteins can be applied externally in the laboratory simply by spraying the mark over the arthropods with any common spray device. They can also be marked internally by feeding them protein-enriched food. For MC studies, the protein markers can be administered to arthropods with conventional spray equipment and are well-retained on most arthropod species. Importantly, the protein markers do not appear to affect an arthropod's biology and behavior. The protein-specific ELISAs used to detect the various types of protein markers are inexpensive, easy to learn, and are also well suited for mass throughput. Thousands of field-collected arthropods can be assayed daily. The diverse array of PIT studies conducted to date amply demonstrate that this procedure can be adapted to study the dispersal behavior of almost any type of arthropod.

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