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A false-positive food chain error associated with a generic predator gut content ELISA

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Abstract

Conventional prey-specific gut content ELISA (enzyme-linked immunosorbent assay) and PCR (polymerase chain reaction) assays are useful for identifying predators of insect pests in nature. However, these assays are prone to yielding certain types of food chain errors. For instance, it is possible that prey remains can pass through the food chain as the result of a secondary predator (hyperpredator) consuming a primary predator that had previously consumed the pest. If so, the pest-specific assay will falsely identify the secondary predator as the organism providing the biological control services to the ecosystem. Recently, a generic gut content ELISA was designed to detect protein-marked prey remains. That assay proved to be less costly, more versatile, and more reliable at detecting primary predation events than a prey-specific PCR assay. This study examines the chances of obtaining a 'false positive' food chain error with the generic ELISA. Data revealed that the ELISA was 100% accurate at detecting protein-marked *Lygus hesperus* Knight (Hemiptera: Miridae) remains in the guts of two (true) primary predators, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) and *Collops vittatus* (Say) (Coleoptera: Melyridae). However, there was also a high frequency (70%) false positives associated with hyperpredators, *Zelus renardii* Kolenati (Hemiptera: Reduviidae), that consumed a primary predator that possessed protein-marked *L. hesperus* in its gut. These findings serve to alert researchers that the generic ELISA, like the PCR assay, is susceptible to food chain errors.

Introduction

Obtaining sufficient and reliable data on an insect predator's prey choice is difficult. Direct field observations of predation are rarely achieved because most insects are small and elusive. Also, most predators do not leave any indirect evidence of an attack because chewing predators usually devour their prey and piercing-sucking predators do not leave specific recognizable feeding wounds in the carcasses of their victims. Molecular gut content analysis of predators has been widely adopted as an indirect means to assess predation. The two contemporary types of gut content assays include the enzyme-linked immunosorbent assay (ELISA) that uses a monoclonal antibody (MAb) to identify a prey-specific protein, and the polymerase chain reaction (PCR) assay that uses prey-specific primers to detect prey-specific DNA (Greenstone, 1996; Sheppard & Harwood, 2005).

The contemporary prey-specific gut assays have proven useful for identifying predators of major pests and for sorting out complex food web interactions (Harper et al., 2005; Sheppard & Harwood, 2005; Gagnon et al., 2011; Hagler & Blackmer, 2015). Although these assays can detect the presence of the insect-specific molecules in a predator's gut, they cannot truly ascertain how the prey was obtained by the predator. The assumption is that if the assay detects the presence of the targeted prey that it was obtained by a primary predator directly feeding on the prey item. However, an erroneous positive assay reaction for a 'predation event' can occur if a predator engages in secondary predation. Secondary predation (also known as hyperpredation and intraguild predation) entails a higher tiered predator consuming a lower tiered (primary) predator (Sabelis, 1992). Unfortunately, if that lower tiered predator had previously

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consumed the pest of interest, there is a chance that the prey-specific protein (for the ELISA) or DNA (for the PCR assay) would pass to the hyperpredator. If so, the primary predator would be abolished from the ecosystem and, in turn, the secondary predator would be erroneously credited for providing the biological control services. Harwood et al. (2001) examined the possibility of such a food chain error in an aphid–spider–carabid system (i.e., pest–primary predator–secondary predator) using an aphid-specific indirect ELISA. That study showed that a food chain error was unlikely to occur under field conditions. However, in an almost identical study using an aphid-specific PCR assay, Sheppard et al. (2005) showed that aphid DNA could be readily detected in carabid beetles after consuming a spider. They concluded that the extreme sensitivity of the PCR assay made detection of hyperpredation more likely. The implication from that study was that the PCR assay was more sensitive than the indirect ELISA. Hence, it was more susceptible to hyperpredation food chain errors.

We have described a versatile prey immunomarking (coined here as the generic gut assay) method that circumvents the need to develop prey-specific assays for gut content evaluations (Hagler & Durand, 1994; Zilnik & Hagler, 2013; Blubaugh et al., 2016; Mansfield & Hagler, 2016). Recently, the accuracy and reproducibility (i.e., each predator sample was assayed in triplicate) of the generic assay was compared with a prey-specific PCR assay. The study revealed that both methods accurately detected the targeted prey in the primary predators for several hours after a meal, but the generic assay was more reproducible (i.e., all three sub-samples yielded the same outcome) than the PCR assay (Hagler et al., 2015). This study examines the prevalence of obtaining secondary predator food chain errors using the generic ELISA procedure. I hypothesized that protein-marked prey remains would be readily detected in the primary predators, but the protein-marked prey remains would not be detected very frequently in the hyperpredators due to degradation through the food chain.

Materials and methods

Insects in the food chain

The insects represented in this food chain study consisted of a pest species, two primary predators, and a single secondary predator. A protein-marked third instar *Lygus hesperus* (lygus) served as the targeted pest, adult *Collops vittatus* (collops) and *Hippodamia convergens* (lady beetle) served as primary predators of the pest, and adult *Zelus renardii* (assassin bug) served as a secondary predator of the primary predators. The primary and secondary predators were collected from an alfalfa field located at The University of Arizona's Maricopa Agricultural Center (Maricopa, AZ, USA).

Pest marking procedure

Third instar lygus were obtained from a laboratory colony reared on the artificial diet described by Debolt (1982). A key ingredient of this diet is chicken egg. As a result, lygus acquire chicken egg protein internally by feeding on the diet (Hagler, 2011). In addition, each individual was removed from the diet and externally marked with chicken IgY (No. I-4881; Sigma Chemical, St. Louis, MO, USA) by placing 5.0 µl of a 1.0 mg ml^{-1} chicken IgY solution over their dorsal surface using a micropipette. The topical mark was allowed to dry for ca. 1 h.

Predator feeding bioassay

A single protein-marked lygus was placed into a 5.5-cm-diameter Petri dish. Then, a starved (for 24 h) primary predator was added to the dish for up to 2 h. The insects were continuously monitored to ascertain the occurrence of a primary predation event. If the primary predator did not feed on the protein-marked lygus within 2 h, the surviving lygus was placed into a 1.5-ml microtube and frozen at −80 °C. These specimens served as the positive protein-marked prey control treatment (to determine the efficacy of the protein marking procedure). If the primary predator consumed a protein-marked lygus, it was immediately placed in another dish containing a starved assassin bug. Similarly, if the assassin bug did not feed within 2 h, then the surviving primary predator was placed in a microtube and frozen. These specimens served as the primary predator treatment (to determine whether the primary predators contained the protein-marked prey). If the assassin bug did feed on the primary predator, it was frozen after it finished its meal. These specimens served as the secondary predator treatment (to determine the frequency of obtaining a false-positive food chain error). Also, the corpse of each primary predator species that had been eaten by an assassin bug was placed in a microtube and frozen. The corpse treatment served as both a primary predator treatment and as a treatment to determine whether all the protein-marked lygus was depleted from its carcass by the piercing-sucking hyperpredator.

Gut content ELISA

Individual insect samples were removed from the freezer and homogenized in 1.0 ml of tris buffered saline (TBS, pH 7.4). Each individual was examined in triplicate by taking three 100-µl aliquots from each sample and assaying

them for the presence of egg protein by the anti-chicken IgY sandwich ELISA described by Hagler et al. (2015). The triplicate assays of each insect sample were performed on different Falcon Microtest Flat Bottom Polystyrene ELISA plates (No. 351172; Becton Dickinson Labware, Franklin Lakes, NJ, USA). The three ELISA absorbance values obtained for each specimen were averaged. It should also be noted that the qualitative (percent positive) and quantitative (ELISA absorbance values) results obtained for the two primary predator species were nearly identical. As such, these data were pooled to simplify the data presentation.

Unmarked controls

Third instar lygus, primary predators, and secondary predators were collected from the alfalfa field described above and frozen at −80 °C. These specimens served as the ELISA negative control treatments. Mean (± SD) ELISA optical absorbance values were calculated for each negative control group (n = 8 per ELISA plate). A primary and secondary predator that consumed a prey item was conservatively scored positive for the presence of the protein mark if its ELISA reading was six standard deviations above that of the negative control mean.

Results

Detection of the protein mark on the target pest

There were 16 protein-marked lygus that survived the 2-h feeding assay with the primary predator. The ELISA detected the protein mark on every lygus. The mean ELISA absorbance value for the protein-marked prey was 19× higher than their unmarked counterparts (Figure 1).

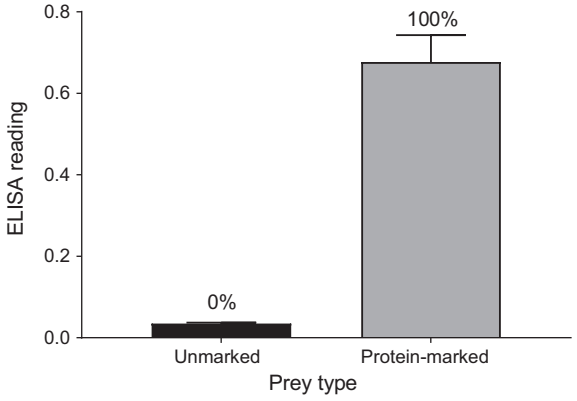

Figure 1 Mean (+ SD) ELISA readings yielded by unmarked (black, n = 8) and protein-marked lygus (gray, n = 16). The percentage above each error bar is based on the number of specimens that scored positive for the presence of the protein mark.

Detection of the protein-marked prey in the primary predators

Protein-marked lygus was detected in every primary predator (i.e., there were no false negatives) that survived the 2-h feeding assay with the secondary predator. The ELISA immunoreactions for these predators were 11× higher than that of the predators that ate an unmarked lygus (Figure 2A). Protein-marked lygus remains were detected in all three sub-samples of 85% of the primary predators (corpses) that did not survive the 2-h feeding assay with the secondary predator. Moreover, the ELISA immunoreactions yielded by the primary predator corpses were similar to the values yielded by those beetles that survived the secondary predator feeding trial (i.e., the secondary predators did not deplete the primary predator of their protein-marked foodstuffs; Figure 2B). Five of the corpses (15%) examined did not contain protein-marked lygus remains (note that all three sub-samples of each of these specimens yielded a negative ELISA response). It is

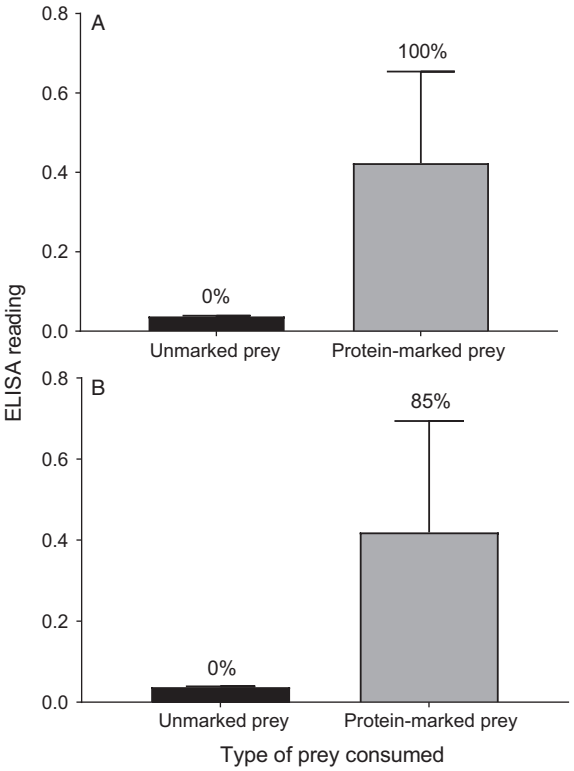

Figure 2 Mean (+ SD) ELISA readings yielded by unmarked primary predators (black, n = 16) and primary predators that consumed (A) a protein-marked lygus (gray, n = 34) after surviving the 2-h secondary predator feeding assay, or (B) a protein-marked lygus (gray, n = 33) that did not survive (corpses) the 2-h secondary predator feeding assay. The percentage above each error bar is based on the number of specimens that scored positive for the presence of the protein-marked prey.

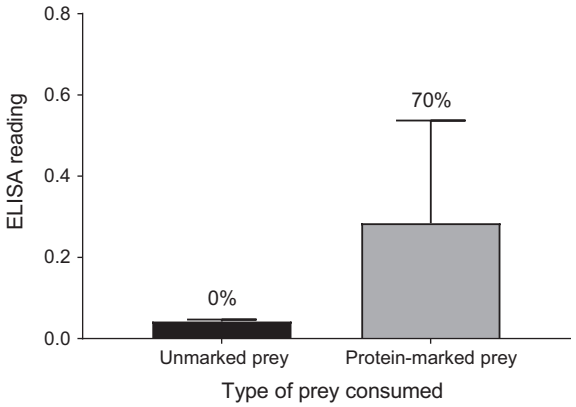

Figure 3 Mean (+ SD) ELISA readings yielded by unmarked secondary predators (black, n = 8) and secondary predators that consumed a primary predator (gray, n = 33). The percentage above each error bar is based on the number of specimens that scored positive for the presence of the protein-marked prey.

highly likely that the protein mark was depleted from these five victims by the secondary predator.

Detection of the protein-marked prey in the secondary predators

Protein-marked lygus remains were detected in all three sub-samples of 70% of the secondary predators that consumed a primary predator. The ELISA reactions were 7× higher than their predator counterparts that fed on an unmarked prey (Figure 3). Ten of the secondary predators (30%) examined did not contain protein-marked lygus remains (note that all three sub-samples of each specimen yielded a negative ELISA response).

Discussion

It is well known that prey-specific ELISA and PCR assays are predisposed to misidentifying certain predation events (Hagler & Naranjo, 1996; Juen & Traugott, 2005; Sheppard & Harwood, 2005; King et al., 2008). For example, prey-specific gut assays will yield a positive response for a 'predation event' regardless whether the prey was attacked while it was alive or dead (scavenged). If a predator species has a proclivity for scavenging, then the biological control services rendered by that predator will be overestimated by the conventional prey-specific assays. However, a recent study conducted in the confines of caged arenas showed that the generic ELISA procedure can be modified to assess scavenging activity. Specifically, Mansfield & Hagler (2016) tagged live lygus with chicken IgY and carrion lygus with rabbit IgG and introduced the protein-tagged specimens to an assemblage of predators on a cotton plant. The generic ELISAs revealed that every so-called lygus 'predator' species examined (as previously determined by an *L. hesperus*-specific PCR assay; Hagler & Blackmer, 2013) preferentially fed on the rabbit IgG-marked carrion.

This study examines the possibility of obtaining another type of false-positive predation error as a consequence of a hyperpredation event (Sheppard et al., 2005). This study and others have shown that the generic ELISA is very effective at detecting prey remains in the guts of primary predators for several hours after a meal (Hagler, 2006, 2011; Hagler et al., 2015). However, this study also shows that the generic ELISA is prone to yielding food chain errors. Specifically, protein-marked lygus remains were detected in 70% of the hyperpredators. It should be noted, however, that the experimental protocol for this study was designed as a 'best case scenario' for detecting a true primary predation event and a false-positive predation event for a secondary predator. Specifically, each primary and secondary predator was killed by freezing immediately after it completed its meal. It has been shown that protein-marked prey can only be qualitatively detected in primary predators for about 6–12 h after a meal and the quantitative ELISA response declines rapidly during this time frame (Hagler et al., 2015; Mansfield & Hagler, 2016). In addition, the simplistic and confining Petri dish feeding arena (e.g., there was only one prey choice and minimal chance for escape) does not mimic these insect's natural habitat. Hence, the high false-positive food chain error rate obtained in this feeding study is unlikely to occur under natural field conditions.

A unique aspect of this study was that each predator sample was assayed in triplicate. To date, the vast majority of predator gut analysis studies has relied on the outcome of a single predator sample to score the specimens for prey remains. The rational for only using one aliquot per sample to judge a predator for the presence of prey remains is uncertain, but it is likely due to the researcher's unsubstantiated faith in the accuracy of the assay coupled with the added expense of conducting multiple assays on each predator specimen. However, Hagler et al. (2015) showed drastic differences in the reliability between the generic ELISA used in this study and a species-specific PCR assay. Specifically, the generic ELISA was more reproducible (e.g., all three sub-samples of each specimen yielded the same outcome) than a species-specific PCR assay (e.g., all three sub-samples rarely yielded the same outcome). The conclusion from that study was that researchers using that species-specific PCR assay should analyze each predator specimen in triplicate, but duplicate sub-samples are not necessary for the generic ELISA. This study also revealed that the generic ELISA was very reliable (reproducible). That is, all three sub-samples for each individual predator specimen always yielded the same outcome for the presence or absence of the protein-marked prey item.

In summary, the generic ELISA procedure is a practical and cost-effective technique to assess predation. The technique has the advantage in that it does not require the development of prey-specific MAbs or DNA probes. Moreover, it provides researchers with a flexible tool for conducting a wide variety of predation studies that cannot be conducted with conventional prey-specific gut assays (see Hagler, 2006, 2011; Mansfield & Hagler, 2016). Finally, the processing of samples is more user-friendly, less costly, and less time-consuming than the PCR assay (Fournier et al., 2008; Aebi et al., 2011; Hagler & Blackmer, 2013). This study also confirms that the generic ELISA is reliable at detecting primary predation events. However, it is also vulnerable to yielding false-positive hyperpredation food chain errors. In short, researchers must be cautious with the interpretation of gut assay results obtained from higher tiered members of the predator assemblage whether they are using conventional prey-specific assays (Calder et al., 2005; Foltan et al., 2005) or the generic ELISA describe here.

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