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A procedure for pinpointing cannibalism, intraguild predation, and life stage-specific feeding events

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Abstract A predator gut analysis technique is described that can simultaneously pinpoint predation events which are life stage-specific, intraspecies-specific (cannibalism) and interspecies-specific (intraguild). The third and fifth larval life stages of green lacewing, *Chrysoperla carnea* Stephens *s.l.* (Neuroptera: Chrysopidae), were marked with rabbit IgG and chicken IgY, respectively. The uniquely marked lacewing life stages were then introduced into caged arenas (n = 59 caged experimental units) containing a cotton plant and an assemblage of generalist predators. The predators released into each arena were recaptured after 6 h and their gut contents were examined for the presence of rabbit IgG- and chicken IgY-marked lacewing remnants by an anti-rabbit and anti-chicken enzyme-linked immunosorbent assay (ELISA), respectively. The predator gut ELISAs detected one cannibalism event and 14 and 8 intraguild predation (IGP) events on third and fifth instar lacewings, respectively. This proof-of-concept study shows that this universal prey immunomarking technique (UFIT), when combined with field cage methods, can be useful for pinpointing cannibalism and life stage-specific predation events.

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Introduction

The molecular identification of prey selection by predators has relied almost exclusively on the use of prey-specific polymerase chain reaction (PCR) technology for two decades (Agustí et al. 1999, 2003; Sheppard and Harwood 2005; Gariépy et al. 2007; King et al. 2008; Hagler and Blackmer 2015; Hagler et al. 2018a). The species-specific PCR approach is useful for qualitative evaluations of predation. However, the approach is not able to distinguish between scavenging and predation, cannot be used to detect intraspecies predation (cannibalism), and cannot pinpoint the life stage preyed upon.

The universal food immunomarking technique (UFIT) is an alternative gut content analysis procedure that can be adapted to study a wide variety of feeding behaviors (Hagler and Durand 1994; Hagler 2019). The procedure requires marking potential prey items (or any food item) with a unique protein and then exposing them to a community of predators for a given amount of time. After exposure, the gut contents of the predators can be examined for the presence of protein-marked prey remnants using a well-established ELISA(s). Recently, the UFIT was adapted to measure

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the proclivity of generalist predators engaging in scavenging versus vivaphagy (Zilnik and Hagler 2013; Mansfield and Hagler 2016). Blubaugh et al. (2016) modified the UFIT to distinguish between omnivorous beetles' feeding activity on rabbit IgG-marked weed seeds and chicken IgY-marked pupal prey. Most recently the UFIT was adapted to pinpoint predation events specifically on the eggs of *Lygus hesperus* (Hagler and Mostafa 2019) and on 5th instar larvae of *Helicoverpa* spp. (Rendon et al. 2018).

The present study describes yet another use for the UFIT. Here, we demonstrate how the UFIT can be modified to measure the penchant for predators to engage in intraspecies (cannibalism) and interspecies (intraguild predation [IGP]) predation on protein-marked *Chrysoperla carnea* (Stephens) *s.l.* (Neuroptera: Chrysopidae) larvae, as well as to distinguish between predation on various life stages of the targeted prey. *Chrysoperla carnea* was targeted for investigation because it is a known cannibal and is vulnerable to IGP by higher-order predators (Duelli 1981; Rojht et al. 2009; Noppe et al. 2012; Hagler and Blackmer 2015).

Materials and methods

Prey marking procedures

The targeted prey consisted of third and fifth instar *C. carnea* larvae that were internally and externally marked with rabbit IgG and chicken IgY, respectively. The larvae used in the feeding arenas were obtained from a laboratory colony reared on an artificial diet described by Ridgway et al. (1970).

Internal marking procedure

Chicken egg is an ingredient of the *C. carnea* artificial diet described by Ridgway et al. (1970). As such, *C. carnea* larvae feeding on this diet reliably obtain a chicken IgY protein mark. In addition, the artificial diet was supplemented with 10 mg of chicken IgY (Sigma Chemical Co., No. I-4881, St. Louis, MO, USA) per 1.0 g of diet to further ensure an adequate amount of internal mark was obtained by the larvae. Cohorts of *C. carnea* were allowed to internally self-mark with chicken IgY by feeding ad libitum on diet as hatchlings until they reached their fifth instar life cycle. Similarly, cohorts of third instar *C. carnea* larvae were internally self-marked by allowing them to feed on a modified version of the artificial diet in which the chicken egg was removed from the recipe, and 10 mg of rabbit IgG (Sigma Chemical Co., St Louis, MO, USA, No. I-8140) per 1.0 g of diet was added.

External marking procedure

One hour before the onset of the feeding tests, the internally-marked third and fifth instar C. *carnea* larvae were immobilized by chilling at 4 °C for approximately 5 min. An individual was then placed on a clean paper towel, and a 10 µl aliquot of a 1.0 mg ml^{-1} rabbit IgG or chicken IgY was topically administered to third and fifth instar larva, respectively. The dual-marked lacewings were dried for at least 20 min prior to release into a feeding arena (see below).

Predator assemblage

The predator assemblage consisted of species commonly encountered in arid-land cotton and alfalfa fields. The species selected for evaluation are regarded as generalist predators and omnivores, many of which are known to prey on *C. carnea* (Hagler and Blackmer 2013, 2015). A listing of the predators introduced into each feeding arena (experimental unit) is given in Table 1. These predators (except for *C. carnea*; see above) were collected from alfalfa and cotton fields located at the University of Arizona's Maricopa Agricultural Research Center (Maricopa, Arizona, USA) the day before each feeding trial. They were isolated in individual Petri dishes and starved overnight. In general, a single individual of each taxon listed in Table 1 was introduced into each of the 59 feeding arenas. Unfortunately, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) and *Sinea confusa* Caudell (Hemiptera: Reduviidae) were rarely encountered in the cotton and alfalfa fields late in the growing season (August). As such, some of the experimental units conducted in August did not contain an *H. convergens* and/or a *S. confusa* specimen. Instead, the predator complex was supplemented with a second *Collops vittatus* (Say) (Coleoptera:

Table 1 A listing of the members of the arthropod assemblage introduced into the feeding arenas

Order	Family	Taxon	Common Name	Lifestage[a]	Mouthpart type	Classification[b]
Neuroptera	Chrysopidae	*Chrysoperla carnea*	Green lacewing	3rd instar 5th instar	Piercing and sucking	Carnivorous
Hemiptera	Geocoridae	*Geocoris punctipes*	Big-eyed bug	Adult	Piercing and sucking	Omnivorous predator
	Miridae	*Lygus hesperus*	Lygus bug	Adult	Piercing and sucking	Omnivorous pest
	Nabidae	*Nabis alternatus*	Damsel bug	Adult	Piercing and sucking	Omnivorous predator
	Reduviidae	*Sinea confusa*	Assassin bug	Adult	Piercing and sucking	Carnivorous
		Zelus renardii	Assassin bug	Adult	Piercing and sucking	Carnivorous
Coleoptera	Melyridae	*Collops vittatus*	Flower beetle	Adult	Chewing	Carnivorous
	Coccinelidae	*Hippodamia convergens*	Lady beetle	Adult	Chewing	Carnivorous
Araneae	Thomisidae	*Misumenops celer*	Crab spider	Adult	Piercing and sucking	Carnivorous

[a]3rd instar and 5th instar *C. carnea* were marked with rabbit IgG and chicken IgY, respectively

[b]The primary feeding niche of each species

Melyridae) to compensate for *H. convergens* and *Zelus renardii* Kolenati (Hemiptera: Reduviidae) for *S. confusa* (see the footnote in Table 2).

It should be noted that the potted cotton plants enclosed in each feeding arena (cage) were infested with whiteflies (*Bemisia tabaci* (Gennadius); Homoptera: Aleyrodidae). Undoubtedly, these pests also served as a potential food source for the predator complex. The density of adult whiteflies (22.7 ± 34.1; $n = 59$) on each plant was estimated 30 min before each feeding study using the leaf turn method described by Naranjo and Flint (1995). Whitefly egg and nymph densities were estimated after the completion of the feeding experiments by the leaf disc method described by Naranjo and Flint (1994). The whitefly egg and nymph densities were 147.0 (± 156.0) and 54.6 (± 64.2) per leaf disc ($n = 59$), respectively.

Caged feeding arenas (experimental units)

Feeding arenas were constructed as needed when the various members of the targeted predator complex became available in the nearby cotton and alfalfa fields from mid-June through mid-August 2015. Depending on the availability of field-collected predators, two to five arenas were erected each day. A predator feeding arena consisted of a single cotton plant (Bayer Crop Science ST5458B2F) placed inside a 30 × 30 × 30 cm^3 cage (BugDorm1, MegaView Science Co Ltd., Taichung, Taiwan) in a climate-controlled greenhouse (maximum temperature was set at 30 °C, RH at 20%). Each cotton plant was grown in the greenhouse in a 10.5 × 10.5 cm^2 wide and 13.0 cm deep pot that contained a standard soil mixture. The plants enclosed in each arena were selected to be as tall as possible without touching the top or sides of the cage. However, in some cases, the plants were slightly bent to fit inside a cage. Glad Press'n Seal Wrap® (Amherst, Virginia, USA) was wrapped across the top of the potted plant, over the soil surface and around the plant stem to reduce the possibility of the predators burrowing into the soil.

One rabbit IgG-marked third instar and one chicken IgY-marked fifth instar *C. carnea* was released into each of the 59 feeding arena by placing them directly onto the plant on opposite leaves at mid-plant height with clean forceps. The protein-marked lacewings were given 15 min to settle on the plant before exposing them to the predators. In general (see footnote in Table 2), one individual of each predator species listed in Table 1 was placed in each of the 59 feeding arenas. The feeding arenas were sealed, and the predators were allowed to roam freely within each cage for 6 h. This time frame was chosen because previous prey-retention studies have shown that a protein-marked lacewing can be detected in a predator's gut for about 6 h after being consumed (Zilnik and Hagler 2013). After 6 h, each feeding arena was transported to a walk-in refrigerator (4 °C), to slow

down the metabolism of the predators, and was thoroughly searched for predators. Upon collection, the predators were counted and scored as alive, dead, or missing. Each predator was collected with forceps that were cleaned with 70% ethanol between each collection, placed into 1.5 ml sample vial, and immediately preserved by freezing at − 80 °C.

Detection of protein-marked prey remains

Sample preparation

The predator samples were removed from the freezer and 1.0 ml of tris-buffered saline (TBS) was added to each vial. Each predator sample was thoroughly homogenized with a clean tissue grinder to expose its gut contents to the buffer. Each predator sample was then examined for presence of protein-marked *C. carnea* remains (third and fifth instars) by the anti-rabbit IgG and anti-chicken IgY-specific sandwich ELISAs described by Hagler (2006). A 100-µl aliquot of sample buffer was used for each ELISA.

Negative control lacewings

Unmarked lacewing larvae were analyzed to establish the level of background noise associated with each protein-specific ELISA. The unmarked negative control specimens were collected from the cotton and alfalfa fields described above. The mean (± SD) ELISA values yielded by the unmarked specimens were calculated for each protein-specific assay. A predator sample was scored positive for the presence of protein-marked *C. carnea* remains if it yielded an ELISA value greater than the critical threshold value (CTV), defined as the mean absorbance value + 6 SD of the negative control lacewings (Boyle et al. 2018; Hagler 2019).

Data analysis

Predator counts were tabulated based on the number living, dead, and missing specimens in each experimental unit ($n = 59$). All the living and dead predator specimens collected after each feeding trial were analyzed for the presence of rabbit IgG- and chicken IgY-marked *C. carnea* remains. The rationale for assaying the dead predator specimens was based on the possibility that they could have consumed a protein-marked lacewing before their demise. It should be noted that none of the dead predators yielded a positive ELISA reaction for the presence of protein-marked lacewing remains. Therefore, only the ELISA data obtained for the living predator specimens is presented. Box-whisker plots were constructed that depict the ELISA results obtained for every surviving predator.

Results

Predator counts

The fate of the various generalist predator taxa placed into the caged feeding arenas is given in Table 2. The fate of the missing individuals could be attributed to: (1) failure to find the specimens at the end of the feeding trial due to observer error, (2) escape from the caged arenas, or (3) being totally devoured by a chewing type predator. The fate of the deceased individuals could be attributed to death by (1) natural causes or (2) being killed by a piercing and sucking predator, or being killed, but not devoured, by a chewing-type predator. Third instar *C. carnea* had the lowest survival rate (47.5%) and percentage of individuals missing (27.1%). The omnivorous pest *L.*

Table 2 Fate of the various arthropod species introduced into the feeding arenas. Note that, in general, a single specimen of each species was introduced into each of the 59 feeding cage arenas

Species	Lifestage	n[a]	Survived	Dead	Missing
C. carnea	3rd instar	59	28	15	16
C. carnea	5th instar	59	40	13	6
L. hesperus	Adult	59	34	18	7
G. punctipes	Adult	59	48	3	8
N. alternatus	Adult	59	38	20	1
Z. renardii	Adult	72	69	3	0
S. confusa	Adult	46	43	2	1
C. vittatus	Adult	65	58	7	0
H. convergens	Adult	53	49	2	2
M. celer	Adult	59	50	8	1

[a]Total specimens released into 59 cages arenas. In general, one specimen of each taxon was released into each cage. In 13 cages, an extra *Z. renardii* was added instead of a *S. confusa*. In six cages, an extra *C. vittatus* was added instead of a *H. convergens*

hesperus had the next lowest survival rate (57.6%) and second highest number of individuals that were deceased (30.5%) at the end of the feeding trials. All the so-called top-tiered predators (e.g., *Z. renardii*, *S. confusa*, *C. vittatus*, and *H. convergens*) had high survival rates (> 89%) and low rates of missing individuals (< 4%).

Gut content assays

A total of 196 field-collected (unmarked negative control specimens) lacewing larvae were assayed to determine the inherent background noise of the two ELISAs. The unmarked lacewings yielded low average ELISA values of 0.045 (± 0.012) and 0.036 (± 0.004) for the rabbit IgG and chicken IgY ELISA, respectively. The calculated CTV (mean + 6 SD) was 0.117 for the anti-rabbit and 0.060 for the anti-chicken ELISA (as depicted by the dotted lines in Figs. 1, 2).

Fidelity of the prey marking procedure

The double (e.g., internal and external) marking method used to biomark the third and fifth instar lacewing life stages was effective. The average (± SE) ELISA reading that the protein-marked third and fifth instar lacewings yielded was 0.91 ± 0.04 and 0.68 ± 0.15, respectively, which produced marking efficiencies of 95.3% and 98.1% (Fig. 1). It should be noted that the data shown in Fig. 1 includes both the living and dead lacewing specimens recovered from the feeding arenas.

Intraspecies and interspecies predation

Intraspecies predation events

The data revealed strong evidence for a single cannibalism event by a fifth instar lacewing larva on a third instar larva (Fig. 2a). Specifically, one specimen yielded an ELISA reaction for rabbit IgG that was almost ten times higher than the calculated CTV. As expected, none of the rabbit IgG-marked third instar lacewings reacted to the anti-chicken IgY ELISA (Fig. 2b). This indicates that there is no indirect evidence of lower-order cannibalism exhibited by the third instar lacewings upon fifth instar lacewings.

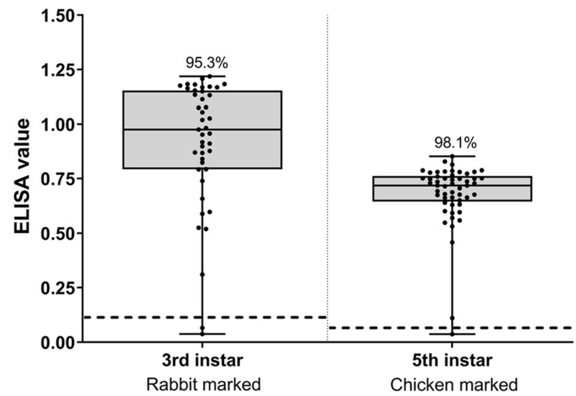

Fig. 1 Box-whisker plots depicting the ELISA summary statistics yielded by the third (n = 43) and fifth (n = 53) instar lacewing larvae marked with rabbit IgG and chicken IgY, respectively. The black dots reveal the ELISA reaction yielded for every lacewing specimen. The whiskers show the extreme ELISA values yielded for each life cycle. The boxes and the horizontal line within each box indicate the quartiles and the median ELISA value for each treatment, respectively. The percentage of positive ELISA reactions for each life stage is given above each plot. The dashed horizontal lines are calculated ELISA critical threshold values yielded by the negative control specimens

Interspecific predation events

Overall, there were 14 interspecies predation events recorded for the third instar *C. carnea* life stage specimens marked with rabbit IgG (Fig. 2a). *Collops vittatus, Z. renardii,* and *N. alternatus* accounted for five, four, and three of these events, respectively. There were only eight probable IGP events recorded for the fifth instar *C. carnea* life stage (Fig. 2b). The *S. confusa*, *N. alternatus*, and *C. vittatus* taxa each contained two individuals that scored positive for fifth instar *C. carnea* remains. One *G. punctipes* and one *N. alternatus* accounted for the remaining IGP events on the fifth instar lacewing life stage.

Discussion

This study and others (Hagler 2006, 2011, 2019; Mansfield and Hagler 2016; Blubaugh et al. 2016; Rendon et al. 2018; Hagler and Mostafa 2019) highlight many of the strengths of the UFIT procedure. They show that the UFIT can be easily adapted to study an array of arthropod feeding behaviors, many of which are not possible with the prey-specific

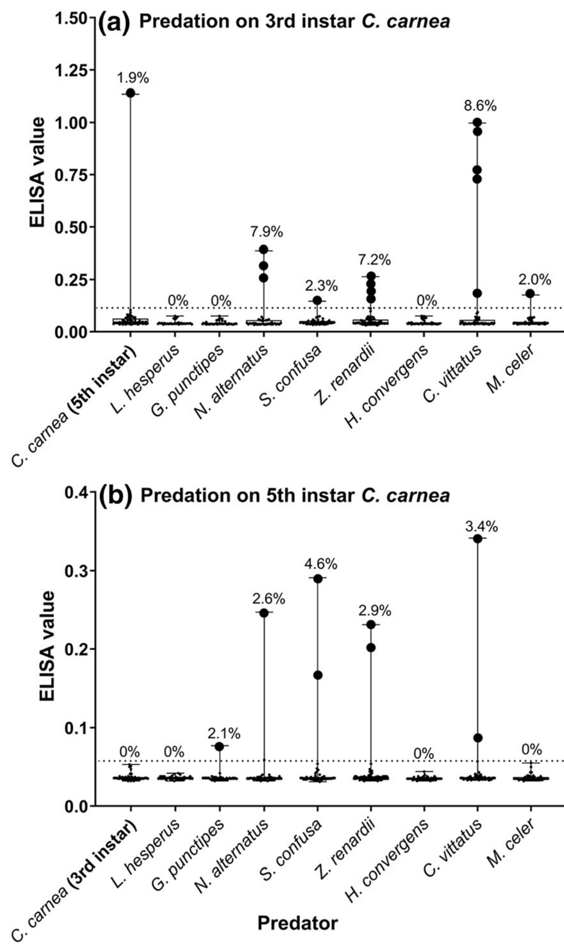

Fig. 2 Box-whisker plots depicting the summary statistics yielded by the predator taxa examined by ELISA for the presence of **a** third instar *C. carnea* marked with rabbit IgG and **b** fifth instar *C. carnea* marked with chicken IgY. The predator sample sizes are given in the number survived column in Table 2. The dashed horizontal lines indicate the critical threshold value for each ELISA. Each black dot shows the ELISA reaction yielded for every predator sample (the small black dots and larger black dots depict the negative and positive predator samples, respectively). The whiskers show the extreme ELISA values yielded for each predator taxon. The percentage of predation events recorded for each predator is given above each plot

molecular gut assay approaches. Here, we described how the UFIT can be used to examine predation simultaneously across: (1) multiple life stages of prey species, (2) within species (cannibalism), and (3) across species (IGP). Some key advantages of using the UFIT for gut analysis research are that the food detection assays have already been developed and optimized, they are inexpensive and easy to learn, and they are well suited for mass throughput. Also, the availability of multiple ELISAs to detect various immunomarkers (e.g., rabbit, chicken, rat sera, etc.) provide an avenue to conduct manipulative studies of a wide variety of predator feeding activities (Hagler 2019). Finally, the sensitivity of the UFIT sandwich ELISA format compares favorably to the prey-specific indirect ELISA and PCR gut assay approaches (Mansfield et al. 2008; Hagler et al. 2015). A potential limitation of the procedure is that, in some cases, the research must be conducted under manipulated field conditions (i.e., in cages). However, we argue that UFIT research, when conducted in concert with field cage methodology, can be advantageous to open-field studies. Specifically, the two experimental approaches can be used in concert to bolster an arthropod feeding experiment providing experimental units, treatments, and replications (Hagler and Mostafa 2019).

As mentioned above, *C. carnea* was selected as the targeted prey for this proof-of-concept investigation because it is known to engage in cannibalism and to be susceptible to IGP. However, most of the previous studies on the subject matter were conducted in highly controlled artificial conditions (Duelli 1981; Rojht et al. 2009; Michaud and Grant 2003; Noppe et al. 2012). In contrast, Rosenheim et al. (1999) conducted a labor-intensive field study where the foraging activity of 136 *C. carnea* larva were visually tracked in cotton. Throughout 448 h of direct field observations, they only documented three instances of "contact" between the focal larvae and conspecifics. None of which resulted in a cannibalism event. Moreover, none of the focal lacewings were observed engaging in IGP. However, they did document nine instances of higher-order predators engaging in IGP on the focal lacewings.

The prey-specific PCR assay approach has also been used to examine IGP on lacewings. Hagler and Blackmer (2015) developed a *C. carnea*-specific PCR assay and then used it to examine the gut contents of 1440 field-collected predators for the presence of lacewing DNA. Overall, about 10% of the predator population contained remnants of lacewing DNA in their guts. The predator taxa with the highest proportion of their population containing lacewing DNA included assassin bugs (*Z. renardii* and *S. confusa*) and orb-weaving spiders (Araneidae). The major advantage of using the prey-specific PCR gut assay approach to assess IGP predation was that the study was

conducted in unmanipulated open field conditions. However, as discussed above, this approach was unable to pinpoint life stage-specific and cannibalism predation events.

The dual (internal and external) marking procedure proved to be very useful for tagging the targeted prey items. Previous studies have revealed that, when possible, prey items targeted for UFIT research should be double-marked (Hagler et al. 2018b). This study also confirms that the UFIT ELISAs are very reliable at detecting protein marks on the selected prey items. Specifically, the ELISAs detected the protein marks on > 95% of the marked lacewing larvae and did not react to any of the non-target predator taxa examined (data not shown), showing there was no incidental marker transfer. Also, none of the rabbit IgG-marked third instar lacewings reacted to the chicken IgY ELISA and only one chicken-marked fifth instar reacted to the rabbit IgG ELISA. The intensity of this reaction strongly suggests that it cannibalized the rabbit IgG-marked lacewing.

In summary, the UFIT has proven useful for identifying complex predator feeding behaviors. The procedure can pinpoint certain predator feeding activities that are not possible with convention (prey-specific) gut assay procedures. Here alone, we showed how the UFIT can be used to study the proclivity of a predator assemblage to engage in intraspecies (cannibalism) and interspecies (IGP) predation. We also proved it can be useful for pinpointing life stage-specific predation events. The UFIT's pinpoint precision coupled with its ease of use (i.e., the assay have already been developed and optimized), mass throughput capability (i.e., hundreds of specimens can be examined per day), and low cost (i.e., ca., US$0.15 per sample) can expidite research aimed at identifying key predators for biological control programs.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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