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Standardization of prey immunomarking: does a positive test always indicate predation?

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Abstract A prey immunomarking procedure (PIP) in combination with generic anti-rabbit and anti-chicken immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs) are used frequently to study arthropod predation. This study was conducted to: (1) further standardize the PIP as a tool for predator gut analysis research, (2) investigate the most effective means for administering IgG marks to prey items, and (3) assess the possibility of the PIP yielding false positive reactions as a consequence of a predator obtaining a mark by incidental contact with, or by a failed predation attempt on, a protein-marked prey item. The pest *Lygus hesperus* Knight (Hemiptera: Miridae) was tagged with either an external rabbit IgG mark, an internal chicken IgG mark, or a double (external rabbit IgG and internal chicken IgG) mark treatment. Then, the variously marked prey items were fed to chewing and piercing-sucking type predators and their gut contents were examined for the presence of IgG remains. Data revealed that all three marking treatments were highly effective at tagging targeted prey. However, ELISA results showed that the prey items should only be marked internally to maximize the likelihood of detecting prey remains while minimizing the risk of obtaining false positive errors. The merits and limitations of using the generic PIP for predator gut analysis research are discussed.

Keywords Predator gut content analysis · *Lygus hesperus* · False positive · Predatory beetles · Predatory bugs · Generic ELISA

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Introduction

Verifying arthropod predation events in their natural habitat is challenging because predators and their prey are often too tiny and elusive to observe directly. Also, predators rarely leave indirect evidence of predation, as chewing type predators (e.g., beetles, earwigs, etc.) usually consume their entire prey and piercing-sucking type predators (e.g., true bugs, spiders, etc.) generally do not leave distinguishable feeding wounds to the bodies of their victims. Even if some evidence of predation remains, supporting observations are needed to identify the predator to species (Scholz et al. 2000; Low et al. 2014). As such, researchers have resorted to

using molecular techniques to identify prey remains in predators' stomachs (reviewed by Greenstone 1996; Hagler and Naranjo 1996; Sheppard and Harwood 2005). The two most common (conventional) molecular gut content analysis methods include prey-specific enzyme-linked immunosorbent assays (ELISAs) and prey-specific PCR assays (Greenstone et al. 2007). However, almost 25 years ago, a standardized gut content analysis method, hereafter referred to as the "generic prey immunomarking procedure" (PIP), was described that used the ELISA format, but did not require the development of a prey-specific monoclonal antibody (Hagler and Durand 1994). In that proof-of-concept study, predators with chewing or piercing-sucking mouthparts were fed a single adult whitefly (*Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) or pink bollworm egg (*Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae)) that had been externally marked with a 1.0 mg ml^{-1} rabbit immunoglobulin G (IgG) solution. The predators were then examined for the presence of the rabbit IgG-marked prey item instead of the prey-specific antigens (proteins), by an established anti-rabbit IgG ELISA (Hagler et al. 1992). The study revealed that the technique was effective at detecting both types of externally marked prey items in the guts of the chewing type predators (> 95% of those examined tested positive), but it was not as effective at detecting prey remains in the piercing-sucking type predators (< 30% tested positive).

Since its development, the generic rabbit IgG-specific ELISA (often used in combination with a generic chicken IgG ELISA) has proven to be a viable alternative to the conventional prey-specific gut assay procedures. The generic PIP has been adapted to study various types of arthropod predator–prey interactions, many of which are not possible with the conventional assay approaches. The two key refinements are: (1) that both internal and external marks have been used to tag the targeted prey and (2) higher concentrations (> 1.0 mg ml^{-1}) of the marking protein(s) have been used to tag the targeted prey items. Specifically, the technique has been used to examine predation on IgG-marked termites (Buczkowski and Bennett 2007), predation on IgG-marked egg masses (Mansfield et al. 2008), trophic level interactions on variously marked prey in the food chain (Hagler 2006; Kelly et al. 2014), granivory of IgG-marked dandelion seeds (Lundgren et al. 2013), omnivore feeding guild preferences (Blubaugh et al. 2016), scavenging activity (Zilnik and Hagler 2013; Mansfield and Hagler 2016), and cannibalism (JRH per. obs.). It has also been used to quantify the predation rates of various predator taxa on *Lygus hesperus* Knight in the cotton agroecosystem (Hagler 2011).

The present study was conducted to refine and further validate the use of the generic PIP for predator gut analysis research. The study consisted of two phases. The first phase was conducted with the goal of identifying the most effective method for tagging prey for PIP research. In the first phase, we fed a single protein-marked prey item to either a chewing or piercing-sucking type predator (defined as a "successful predation event"). The prey item offered to each individual predator contained one of three mark treatments. The treatments consisted of a *L. hesperus* marked: (1) internally with chicken IgG, (2) externally with rabbit IgG, or (3) internally and externally marked (double marked) with both IgGs. Predators that consumed a protein-marked prey item were then assayed by generic (i.e., standardized) anti-chicken and anti-rabbit IgG ELISAs (Hagler 1997).

The second phase of the study, in which the predators "failed" in their predation attempt on the variously marked prey treatments, was designed to determine if each ELISA could detect the transfer of the mark from the prey to the predator as a consequence of an unsuccessful predation event (i.e., a false positive gut assay reaction). In this phase, the predators purposefully were not allowed to kill and consume the protein-marked prey. Specifically, they were only permitted to: (1) briefly touch their prey by incidental contact or (2) briefly grab and bite, but not kill or devour their prey. The incidence of false positives due to contact or failed predation were analyzed for different marking methods and predator–prey interaction scenarios. Finally, the merits and limitations of using the generic PIP for studying a wide variety of predator–prey interactions are considered.

Materials and methods

Prey marking treatments

Unmarked prey control treatment

Third-instar *L. hesperus* were collected with a 38 cm diameter sweep net from an alfalfa field located at the

University of Arizona's Maricopa Agricultural Center located near Maricopa, Arizona, USA. An unmarked *L. hesperus* was placed into a 35 mm diameter × 10 mm tall Petri dish (the feeding arena) that contained a predator (see below) that had been starved for 24 h. After each predator species (n = 15) consumed the unmarked *L. hesperus*, the predator was frozen immediately for later analysis by the ELISAs described below. Also 15 *L. hesperus* nymphs, serving as negative control prey, were removed from the sweep net and frozen for later analysis by the ELISAs described below.

Externally marked prey treatment

Third instar *L. hesperus* were collected from the field as described above. Individuals were marked topically (drenched) by placing 10 µl of a 1.0 mg ml^{-1} rabbit IgG solution over their dorsal surface using a micropipette. Each prey item was allowed to dry for at least 1 h. Then, a rabbit IgG-marked prey item was placed into a feeding arena that contained a predator (see below) that had been starved for 24 h. After each predator species (n = 15) consumed the externally-marked prey, it was frozen immediately for later analysis by the ELISAs described below. Also, 15 *L. hesperus* nymphs, serving as positive control prey items (to validate the efficacy of the external marking procedure), were marked and frozen for later analysis by the ELISAs described below.

Internally marked prey treatment

Third instar *L. hesperus* were obtained from a laboratory colony reared on artificial diet (Debolt 1982). A key ingredient of the diet is chicken egg. As a result, about half of the nymphs removed from the colony already possessed chicken egg (IgG) protein in their gut (i.e., an internal mark) by ingestion of the diet (pers. obs). Nymphs ($n \approx 45$) were removed early each morning of the feeding observations from the colony and placed in 10 × 10 × 1.5 cm square plastic dish with no diet pack inside. They were starved for 6–8 h, and then a fresh diet packet that was slightly smaller than the square dish was placed inside the dish. The nymphs were allowed to feed freely overnight. The next day, an individual *L. hesperus* nymph, typically directly observed feeding on the diet packet, was removed from the square dish and then this internally marked prey item was placed in a feeding arena containing a starved predator (see below). After each predator species (n = 15) consumed an internally marked prey item, it was frozen immediately for later analysis by the ELISAs described below. Also, 15 *L. hesperus* nymphs, serving as positive control prey items (to validate the efficacy of the internal marking procedure), were removed from the feeding packet and frozen for later analysis by the ELISAs described below.

Double marked prey treatment

Third instar *L. hesperus* were collected from the laboratory colony and internally marked with chicken egg (IgG) and externally marked with rabbit IgG as described above. Then, a double-marked prey item was placed into a feeding arena that contained a predator (see below) that had been starved for 24 h. After each predator (n = 15 per species) consumed the double-marked prey item, it was frozen immediately for later analysis by the ELISAs described below. Also, 15 *L. hesperus* nymphs, serving as positive control prey items (to validate the efficacy of the external and internal marking procedures), were removed and frozen for later analysis by the ELISAs described below.

Predator feeding trials

Monitoring of a successful predation event

The predators examined included field-collected adult *Geocoris punctipes* (Say) (Hemiptera: Geocoridae), *Zelus renardii* Kolenati (Hemiptera: Reduviidae), and *Collops vittatus* (Say) (Coleoptera: Melyridae). The *G. punctipes* and *Z. renardii* represent piercing-sucking type predators and the *C. vittatus* represents a chewing type predator. The predators were collected in the alfalfa field described above and starved for at least 24 h before each feeding trial. An individual predator (n = 15) was fed a single *L. hesperus* nymph that had received one of the four protein mark treatments (no mark, external mark, internal mark, or double mark) described above. The *G. punctipes* and *C. vittatus* were reluctant to feed on live prey. Hence, protein-marked *L. hesperus* were killed by freezing at − 80 °C. The frozen prey were then air dried and presented to the predators. The feeding arenas were continuously

monitored to ascertain the occurrence of a feeding event. Once a predator attacked the prey item, its feeding duration was recorded. Immediately after the feeding ceased, the predator was placed in a 1.5 ml microtube and frozen at − 80 °C. Individual predators were removed from the freezer, homogenized in 1000 µl of tris buffered saline (TBS, pH 7.4), and assayed for the presence of the rabbit IgG and chicken IgG marked prey by the ELISAs described by Hagler (2011). Each insect homogenate was assayed in triplicate (a 100 µl aliquot per sample). The assays proved to be 100% reproducible (i.e., they yielded very similar ELISA reactions) therefore data presented are averages of the three sub-samples from each predator specimen. The average value yielded by each sample was then used to score the predator specimen as positive or negative for the presence of the targeted prey (protein) remains.

Monitoring of an unsuccessful predation event

The chewing predators examined included adult *C. vittatus* and *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae). The piecing-sucking predators examined included late instar (4th and 5th) and adult *G. punctipes*, *Z. renardii*, *Sinea confusa* Caudell (Hemiptera: Reduviidae), and *Nabis alternatus* Parshley (Hemiptera: Nabidae). All predators were collected and starved as described above. Again, predators were exposed to a single *L. hesperus* nymph that had received one of the mark treatments. For data analysis, the predator treatments were pooled by mouthpart morphology (chewing or piercing-sucking) to bolster the sample sizes. Again, some predator taxa were reluctant to feed on live prey (i.e., all *C. vittatus* and *H. convergens*, and some *G. punctipes* and *N. alternatus*). Instead these predators were offered protein-marked cadavers that were prepared as described above. The "unsuccessful feeding trials" differed from the "successful feeding trials" in that each feeding trial lasted 10 min or until a predation attempt, whichever occurred first. The unsuccessful feeding trials were also placed in one of two treatment categories: (1) a predation "attempt" treatment or (2) a "contact by touch" only treatment, respectively. Additionally, each of the unsuccessful treatments had two sub-treatments: (1) a short handling duration treatment or (2) a long handling duration treatment. For the predation attempt treatment, the predator attempted to attack and feed on the prey item within the 10 min feeding trial. In this case, the feeding trial ceased, and the predator and protein-marked prey item were separated as quickly as possible with a clean toothpick or tweezers. Separating the predator from its prey item sometimes proved difficult. Any delay between the start of feeding and the separation from the prey item was noted as either a short handling duration ($\leq$ 5 s; the average $\pm$ SD short handling duration was 2.8 $\pm$ 1.3 s) or a long handling duration ($>$ 5 s; the average $\pm$ SD long handling duration was 13.0 $\pm$ 11.5 s). Alternatively, for the "contact only" treatment, the full 10 min allotted for the feeding trial elapsed with no predation attempt. However, during this time frame the predator and prey typically engaged in bodily contact (e.g., brief touches). In general, a single touch lasted about 0.5 s. The number of touches occurring over the 10 min period was recorded by the observer and the total time was summed. The "contact only" treatment was further classified as either a short ($\leq$ 5 s) or long duration ($>$ 5 s), based on total contact time. In either case, immediately at the end of each unsuccessful predation attack, and after separation (if necessary), predator and prey were removed from the arena, transferred to individual 1.5 ml microtubes, and frozen at − 80 °C. Each predator specimen was removed from the freezer, homogenized in 1000 µl of TBS, and examined for the presence of the rabbit IgG and chicken IgG marked prey by the ELISAs described by Hagler (2011). Frozen prey were also assayed to confirm the success of the prey marking procedure. Note that only a single aliquot of sample buffer was used from each specimen (i.e., we did not run these samples in triplicate) due to the high reproducibility yielded from the "successful predation" assays described above, and also that reported by Hagler et al. (2015).

Scoring a predation event

Mean ($\pm$ SD) ELISA readings (optical density) were calculated for each predator taxa that fed on an unmarked *L. hesperus* nymph. An individual predator was scored positive for the presence of protein-marked prey remains if its ELISA reading value was six SD above that of the negative control mean value (the critical ELISA threshold value; Hagler 2011). The percentage of positive results from each predator treatment was calculated. All percent values reported are rounded to a whole number.

Results

Prey marking treatments

No mark prey treatment

None of the field-collected (unmarked) *L. hesperus* samples tested positive for the presence of rabbit IgG or chicken IgG (Fig. 1a). The average anti-chicken and anti-rabbit ELISA readings yielded by the unmarked specimens were essentially the same as the readings yielded by the TBS buffer controls.

External mark prey treatment

Rabbit IgG was detected on 87% of the externally marked prey (Fig. 1b). However, two of the 15 individuals that were known to be topically marked with rabbit IgG yielded a false negative reaction. As expected, none of the rabbit IgG marked samples screened positive for the presence of chicken IgG.

Internal mark prey treatment

Chicken IgG was detected in all the *L. hesperus* samples that had fed on the chicken IgG enriched diet (Fig. 1c). As predicted, none of these samples tested positive for the presence of rabbit IgG.

Double mark prey treatment

All of the double marked prey samples yielded very strong immunoreactions for the presence of both the external rabbit IgG and internal chicken IgG marks (Fig. 1d).

Predator feeding trials

Prey detection of a successful predation event

None of the predators that consumed an unmarked *L. hesperus* prey item reacted to the anti-chicken IgG and anti-rabbit IgG ELISA (Fig. 2a). The majority of *C. vittatus* (a chewing type predator, 13 out of 15 individuals) that ate an externally marked *L. hesperus* nymph yielded a positive ELISA reaction for the presence of rabbit IgG-marked prey remains (Fig. 2b). However, the rabbit IgG ELISA was not very effective at detecting externally marked *L. hesperus* remains in the two piercing-sucking feeding predator species (*Z. renardii* and *G. punctipes*, only one of 15 predators of each species tested positive). The chicken IgG ELISA was 100% effective at detecting *L. hesperus* that were internally marked with chicken IgG in the chewing predator species and the two piercing-sucking predator species (Fig. 2c). The internal chicken IgG mark and the external rabbit IgG mark prey remains were readily detected in the stomach contents of the chewing

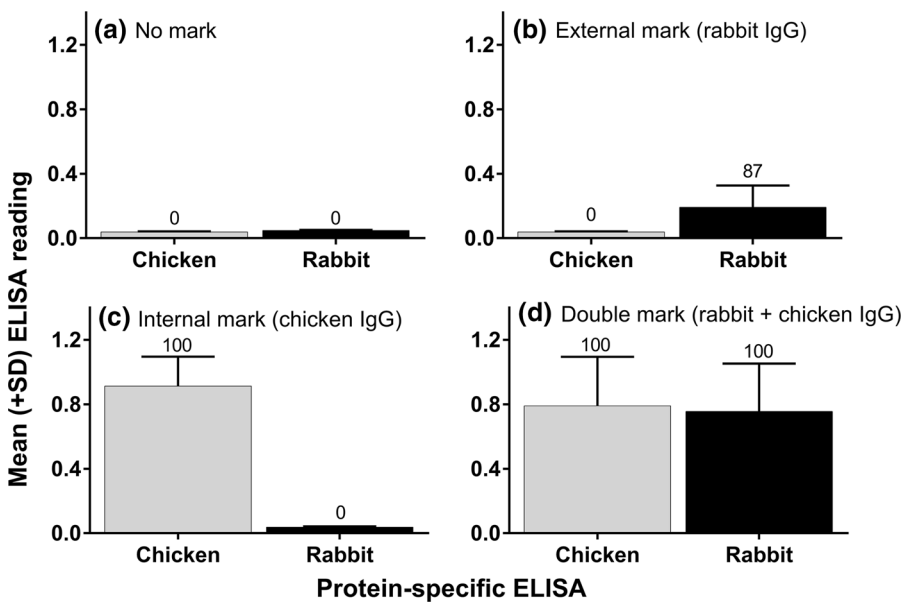

Fig. 1 Mean (+SD) ELISA values (n = 15 per treatment) and percentage of *Lygus hesperus* (prey) scoring positive (% values given above each error bar) for the presence of a rabbit IgG (black bars) and chicken IgG (grey bars) protein mark. The *L. hesperus* examined contained **a** no mark, **b** an external rabbit IgG mark, **c** an internal chicken IgG mark, or **d** an external rabbit IgG and an internal chicken IgG mark. Each *L. hesperus* specimen (n = 15 per treatment) was analyzed by an anti-rabbit IgG and anti-chicken IgG ELISA, respectively

predator *C. vittatus* that consumed a double marked prey item (Fig. 2d). Moreover, the chicken IgG mark (internal mark) was detected in most of the piercing-sucking predators that consumed a double marked prey item with only three *Z. renardii* giving false negatives (Fig. 2d). However, the anti-rabbit IgG ELISA efficiency for detecting rabbit IgG prey remains (i.e., an external mark) for those piercing-sucking predators that consumed a double marked prey item was erratic. Specifically, the external mark was detected in 11 *Z. renardii* and only 1 *G. punctipes* that were fed double marked prey (Fig. 2d). Also, it should be noted that the anti-chicken and anti-rabbit ELISAs proved to be highly specific as there were no false positive ELISA reactions yielded by the predator samples examined (Fig. 2a–c).

Prey detection of an unsuccessful predation event for chewing predators

In total 30 out of 179 chewing predators (17%) returned false positive ELISA results due to lateral transfer of the protein marks. None of the chewing predators that briefly (< 5 s) contacted or attempted predation on an externally rabbit IgG-marked *L. hesperus* tested positive. For longer interactions (> 5 s) only one chewing predator that had either made contact or attempted predation then yielded a weak but positive reaction for the external mark (Fig. 3a, b). The internal chicken IgG mark was not obtained by any of the chewing predators from contact alone, regardless of the handling time (Fig. 3c) and only one chewing predator obtained the internal mark from a short predation attempt. However, six chewing predators (out of 21) that completed a long predation attempt did test positive for the internal chicken IgG mark (Fig. 3d). There was a low frequency (1–3 individuals) of the external rabbit IgG protein mark transferring from a double marked prey item to a chewing predator that either came into brief or extended contact with the prey (Fig. 3e) or had a brief (< 5 s) predation attempt. However, over half of those predators (ten out of 18) that completed a long predation attempt on double marked prey also tested positive for the rabbit IgG mark (Fig. 3f). Again, the internal chicken IgG mark was not obtained by any of the chewing predators from contact alone, regardless of the handling time (Fig. 3e). No chewing predators

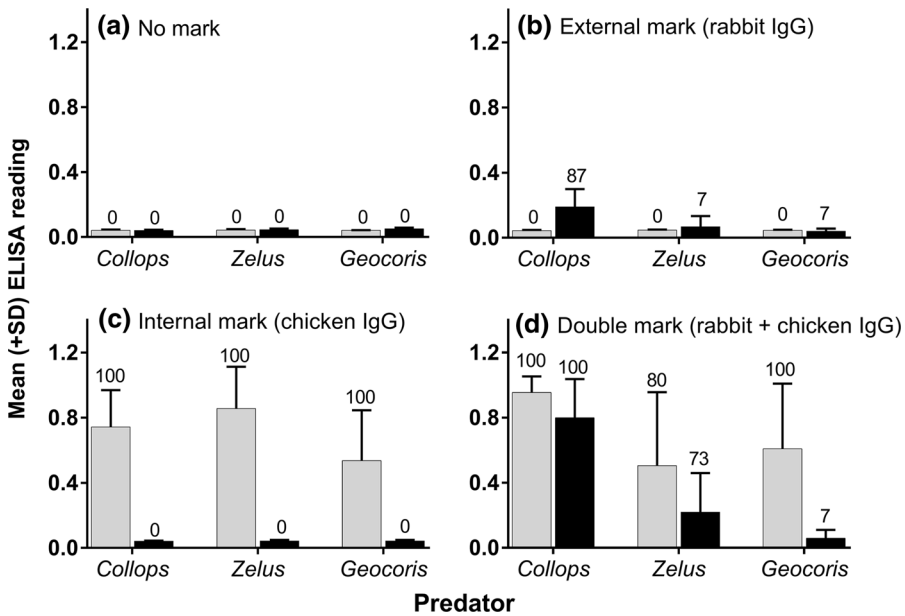

Fig. 2 Mean (+SD) ELISA readings and percentage of predator species scoring positive (% positive is given above the error bars) for the presence of rabbit IgG (black bars) and chicken IgG (grey bars) for the presence of protein-marked *Lygus hesperus* remains. Each predator consumed a prey item containing **a** no mark, **b** an external rabbit IgG mark, **c** an internal chicken IgG mark, and **d** an external rabbit IgG and an internal chicken IgG mark. Each predator (n = 15 per treatment) was analyzed by an anti-rabbit IgG (black bars) and anti-chicken IgG (grey bars) ELISA, respectively

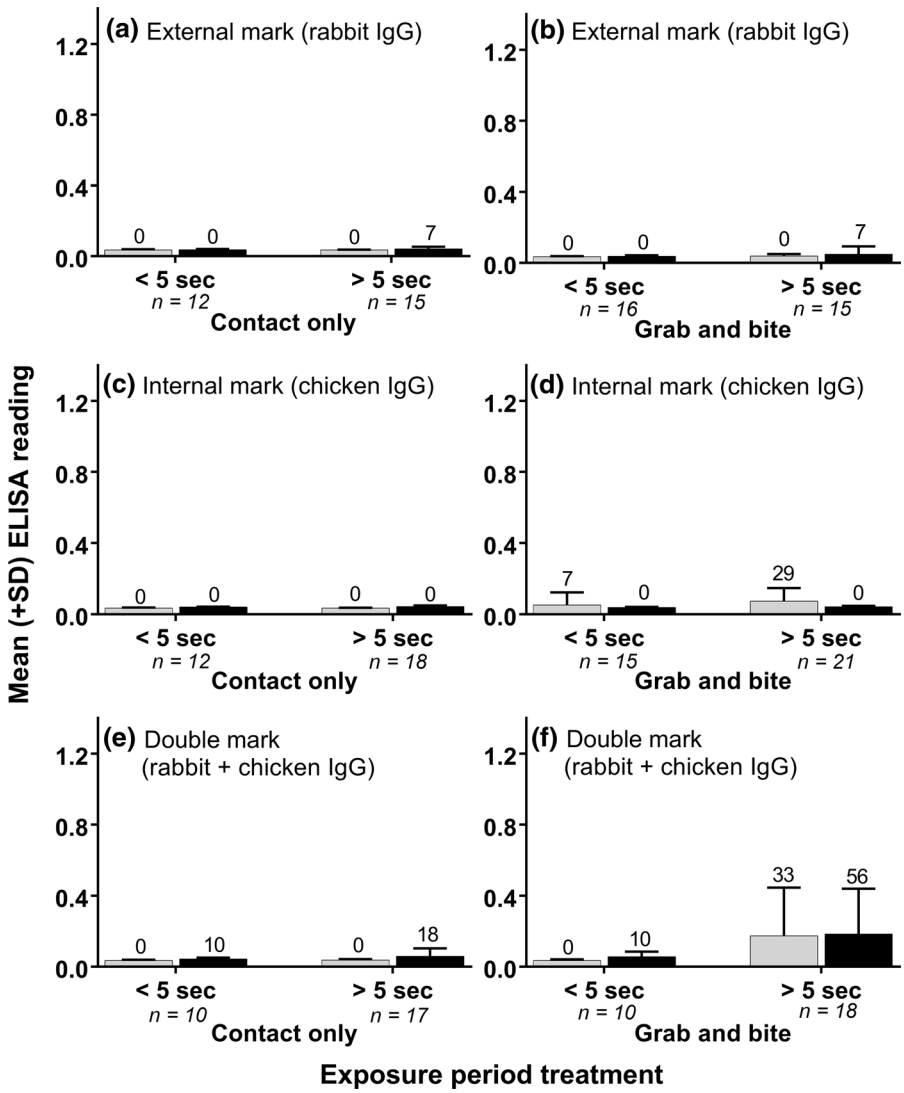

Fig. 3 Mean (+SD) ELISA readings and percentages of chewing predators scoring positive (% positive is given above the error bars) for the presence of rabbit IgG (black bars) and chicken IgG (grey bars) marked *Lygus hesperus* remains. Each chewing predator either **a** contacted (for < 5 or > 5 s) or **b** grabbed and bit (for < 5 or > 5 s) a prey item that was externally marked with rabbit IgG, **c** contacted or **d** grabbed and bit a prey item internally marked with chicken IgG, or **e** contacted or **f** grabbed and bit a prey item externally marked with rabbit IgG and internally marked with chicken IgG. Sample sizes are given below the x-axis for each exposure period treatment

obtained the internal mark from short predation attempts but six out of 18 individuals obtained the internal mark from long predation attempts (Fig. 3f).

Prey detection of an unsuccessful predation event for piercing-sucking predators

The frequency of lateral transfer of the external and internal protein marks to the piercing-sucking predators because of either contact only or an unsuccessful predation attempt (i.e., a false positive assay response) was much lower (3%, six out of 213 individuals) than that exhibited by the chewing predators (Fig. 4). The only treatment that yielded a relatively high frequency of false positive reactions (three out of 15 individuals) was for piercing-sucking predators that completed a long predation attempt on externally marked *L. hesperus* (Fig. 4b).

Discussion

This study was conducted to identify techniques to further improve and standardize the use of the generic PIP as a tool for predator gut analysis research. The obvious first step for conducting a successful PIP research project is to ensure that the targeted protein is administered successfully to the prey of interest. Our

research showed that the internal, external, and internal/external (double) mark application treatments were all effective means for tagging the third-instar *L. hesperus* prey items. This concurs with previous studies that employed a variation of the immunomarking procedure for tagging insects for dispersal research. Those studies showed that protein markers are persistent on the internal and external surfaces of a wide variety of insect taxa (Hagler et al. 1992, 2002, 2009; Hagler 1997; Hagler and Jackson 1998; Blackmer et al. 2004; Buczkowski and Bennett 2006; Jasrotia and Ben-Yakir 2006; Jones et al. 2006; Boina et al. 2009; Janke et al. 2008; Hagler and Jones 2010).

The second essential step for conducting a successful PIP gut analysis study is to ensure that the generic ELISA can faithfully detect the protein-tagged meal in the predator's gut. The proof-of-concept study describing the generic PIP showed that externally marked prey remains were detectable in the guts of 99% of the chewing type predators examined, but in only 30% of the piercing-sucking type predators (Hagler and Durand 1994). In an ensuing study, the generic ELISA was tested for efficacy at detecting protein-marked prey remains in large chewing type (earwig) and small piercing-sucking type (minute pirate bug) predators. For that study, large earwigs (*Labidura riparia* (Pallus)) were allowed to consume a relatively large pink bollworm (*P. gossypiella*) larva that was internally marked and minute pirate bugs (*Orius tristicolor* (White)) were allowed to consume a very small whitefly parasitoid (*Eretmocerus* sp.) that

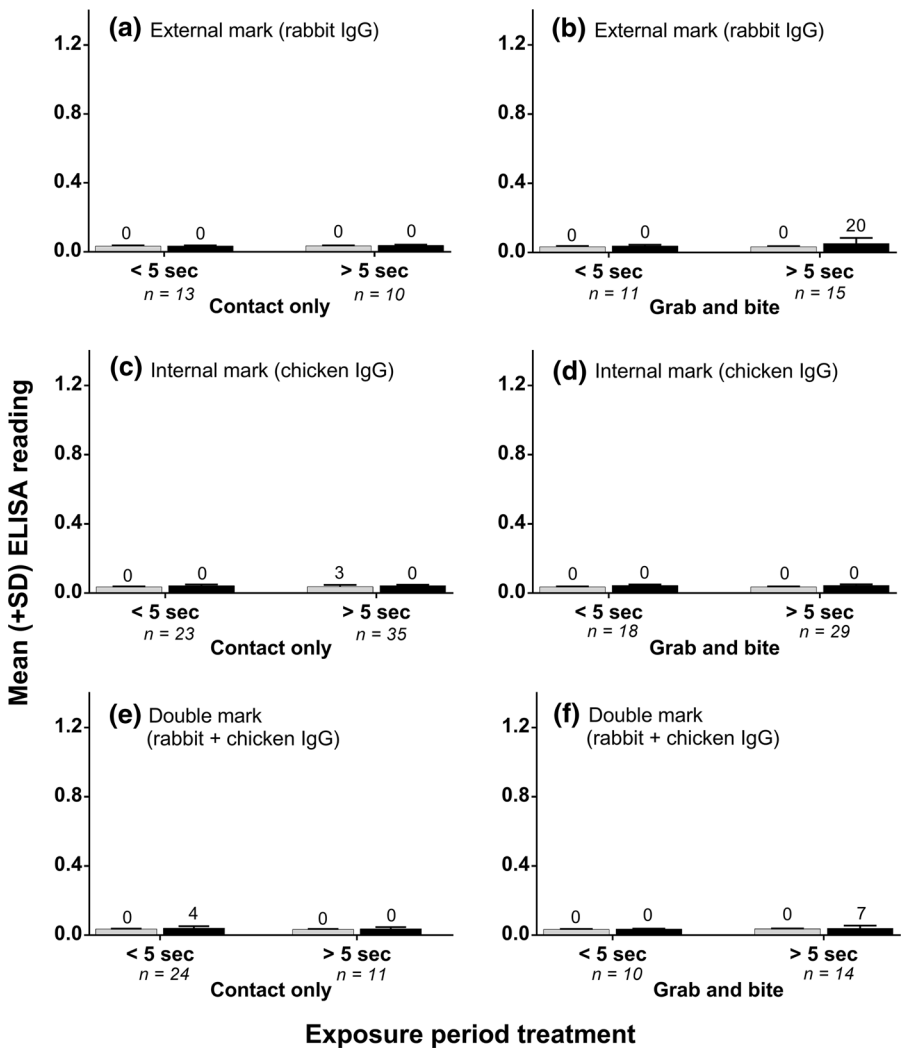

Fig. 4 Mean (+SD) ELISA readings and percentages of piercing-sucking predators scoring positive (% positive is given above the error bars) for the presence of rabbit IgG (black bars) and chicken IgG (grey bars) marked *Lygus hesperus* remains. Each piercing-sucking predator either **a** contacted (for < 5 or > 5 s) or **b** grabbed and bit (for < 5 or > 5 s) a prey item that was externally marked with rabbit IgG, **c** contacted or **d** grabbed and bit a prey item internally marked with chicken IgG, or **e** contacted or **f** grabbed and bit a prey item externally marked with rabbit IgG and internally marked with chicken IgG. Sample sizes are given below the x-axis for each exposure period treatment

was internally and externally (double) marked. The generic ELISA proved effective at detecting protein-marked prey remains in both types of predators for several hours after the meal (Hagler 2006). Subsequently, the PIP was refined to test whether the generic ELISA could detect double marked prey remains in both chewing and piercing-sucking predators. Overall, $\geq$ 90% of predators examined, regardless of their mouthpart morphology, tested positive by ELISA for up to a day after they fed on the protein-marked prey item (Hagler 2011). It can be surmised from these previous studies that the generic ELISA can reliably detect both internally or externally marked prey remains in the guts of chewing type predators, but an internal prey mark is needed for piercing-sucking type predators. The data yielded from the present study verified that the external only, internal only, and double prey marking treatments are highly effective for gut assay research dedicated to chewing type predators. However, the application of an internal mark to targeted prey items is essential for a successful PIP gut content evaluation of piercing-sucking predators.

Another essential step for conducting a successful predator gut analysis research project, which to date has been largely overlooked by the scientific community, is to ascertain the risk of obtaining a false positive gut analysis response (i.e., a type I statistical error). The fact that a field-collected predator sample tests positive for the presence of prey remains by any given gut content analysis procedure does not necessarily equate to a successful predator attack (i.e., death of the target species). In such a case, the predator would get falsely credited for providing a biological service to the agroecosystem. Perhaps the two most common types of false positive assay errors are a consequence of a secondary predation event or a scavenging event (Hagler and Naranjo 1996; Sheppard and Harwood 2005; King et al. 2008). A false positive secondary predation error (also known as a food chain error) can occur if a higher tiered predator feeds on a lower tiered predator that had previously attacked and consumed the prey of interest (typically a herbivore pest species). In such a case, the higher tiered predator would be erroneously credited for the biological control services rendered on the targeted prey. Limited research has shown that the prey-specific assay (e.g., PCR and ELISA) approaches and the PIP described here are vulnerable to yielding false positive food chain errors (Harwood et al. 2001; Sheppard et al. 2005; Hagler 2016). Furthermore, predators that engage in feeding on carrion (scavenging) are also prone to yielding false positive gut analysis errors. Unfortunately, the prey-specific assays are not able to differentiate between a scavenging (necrophagy) and true predation (viviphagy) event (Calder et al. 2005; Foltan et al. 2005). As such, data yielded from prey-specific gut analyses likely are overestimating the impact of an apparent predator for those predators that are facultative scavengers.

Recently, Zilnik and Hagler (2013) demonstrated that the PIP can be modified and used as a research tool to differentiate between necrophagy and viviphagy. Mansfield and Hagler (2016) then used the PIP to examine the gut contents of three "predator" species that were exposed to chicken IgG-marked live *L. hesperus* and rabbit IgG-marked *L. hesperus* cadavers using generic chicken IgG and rabbit IgG ELISAs, respectively. That study showed that necrophagy was much more prevalent than expected for all three predator species. Specifically, 75% of the positive "predator" gut assay responses yielded in that study were for the presence of the carrion prey items. These data were alarming in that previous research, using prey-specific ELISA and PCR gut assay methodology, indicted that some of these predators were key "predators" of *L. hesperus* (Hagler and Blackmer 2013). These data are a warning to predator gut analysis researchers, who use prey-specific assays, that scavenging activity might be more prevalent than expected and deserves more thorough investigation.

There is a third possible type of false positive assay error that can occur with the molecular gut analysis research approach that has been neglected in previous research. Specifically, it is conceivable that a gut content assay (prey-specific or generic) could yield a false positive reaction for a predation event as a consequence of the predator obtaining prey remnants (e.g., prey DNA, a prey protein, or an IgG mark) by incidental contact or a failed predation attempt in which the prey survived the attack (and thus in which the predator failed to provide biological control). Data revealed that there was a low frequency of false positive errors obtained by chewing and piercing-sucking type predators that only had incidental contact (brief and extended) with a protein-marked prey item. Moreover, a type I error in the case of an internal mark was observed occasionally from those piercing-

sucking predators that were permitted to grab and bite the protein-marked prey. However, the frequencies of false positive assay reactions yielded by chewing predators that were allowed to grab and bite their prey for an extended time (i.e., > 5 s) were unacceptably high (29–56%). Furthermore, the false-positive frequency for the external mark in piercing-sucking predators which grabbed and bit for > 5 s was 20% in the case of external-only prey and 7% in the case of dual marked prey. These findings show that the internal prey-marking method is clearly superior in terms of minimizing false positives based on contact or incomplete predation, especially when handling time exceeded 5 s. Whether those prey items that were grabbed and chewed for > 5 s would actually have been killed, had the two insects not been separated deliberately, is not known. The impact of unsuccessful predation attempts on subsequent survival of the affected prey warrants further investigation. Moreover, whether prey remnants acquired by predators through incidental contact or failed predation attempts are detectable by the conventional prey-specific assay methods are unknown and is also a topic in need of investigation.

The methods here provide further refinements to the PIP and generic ELISA approach for studying predator foraging behavior. The PIP and generic ELISAs have many features that make them practical tools for molecular gut content analysis research. First, the generic ELISAs have been standardized and are less expensive and better suited than the PCR assay for mass screening of field-collected predator specimens (Fournier et al. 2008). At the time of writing, we estimate that it cost about US $0.12 to analyze a single predator specimen (JRH pers. obs.). Moreover, we have optimized the procedure so that about 2000 predators can be analyzed per day so sample size is not constrained by the analysis process. Second, the generic PIP does not require the development of a prey-specific assay. As discussed above, this attribute gives researchers enormous flexibility in the type of study they conduct. That is, the generic PIP can be modified slightly to conduct studies of carnivory, omnivory, herbivory, or other feeding behaviors. Third, the sensitivity and reproducibility of the generic ELISA compare favorably to the prey-specific ELISA (Mansfield et al. 2008) and PCR gut assay approach (Hagler et al. 2015). Finally, the generic PIP has been proven effective for studying certain aspects of predation that are not achievable with prey-specific assays. For example, it has been used in combination with field cage inclusion and exclusion methods to identify the diel feeding patterns of predators (Hagler 2006), to quantify prey consumption (Hagler 2011), and, as mentioned above, to differentiate between active predation and scavenging behaviors (Mansfield and Hagler 2016). The data presented here further increase the credibility of using the PIP for predator gut analysis research.

In conclusion, this study and others show that the PIP (and the associated generic ELISAs) is a viable alternative to prey-specific assays for investigating food webs. We recommend that in most cases the targeted prey items be marked internally to maximize the likelihood of detecting prey remains in both chewing and piercing-sucking type predators while minimizing the risk of obtaining false positive assay errors as consequences of incidental contact or failed predation attempts. The application of an internal mark to the prey item can be achieved by simply augmenting their foodstuffs (e.g., artificial diet, natural diet, etc.) with the protein markers (Hagler and Jackson 1998; Hagler 2011). The general flexibility of the PIP in combination with the generic ELISAs are well-suited for further modifications and refinements for studying various aspects of predation that are unattainable (such as cannibalism; JRH in prep.) or too costly when using the prey-specific ELISA and PCR gut assay approaches.

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