

# A Gut Analysis Technique for Pinpointing Egg-Specific Predation Events

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## Abstract

A universal food immunomarking technique (UFIT) is described for postmortem gut analysis detection of predation on the egg stage of *Lygus hesperus* Knight (Hemiptera: Miridae). *Collops vittatus* Say (Coleoptera: Melyridae) and *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) were fed a single *L. hesperus* egg that was marked with rabbit and chicken sera proteins. The protein-marked egg remnants were detectable in the guts of the majority of the predators by each sera-specific enzyme-linked immunosorbent assay (ELISA) for 3 to 6 h after a feeding event. A novel technique was then developed to expose protein-marked eggs to predators that simulated the *L. hesperus* endophytic oviposition behavior. The procedure entailed embedding *L. hesperus* eggs in an artificial substrate that mimicked the stem of a plant. A predator feeding choice study was then conducted in cages that contained a cotton plant and artificial stems containing endophytic (concealed) and exophytic (exposed) egg patches. The endophytic and exophytic egg treatments were marked with chicken and rabbit protein, respectively. The gut analyses revealed that higher proportions of both predator populations contained remnants of the exophytic egg treatment and *L. hesperus* eggs were more vulnerable to *C. vittatus* than *H. convergens*. This study shows how the UFIT can be used to pinpoint stage-specific feeding activity on two distinct egg exposure treatments (endophytic and exophytic) of the same species.

**Key words:** immunomarking, enzyme-linked immunosorbent assay, western tarnished plant bug

The western tarnished plant bug, *Lygus hesperus* Knight (Hemiptera: Miridae), and other *Lygus* spp., are harmful to a wide variety of economically important crops (Scott 1977). The immature and adult stages can reduce crop yields or commercial values via fruit deformation or abscission by feeding preferentially on the fruiting structures of plants (Strong 1970, Kelton 1975, Swezey et al. 2007, Goodell 2009, Cooper and Spurgeon 2013). Future identification and then conservation of key predators of the *Lygus* spp. egg stage could help alleviate the destructive damage caused by the juvenile and adult life stages of this pest.

Many methods have been used to identify *Lygus* spp. predators. One such method is by postmortem gut content analyses (Greenstone 1996, Symondson 2002, Sheppard and Harwood 2005, King et al. 2008). The two most common postmortem gut assays include the prey-specific enzyme-linked immunosorbent assay (ELISA) and the prey-specific polymerase chain reaction (PCR) assay. Over a quarter of a century ago, an egg-specific monoclonal antibody (MAb)-based ELISA was developed to detect *L. hesperus* predation events (Hagler et al. 1991). That ELISA was effective at identifying *L. hesperus* egg (protein) remnants in heteropteran predators (Hagler et al. 1992a). However, the egg-specific ELISA was not useful for detecting

predation events on the juvenile and adult male life stages. A PCR assay was also developed to detect *L. hesperus*-specific DNA remnants in predators (Hagler and Blackmer 2013, Hagler et al. 2018). The PCR assay proved effective at detecting *Lygus*-specific predation events, but it was incapable of pinpointing stage-specific feeding events.

The universal food immunomarking technique (UFIT) is the third type of assay that is gaining popularity as a viable method for postmortem gut analysis. The procedure consists of simply tagging potential prey items with a unique protein biomarker before the study (Hagler and Durand 1994). The technique was recently coined as ‘universal’ because the same protein-specific ELISAs, developed over two decades ago, have been applied to study a wide variety of predator feeding activities (see Hagler 2019 for a review).

The goal of this study was to demonstrate how the UFIT can be used to pinpoint predation events specifically on the *L. hesperus* egg stage. Identifying egg-specific predation events is challenging because *Lygus* spp. display an endophytic oviposition behavior (Wheeler 2001). That is, a *Lygus* bug inserts her eggs deep into plant tissue. It has been suggested that this oviposition behavior serves to shield the eggs from natural enemies (Tallamy and Schaefer 1997,

Wheeler 2001). Here, we developed a UFIT designed to present protein-marked *L. hesperus* eggs to predators that mimic its endophytic oviposition behavior. We then conducted a proof-of-concept predator feeding choice study to validate the UFIT. Specifically, we provided cohorts of two predator species with a dual food choice consisting of either fully exposed (exophytic) or concealed (endophytic) *L. hesperus* egg treatments. The exposed and concealed egg treatments were marked with rabbit and chicken serum, respectively. The UFIT allowed us to simultaneously test our hypothesis that endophytic oviposition behavior protects eggs from predators. Ultimately, the methods described can be used in concert with field cage methods (Hagler 2011) to precisely identify indigenous predators of the *L. hesperus* egg stage.

## Materials and Methods

### Prey Detection Study

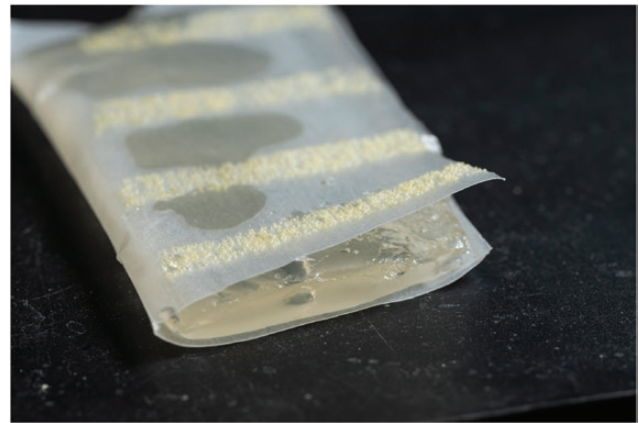
A prey detection study was conducted to determine the temporal effects of the UFIT. The goal was to determine how long the remnants of a single protein-marked *L. hesperus* egg could be reliably detected in predators after a feeding event. The predators examined included *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) and *Collops vittatus* Say (Coleoptera: Melyridae). These predators were selected for data presentation because they readily fed on fully exposed *L. hesperus* eggs in the prey detection study (note that focal piercing-sucking type predators were reluctant to feed on the eggs). The predators were collected the day before the experiments from alfalfa fields located at the University of Arizona, Maricopa Agricultural Center, Maricopa, Arizona, United States and starved overnight.

### Prey Marking Procedure

*Lygus hesperus* eggs were obtained from a laboratory colony reared on artificial diet (Debolt 1982) and fresh green beans. The rearing protocol consisted of providing gravid females with an oviposition substrate containing agarose gel enclosed in parafilm packets. This substrate serves to mimic plant tissue, which is essential for a successful *L. hesperus* oviposition event. Oviposition packets were prepared as described by Debolt (1982). Before exposure to the *L. hesperus* colony, the packets were masked with a plastic shield so that females could only deposit their eggs into 4.0-cm long by 0.4-cm wide strips of parafilm on each packet. The modified packets were then placed in the *L. hesperus* colony for 2–4 h. After eggs were laid, the plastic shield was removed from the parafilm, and the parafilm strip was cut with a clean razor blade and peeled away from the gelatinous agarose to expose the eggs (Fig. 1). The exposed eggs, while still embedded in the parafilm, were then marked with a dual protein marking solution consisting of a 1:1 mixture of pure rabbit serum (#16120099; Fisher Scientific, Gibco; Waltham, MA) and chicken serum (#C5405; Sigma Aldrich; St. Louis, MO). Each egg was topically marked by dipping an artist's #6 fine-tipped camel hair paintbrush into the marking solution and then lightly dabbing it with the brush. The protein-marked eggs were dried at room temperature for  $\geq 1$  h. After 1 h, the parafilm was stretched in multiple directions over wax paper to free the eggs from the parafilm.

### Monitoring a Predator Feeding Event

Approximately 20 protein-marked eggs were evenly distributed on the bottom of a 3.5-cm diameter Petri dish (feeding arena). An individual predator was then placed in a feeding arena and continuously observed until it devoured a single protein-marked egg or 15 min



**Fig. 1.** An agarose-filled packet showing *Lygus* eggs deposited in 4.0-cm long by 0.4-cm wide strips of parafilm. It should be noted that the egg densities in the photograph is much greater than the densities used in the predator feeding choice study to better highlight the eggs embedded in the parafilm.

had elapsed. Predators that consumed an egg were immediately removed from the feeding arena and either frozen (for the 0 h post-feeding treatment) or held at 27°C in individual Petri dishes (holding arenas) for 3 or 6 h. At the end of each post-feeding time interval, the predators were preserved by freezing at –80°C. Those predators that did not consume a protein-marked egg within the 15-min time allotment were also frozen at –80°C. These individuals served as the negative control predator treatment (note that these predators had frequent incidental contacts with the marked eggs). Two un eaten (protein-marked) eggs, serving, and the positive control egg treatment were randomly selected from each feeding arena for protein analysis. Finally, *L. hesperus* eggs serving as the unmarked negative control egg treatment were collected from the laboratory colony for protein analysis.

### Sample Preparation

Each predator and egg specimen was removed from the freezer, placed into a 1.6-ml microcentrifuge tube, and homogenized with a clean tissue grinder in 1,000- $\mu$ l of tris-buffered saline (TBS). The samples were analyzed for the presence of rabbit and chicken sera marks by the protein-specific ELISAs described below.

### Feeding Choice Study

A predator choice study was conducted that consisted of two distinct *L. hesperus* egg treatments. The prey choices included ‘concealed’ eggs marked with chicken serum and ‘exposed’ eggs marked with rabbit serum. The concealed egg treatment served to mimic *Lygus* bug’s instinctive (endophytic) oviposition behavior. The exposed egg treatment (exophytic) served as a comparative sham control treatment. That is, the sham eggs were fully exposed to the environment and to the foraging predator population.

### Preparation of the Protein-Marked Egg Sachets

Patches (ca. 100 eggs per patch) of *Lygus* eggs were obtained from the 4.0-cm long by 0.4-cm wide parafilm strips as described above and shown in Fig. 1. The eggs designated for the exposed egg treatment and the concealed egg treatment were distinctly marked (as described above) with either pure rabbit serum or chicken serum, respectively. The rabbit-marked eggs serving as the exposed egg treatment (exophytic) were wrapped around a wood dowel so that the eggs embedded in the parafilm were facing outward (Fig. 2A). The

chicken-marked eggs embedded in parafilm were wrapped around a wooden dowel with the eggs facing inward to simulate an endophytic oviposition pattern (Fig. 2B). The square dowel was 1.27-cm wide on each side and 15.24-cm long. Each dowel had two, 0.32-cm wide grooves that were also 0.32-cm deep, cut around its circumference (Fig. 2). The grooves were located 5.08-cm from the top and bottom of each dowel (i.e., the upper third and lower third of each dowel). The grooves served to keep the concealed eggs intact while embedded in the artificial stem.

#### Caged Feeding Arena

The study consisted of 20 caged feeding tests (experimental units). Cotton plants ('Delta Pine 5415') were grown in 15.24-cm diameter pots in a climate-controlled greenhouse (32°C, 30% RH). A single cotton plant, approximately 60-cm tall, was placed in a screen cage. The screen consisted of an 18.9-liter plastic bucket paint strainer (SuperTuff Elastic-Top Strainer, Morrisville, NC) purchased from a local hardware store. One to three (depending on predator availability) cages (experimental units) were erected each day at approximately 10:00 a.m. in the greenhouse. Two artificial cotton stems, as described above and in Fig. 2, were inserted into the potting soil so they were adjacent and parallel to the main cotton stem. Each stem contained an exposed and concealed *L. hesperus* egg patch treatment. Again, the egg patch treatments were located one-third from the top and one-third from the bottom of each dowel, respectively. The two dowels in each cage were arranged so that a concealed egg patch was on the upper part of one stem and the lower part of the other stem. By default, the exposed egg patch treatments were vice versa on each artificial stem. Adult *C. vittatus* and *H. convergens*

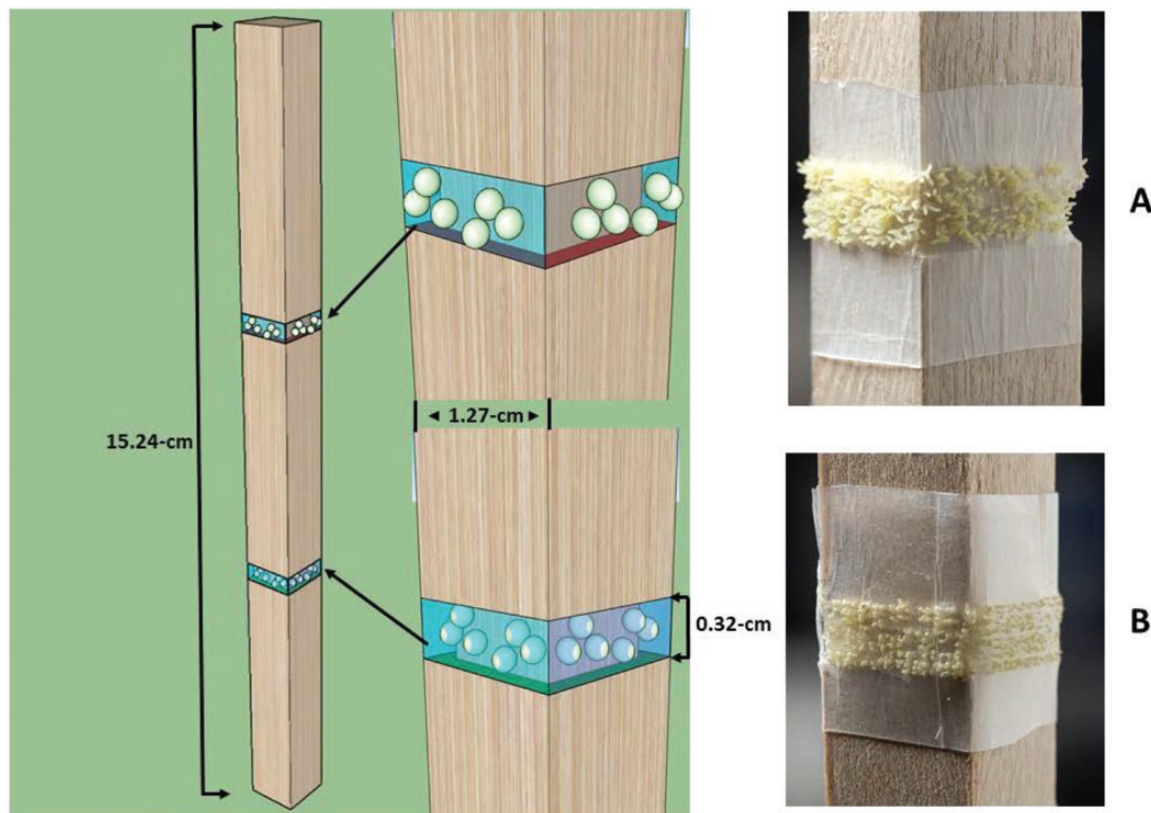
( $n = 20$  of each species) were released into each cage. The predators were allowed to roam freely within the arenas for 4 h. After 4 h, the entire contents of each cage were snap-frozen at  $-80^{\circ}\text{C}$  to preserve the predators and protein-marked eggs for protein analysis by ELISA.

#### Sample Preparation

Predators from each experimental unit (cage) were removed from the freezer, placed into individual 1.6-ml microcentrifuge tubes, and homogenized in 1,000- $\mu\text{l}$  of TBS. Also, two to three eggs from each parafilm sachet were randomly selected for protein analysis. These samples served to determine the fidelity of the prey marking procedure over the 4 h duration of the study. Each predator and egg specimen was analyzed for the presence of the rabbit and chicken sera marks by the protein-specific ELISAs described below.

#### Gut Content ELISAs

The anti-rabbit IgG and chicken IgG ELISAs described originally by Hagler and Durand (1994) and Hagler (1997) were used to analyze the predator and egg samples for the presence of protein-marked egg remains. These ELISAs were initially developed to detect the highly purified and expensive rabbit and chicken IgG proteins, respectively. However, recent research has shown that the ELISAs are equally effective at detecting crude and inexpensive whole rabbit and chicken sera (J.R.H., personal observations). A predator or *L. hesperus* egg sample was scored positive for the presence of the respective protein-marked egg treatment if its ELISA value was greater than the mean absorbance + 6 SD of the negative control treatment.



**Fig. 2.** Schematic diagram of the 15.2-cm long wood dowel containing an (A) exophytic (exposed) and (B) and endophytic (concealed) patch of protein-marked *Lygus* eggs. It should be noted that the egg densities in the photographs are much greater than the densities used in the predator feeding choice study to better highlight the egg exposure treatments.

## Statistical Analysis

The proportion of each predator population ( $n = 20$  individuals per species) in each experimental unit ( $n = 20$  cages) scoring positive for protein-marked egg remnants was determined. A one-way analysis variance (ANOVA) on non-transformed data was used to test for significant differences in the feeding frequencies yielded by each predator species on each prey type (JMP Ver. 12.2, SAS Institute Inc., Cary, NC). The Tukey's mean separation test was used to identify significant differences between predator feeding frequency on each egg treatment.

## Results

### Prey Detection Study

#### Fidelity of the Prey Marking Procedure

The method used to topically apply the two marks onto the eggs was very effective. Both protein-specific marks were detected on 61 of the 62 (98.4%) individual eggs examined (Fig. 3). Only one egg specimen failed to yield a positive ELISA response for the presence of either marker. This is probably due to a human error during the marking process.

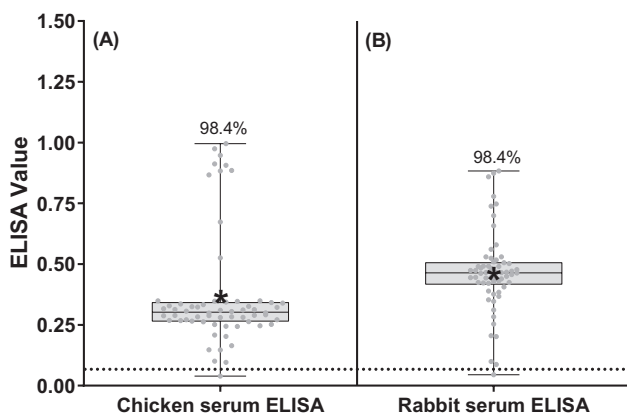
#### Detection of a Protein-Marked Egg in Predators

##### *Hippodamia convergens*.

None of the negative control *H. convergens* exposed to protein-marked eggs in the feeding arenas responded to either protein-specific ELISA (Fig. 4A and B). The remnants of a single chicken and rabbit sera-marked *L. hesperus* egg were detected in every *H. convergens* examined immediately after a feeding event. In general, about 75% of the *H. convergens* examined up to 6 h after feeding yielded a positive ELISA reaction for the presence of protein-marked egg remnants.

##### *Collops vittatus*.

None of the negative control *C. vittatus* exposed to protein-marked eggs in the feeding arenas responded to either protein-specific ELISA (Fig. 5A and B). The remnants of a single *L. hesperus* egg marked



**Fig. 3.** Distribution of the ELISA values yielded by every egg specimen (gray dots,  $n = 62$ ) examined for the presence of (A) chicken serum and (B) rabbit serum. The percentage of positive ELISA reactions yielded for each protein mark is given above the boxplots. The upper and lower whiskers depict the extreme ELISA values yielded for each treatment. The median and mean ELISA readings are depicted by the solid horizontal line (in each gray box) and asterisk in each plot, respectively. The dotted horizontal line is the critical ELISA threshold value used to score the egg samples for the presence of each protein mark.

with chicken and rabbit sera was detected in 100 and 80% of the *C. vittatus* examined at 0 and 3 h after a feeding event, respectively (Fig. 5A and B). However, chicken and rabbit sera-marked egg remnants were detected in only 20 and 7% of the *C. vittatus* examined 6 h after a feeding event, respectively.

### Feeding Choice Study

#### Fidelity of the Protein-Marked Eggs

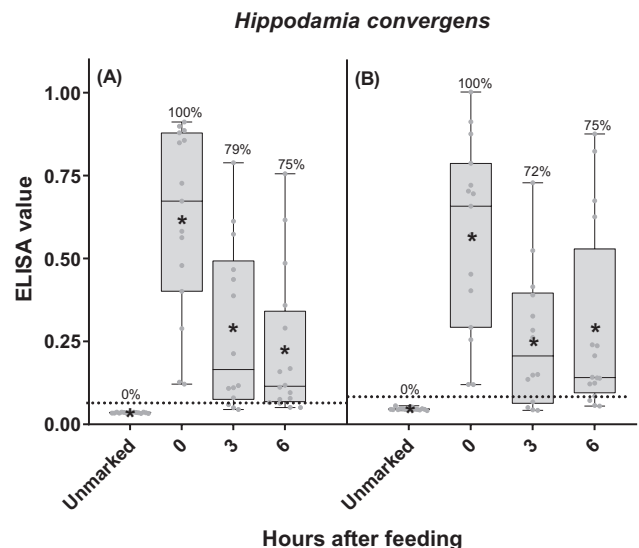
Both protein marks were well retained on the *Lygus* eggs examined at the end of the 4-h feeding study. The marks were detected 98 (e.g., there was only one false positive ELISA reaction) and 100% of the chicken and rabbit sera-marked eggs, respectively (Fig. 6). Moreover, none of the egg samples yielded a false positive ELISA reaction. That is, none of the rabbit serum-marked eggs reacted to the anti-chicken ELISA and vice versa.

#### Predator Feeding Choice

The gut assay results yielded by the predators collected at the end of the caged feeding studies revealed that a higher proportion of both predator populations contained egg remnants of the exposed egg treatment. On average, 12.4 and 1.8% of the *H. convergens* and 81.5 and 27.6% of the *C. vittatus* contained remnants of exposed (rabbit sera) and concealed (chicken sera) eggs, respectively (Fig. 7). These data also showed that that *L. hesperus* eggs, regardless of the exposure treatment, were more vulnerable to *C. vittatus* compared with *H. convergens* (Fig. 7).

## Discussion

The versatility of the UFIT is just one of the major factors that make it a unique tool for postmortem predator gut analysis. It has been used, usually in concert with field cage methodology, to study various



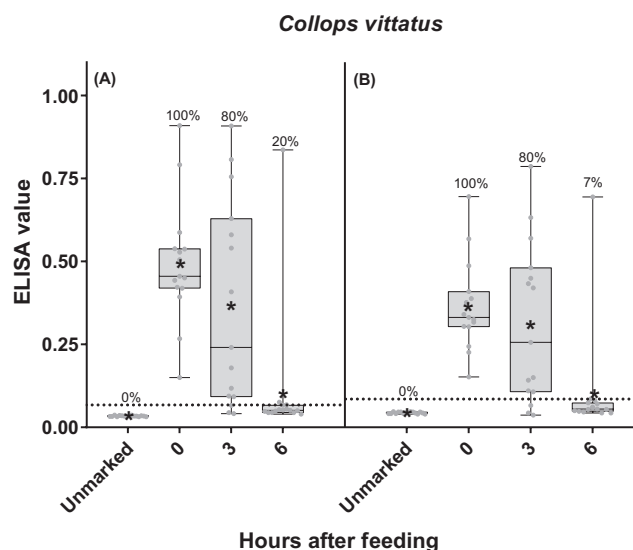
**Fig. 4.** Distribution of the ELISA values yielded by every *Hippodamia convergens* specimen (gray dots,  $n = 14$  to 16 per time interval) examined for the presence of (A) chicken serum and (B) rabbit serum egg remnants. The percentage of positive ELISA reactions is given above the boxplots. The upper and lower whiskers depict the extreme ELISA values yielded for each treatment. The median and mean ELISA readings are represented by the solid horizontal line (in each gray box) and asterisk in each boxplot, respectively. The dotted horizontal line shows the critical ELISA threshold value used to score the predators for the presence of protein-mark egg remnants.

aspects of arthropod carnivory (including scavenging, trophic level interactions, etc.), omnivory, and herbivory (Hagler 2006, Lundgren et al. 2013, Mansfield and Hagler 2016, Blubaugh et al. 2016). The UFIT has also been used, in manipulated field studies (i.e., field cages), to quantify predation rates on individually marked *L. hesperus* nymphs (Hagler 2011). Moreover, the UFIT has been adapted to pinpoint predation events on every insect life stage in both manipulated and open field settings (Hagler and Durand 1994; Hagler

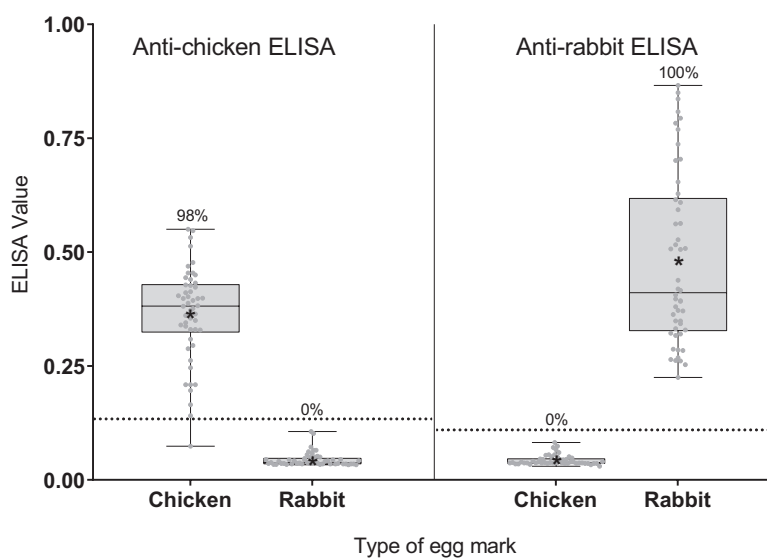
2006, 2011; Mansfield et al. 2008; Zilnik et al. 2013; Kelly et al. 2014; Blubaugh et al. 2016; Rendon et al. 2018).

The present study demonstrates how the UFIT can be modified to study egg-specific predation events on an insect that displays a complex endophytic oviposition behavior. First, we showed that *L. hesperus* eggs can be marked by simply applying a dab of protein solution onto the surface of an egg. External protein marks have proven effective for tagging prey items in other UFIT studies (Hagler 2019). Second, we determined that the remnants of a single protein-marked egg were detectable by protein-specific ELISAs in most predators examined up to 3 h after a feeding event. This prey detection interval is comparable to other postmortem gut assay studies employing UFIT, prey-specific ELISA, and prey-specific PCR gut analysis approaches (Hagler and Durand 1994, Hagler 1998, Harwood et al. 2007, McMillan et al. 2007, Fournier et al. 2008, Kuusk et al. 2008, Eskelson et al. 2011, Mansfield and Hagler 2016). The results from the prey retention study provide a time frame of effectiveness (ca. 3 h) for future studies that employ this technique. Third, we described a novel method to present *L. hesperus* eggs to predators that simulate its natural (endophytic) oviposition pattern. This is a critical requirement for conducting meaningful studies of egg predation in the future. Finally, we conducted a proof-of-concept feeding study using the simulated oviposition sachets. The use of two distinct protein marks allowed us to distinguish between predation events on two different egg treatments. The eggs were either fully exposed (exophytic) to, or concealed (endophytic) from, the predators. The feeding bioassay revealed that the endophytic oviposition treatment yielded a lower frequency of predator attack by both predator species. These data support the hypothesis that endophytic oviposition behavior serves to protect insect eggs from natural enemies (Tallamy and Schaefer 1997, Wheeler 2001). The bioassay also revealed that *L. hesperus* eggs, regardless of the exposure treatment, were more vulnerable to *C. vittatus* than *H. convergens*.

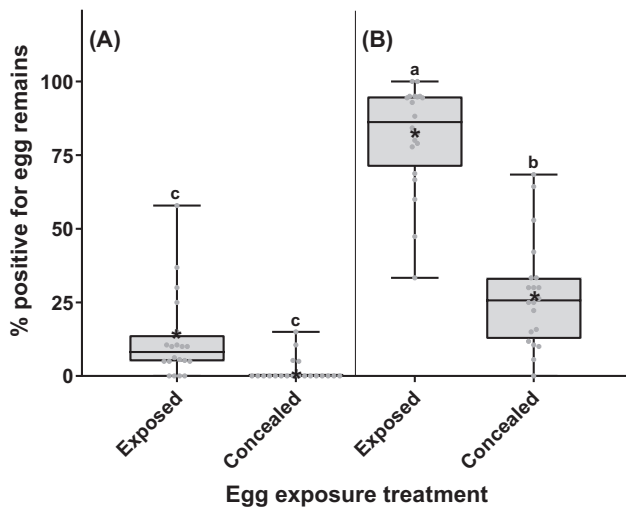
The UFIT is a powerful tool that can be used to complement other types of predator assessment. We plan to use the UFIT described here in concert with the field cage method described by



**Fig. 5.** Distribution of the ELISA values yielded by every *Collops vittatus* specimen (gray dots,  $n = 15$  per time interval) examined for the presence of (A) chicken serum and (B) and rabbit serum egg remnants. The percentage of positive ELISA reactions is given above the boxplots. The upper and lower whiskers depict the extreme ELISA values yielded for each treatment. The median and mean ELISA readings are represented by the solid horizontal line (in each gray box) and asterisk in each boxplot, respectively. The dotted horizontal line shows the critical ELISA threshold value used to score the predators for the presence of protein-mark egg remnants.



**Fig. 6.** Distribution of the ELISA values yielded by the uneaten protein-marked egg specimens (gray dots,  $n = 50$  per mark) examined for the presence of (A) chicken serum and (B) and rabbit serum. The percentage of positive ELISA reactions is given above the boxplots. The upper and lower whiskers depict the extreme ELISA values for each treatment. The median and mean ELISA readings are represented by the solid horizontal line (in each gray box) and asterisk in each boxplot, respectively. The dotted horizontal line shows the critical ELISA threshold value used to score the egg samples for the presence the protein marks.



**Fig. 7.** Frequency distribution ELISA reactions (based on 20 predators per gray dot) yielded by (A) *Hippodamia convergens* and (B) *Collops vittatus* (dots,  $n = 20$  experimental units) scoring positive for the presence of exposed and concealed *Lygus* eggs. The upper and lower whiskers depict the extreme frequencies of positive ELISA reactions for each egg exposure treatment. The median and mean frequencies are represented by the solid horizontal line (in each gray box) and asterisk in each boxplot, respectively. Different lowercase letters indicate significant differences in the frequency of feeding responses yielded by each predator species for each egg treatment as determined by the Tukey multiple comparison test ( $P < 0.05$ ).

Hagler (2011) to identify indigenous predators of the *L. hesperus* egg stage. The whole plant cage is designed to be pulled over an entire plant and secured in a matter of seconds. The speed of the caging process ensures that the native arthropod fauna on the plant is enclosed within the cage. Then, the protein-marked prey can be introduced into the cage (experimental unit) for a fixed amount of time. A key feature of the caging procedure is that the entire contents of each cage can be rapidly collected and immediately frozen to preserve the predator specimens for counting and gut content analysis at a later time. Previous work has shown that it is possible to obtain hundreds of experimental units for any given study (Hagler 2011).

Along with its versatility, the UFIT has many other attributes that make it a practical tool for postmortem gut analysis research. First, two protein-specific assays (rabbit and chicken sera-specific ELISAs) have already been developed and optimized (Hagler et al. 1992b; Hagler 1997, 2019). As such, no assay development is required. Second, the detection of prey in predators by ELISA is cost-effective (<US\$ 0.12 per sample) and well suited for mass throughput (e.g., >2,000 samples per day) (Hagler and Machtley 2016, Hagler 2019). Third, studies have shown that the UFIT sandwich ELISA compares well in dependability to prey-specific indirect ELISA and PCR assays. Specifically, the sandwich ELISA format is less prone to yielding false negative assay responses (Hagler 1998, Mansfield et al. 2008, Hagler et al. 2015).

In summary, we described a UFIT method that can be used to pinpoint predation events on the egg stage of an insect pest that displays an endophytic oviposition behavior. The flexibility of the UFIT also allowed us to compare predator activity on *L. hesperus* eggs deposited naturally (endophytic) to a sham control egg treatment (i.e., fully exposed eggs). The data supported our hypothesis that endophytic oviposition behavior protects eggs from predators. Moreover, the UFIT yielded data that indicates that *L. hesperus* eggs are more vulnerable to *C. vittatus* than *H. convergens*. The UFIT can

be used to study a wide range of predator feeding behaviors (Hagler 2019), many of which are not possible using the prey-specific PCR assay approach. We plan to apply this method, in concert with field cage methods, to identify key predators of the *L. hesperus* egg stage in various agroecosystems.

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## Disclaimer

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