

Accuracy of a prey-specific DNA assay and a generic prey-immunomarking assay for detecting predation

James R. Hagler*, Felisa Blackmer and Dale W. Spurgeon

Arid Land Agricultural Research Center, USDA-ARS, 21881 North Cardon Lane, Maricopa, AZ 85138, USA

Summary

1 Predator gut examinations are useful for detecting arthropod predation events. The accuracy and reproducibility of two different gut assays are tested on various predator species that consumed *Chrysoperla carnea* (Stephens) that was externally labelled with rabbit immunoglobulin (IgG).

2 Each predator homogenate was examined in triplicate for prey remains by both a conventional PCR assay to detect for *C. carnea* DNA and a generic ELISA to detect for rabbit IgG-marked prey. The ability of each method to detect predation over time was compared among predators, and between assay types were determined using a novel three-dimensional contingency table approach.

3 Both assays reliably detected prior predation (e.g. at least one of the three subsamples yielded a positive reaction) for 6–12 h after feeding. However, the generic ELISA was more reproducible (e.g. all three subsamples yielded the same outcome) than the PCR.

4 This shows that it was important to assay the predators in triplicate by PCR to avoid a high occurrence of false-negative reactions. Conversely, reproducible results from the ELISA procedure were not dependent on duplicate subsamples. Overall, the generic immunomarking gut assay procedure proved an effective method to assess predation.

Key-words: *Chrysoperla carnea*, ELISA, gut analysis, PCR, predator–prey interactions, statistical analysis

Introduction

Obtaining field data on predator feeding activity is difficult because arthropods are often too small and elusive to directly observe in their natural habitat. In addition, arthropod predators rarely leave evidence of attack; chewing predators devour their prey and piercing – sucking predators rarely leave recognizable feeding wounds. Hence, molecular post-mortem techniques to identify prey remain in predator stomachs have proven useful for detecting predation (Greenstone 1996; Hagler & Naranjo 1996; Sheppard & Harwood 2005). The two most widely used molecular gut content analysis methods include the enzyme-linked immunosorbent assay (ELISA) that uses a prey-specific monoclonal antibody (MAb) and the polymerase chain reaction (PCR) assay that uses prey-specific primers (Greenstone *et al.* 2007). Of these, the PCR assay has been touted as the method of choice for molecular gut content analysis (King *et al.* 2008). This is due, in part, to the fact that PCR assays are relatively inexpensive and easy to develop when compared to developing the prey-specific MAb needed for an ELISA (Greenstone & Shufran 2003; Monzó *et al.* 2010). However, PCR is not well suited for mass screening predators because it is expensive (Fournier *et al.* 2008). In short, prey-specific ELISAs require more time and resources

to develop and prey-specific PCR assays require more time and resources to process.

Over two decades ago, a gut analysis technique was described that used the ELISA format, but did not require a prey-specific MAb (Hagler & Durand 1994). The technique consists of tagging prey items with a protein such as rabbit immunoglobulin G (IgG). In turn, a protein-marked prey can be detected in a predator's stomach by an anti-rabbit IgG ELISA. The advantages of this approach are that the rabbit IgG antibody is commercially available at an affordable price, and the ELISA is well suited for mass screening predators (Hagler 2011). Here, we examine the gut contents of four predator taxa that fed on a *Chrysoperla carnea* (Stephens) larva that was externally marked with a small amount of rabbit IgG. Each individual was examined, in triplicate, by a *C. carnea*-specific PCR assay and then by a rabbit IgG-specific ELISA. The accuracy and reproducibility of each assay was assessed, and the merits and limitations of using these assays are discussed.

Materials and methods

PREY MARKING PROCEDURE

Chrysoperla carnea were reared on artificial diet using the procedure described by Ridgway, Morrison & Badgley (1970). A 167- μ L aliquot of 11.9 mg mL⁻¹ rabbit IgG solution (Sigma Cat. No. I-8140; Saint Louis, MO, USA) was topically applied to cohorts ($n \approx 10$ per cohort)

*Correspondence author. E-mail: james.hagler@ars.usda.gov

of *C. carnea* larvae in a 1-L polypropylene container using a medical nebulizer (Hudson RCI®, Micro Mist® Nebulizer, Research Triangle Park, NC, USA) as described by Hagler *et al.* (2002). The marked prey were air dried for ≥ 1 h at 27°C before they were presented to predators.

PREDATOR FEEDING STUDIES

A protein-marked *C. carnea* larva was placed into a 35 × 10-mm Petri dish containing one of the following predators: *Hippodamia convergens* Guérin-Ménéville, *Collops vittatus* (Say), *Geocoris punctipes* (Say) or *Zelus renardii* Kolenati. The age of the marked larva presented to the predators depended on the largest larval instar that each respective predator could eat without being counterattacked. As such, *C. vittatus* was fed 1st or early 2nd instars, *H. convergens* and *G. punctipes* were fed 2nd instars, and *Z. renardii* was fed 3rd instars. Observations were taken every 20 min to ascertain the occurrence of a feeding event. Predators were allowed to complete the feeding event prior to the removal of the prey item from the Petri dish. Predators were frozen (−80°C) at various timed intervals after ingesting the marked prey. Twenty individuals of each species were tested for each prey retention interval. Each predator was then analysed first in triplicate for the presence of *C. carnea* DNA by PCR and then again in triplicate for rabbit IgG by ELISA.

CHRYSOPERLA CARNEA-SPECIFIC PCR ASSAY

DNA extraction

The *C. carnea*-fed predators were placed individually in sterile 2.0-mL microtubes and homogenized in 180- μ L of phosphate-buffered saline (PBS, pH 7.2) using sterile 5-mm stainless steel beads and Qiagen's TissueLyser (1 min at 30 Hz). A maximum of 50 mg of tissue was individually processed; specimens that weighed over 50 mg were homogenized in 360 μ L of PBS. The homogenates were then centrifuged (3935 × *g*) at 4°C for 4 min. A 20- μ L homogenate of each sample was stored at −80°C for the subsequent ELISA described below. The DNA was then extracted from the samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). Samples that were homogenized in 360 μ L of PBS were split between two DNeasy mini spin columns. Total DNA was eluted twice in the 30- μ L AE buffer provided by the manufacturer. The stock DNA extracts were stored at −80°C.

DNA quantification and normalization

DNA extracts were quantified and normalized prior to PCR amplification to control for PCR amplification variation and quenching. A 1.5- μ L aliquot of each DNA sample was taken for quantification using Thermo Scientific's Nanodrop 1000 (West Palm Beach, FL, USA). Each quantified sample was then normalized to a concentration of 40 ng μ L^{−1}, using sterile TE Buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0).

PCR amplification

All DNA extracts were subjected to triplicate PCR assays to determine the reproducibility of PCR results. A primer set was designed for the *C. carnea* cytochrome oxidase subunit I gene (5'-CCTATTG-TAATTGGAGGTTTTGG, 5'-TCCAGCATGAGCAATTCTTG, GenBank Accession Number AY743792). Previous PCR tests confirmed that this primer set is highly specific to *C. carnea* as it did not

cross-react with 30 other arthropod species tested (R.H. James, F. Blackmer, unpub. data). The PCR amplifications were performed in a 10- μ L reaction volume containing 3 μ L of 40 ng μ L^{−1} DNA extract, 1 μ L of each primer (2.5 μ M) and 5 μ L of HotStarTaq Master Mix (Qiagen Inc.). Samples were amplified in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Westbury, NY, USA) beginning with an initial denaturing step of 95°C for 15 min followed by 10 cycles of touchdown PCR at 94°C for 30 s, 69°C for 30 s and 72°C for 30 s, −1°C per cycle. Touchdown PCR was followed by 50 cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 30 s. The PCR was finished with a 10 min extension at 72°C.

PCR products were separated by electrophoresis in 2% agarose gels (120 volts, 25 min). Each gel was stained with ethidium bromide, and the bands on the gel were visualized using Quantity One Software™ (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR gel contained lanes dedicated for a negative TE control, a positive *C. carnea* control and a negative predator control.

Protein-specific ELISA

A sandwich anti-rabbit IgG ELISA was performed on each predator sample in triplicate using the method described by Hagler (1997). Triplicate assays of each predator sample were performed on different ELISA plates (Falcon® Microtest™ Flat Bottom Polystyrene Plate, No. 351172; Becton Dickinson Labware, Franklin Lakes, NJ, USA). A 50- μ L aliquot of goat anti-rabbit IgG (Sigma No. R2004) diluted 1:500 in a solution consisting of 1% non-fat dairy milk was added to each well and incubated for 1 h at 27°C. Wells were blocked with 260 μ L of 1% non-fat dry milk for 30 min at 27°C. The milk was emptied from the ELISA plates, and a 50- μ L aliquot of diluted insect homogenate (original 20 μ L of insect homogenate collected during DNA extraction was diluted with TBS to a total volume of 200 μ L) was added to each well and incubated for 1 h at 27°C. Plates were then emptied and washed three times with TBST and twice with TBS before adding to each well 50 μ L of goat anti-rabbit IgG (Sigma No. A6154) conjugated to horseradish peroxidase diluted 1:1000 in 1% non-fat dairy milk. Plates were incubated again for 1 h at 27°C, emptied and washed three times with TBST and twice with TBS before applying 50 μ L of TMB substrate to each well. Plates were incubated for 10 min at 27°C, and the ELISA optical density was measured with a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) set at 650 nm.

Negative controls

Starved predators, serving as negative PCR and ELISA controls, were also assayed in triplicate as described above to ensure that there was no cross-reactivity of each assay to the various predator species. Each PCR subsample was scored positive by visual detection of a band corresponding to 207 bp in size on a 2% agarose gel. Each ELISA subsample was scored positive if the optical density reading exceeded the mean negative control reading by six standard deviations (Hagler 2011).

Assay reproducibility ratios

The number of subsamples of each predator specimen yielding a positive assay response for the prey was tallied. Reproducibility ratios yielded by the PCR and ELISA were calculated for each taxon at each prey retention interval by summing the number of samples for which

subsamples were all positive (e.g. the target molecule was detected in all three subsamples) or all negative (e.g. the target molecule was not detected in any of the three subsamples) and dividing this sum by the predator sample size ($n = 20$) for each prey retention interval. As such, calculated reproducibility ratios near 1.0 identify the most reproducible assays.

Scoring predation events using various threshold level criteria

To date almost all gut analysis, studies have reported results obtained from a single predator sample unit (aliquot). A unique aspect of this study is that each individual predator homogenate was assayed in triplicate for targeted prey remains by both PCR and ELISA. Here we report on the accuracy of each assay type by calculating the percentage of predators testing positive for prey remains using a loose, moderate and strict threshold level criteria (TLC). The loose, moderate and strict TLC required that 1 or more, at least 2, and all 3 of the subsamples from each predator homogenate were needed to identify a predation event, respectively. In addition, we randomly selected one of the three subsamples of each individual predator to score a predation event. This was done to simulate the ramifications of using a single sample to judge the predators for prey remains. We coined this procedure as the conventional TLC. The assay results yielded using the conventional TLC were the data used for the statistical analyses described below. Except for controls, all of the assayed predators were previously observed feeding on the targeted prey. Therefore, perfect accuracy by any of the criteria would be reflected by 100% of samples testing positive. However, due to a predator's natural digestion process, it is expected that the rate of 'false-negative' assay reactions will increase as the time since feeding increases. Hence, for the purposes of comparing the influences of the various TLCs on temporal patterns of predation detection, PCR and ELISA assays were designated as sufficiently accurate until detection of predation in non-control samples declined to <75%.

STATISTICAL ANALYSIS

The ability of each method (ELISA, PCR) to detect predation over time using the conventional TLC was compared among predators using three-dimensional contingency tables. These analyses were conducted using the SAS GLIMMIX procedure (SAS Institute 2012). The dimensions in the tables corresponding to each method were represented by predator, time after predation and assay status (positive or negative for predation) where the number of assayed predators in each status category was treated as a Poisson variable. This approach allows for the simultaneous testing of multiple effects adjusted for other effects in the model (Stroup 2013). Significance of the interaction between predator, time and status indicates that changes in the numbers of positive assay result over time differ among predators. Where this three-way interaction is not significant, significance of interactions between predator and status, and between time and status indicates the numbers of positive assays differ among predators and times, respectively. Because data for some predator taxa were only collected up to 24 h after the predation event, time intervals >24 h after predation were excluded from these analyses. In separate analyses, the abilities of the two assay methods to detect predation over time were compared for each predator also using the three-dimensional contingency table approach. Dimensions in each table were represented by assay method, time after predation and assay status. A significant three-way interaction indicates a difference between methods in their ability to detect

predation over time. Where this interaction was not significant, tests of the assay method \times status and time \times status interactions evaluate the respective effects of assay method and time after predation on the numbers of positive assays. Statistical comparisons among the various TLCs were not attempted because the data for each TLC were not independent from each other.

Results

COMPARISONS AMONG PREDATOR SPECIES USING THE PCR METHOD

Analyses of the PCR data using the conventional TLC indicated an overall difference in the number of positive assays among predator species ($\chi^2 = 11.6$, d.f. = 3, $P < 0.01$) but no effect of time on the number of positive assays ($\chi^2 = 5.3$, d.f. = 4, $P = 0.26$). However, interpretation of these tests is contingent on a non-significant three-way interaction (predator \times time \times status). The P -value of the three-way interaction was not significant at $\alpha = 0.05$ ($\chi^2 = 19.63$, d.f. = 12, $P = 0.07$) but was low enough to warrant examination of the interaction slices. Slices of the interaction suggested the PCR method was more effective for detecting predation by *H. convergens* than for other predators at 12 (*H. convergens* positive assays = 18, other species range from 6 to 10) and 24 h after the predation event (*H. convergens* positive assays=18, other species range from 5 to 7; Fig. 1). Therefore, the high number of positive assay results observed for *H. convergens* at 12 and 24 h after predation was likely responsible for failure to detect an overall decline in the number of positive PCR assays with increasing time.

COMPARISONS AMONG PREDATOR SPECIES USING THE ELISA METHOD

The contingency table analyses of the conventional TLC indicated a significant interaction between time since the predation event and status of the assay ($\chi^2 = 45.3$, d.f. = 4, $P < 0.01$). This test simply showed that, as expected, the ability of ELISA to detect predation declined over time (Fig. 1). Although the predator \times time \times status interaction was not significant at $\alpha = 0.05$ ($\chi^2 = 17.8$, d.f. = 12, $P = 0.12$), when an interaction between model effects is contained within only a few cells of the table, the overall P -value may not detect the interaction (Stroup 2013). Examination of individual cell counts by slices of the three-way interaction suggested the number of positive ELISA assays at 6 h after predation was lower for *G. punctipes* (5 positive) than for the other predator species (ranging from 17 to 20 positive; Fig. 1). Slices at 12 h after predation suggested the ELISA more effectively detected predation in *Z. renardii* (20 positive assays) than for *G. punctipes* (0 positive assays) or *H. convergens* (1 positive assay). The number of positive assays for *C. vittatus* at 12 h after predation was intermediate (10 positive assays) compared with the other predators. Slices of the three-way interaction did not suggest differences among the predator species at any other time intervals after predation.

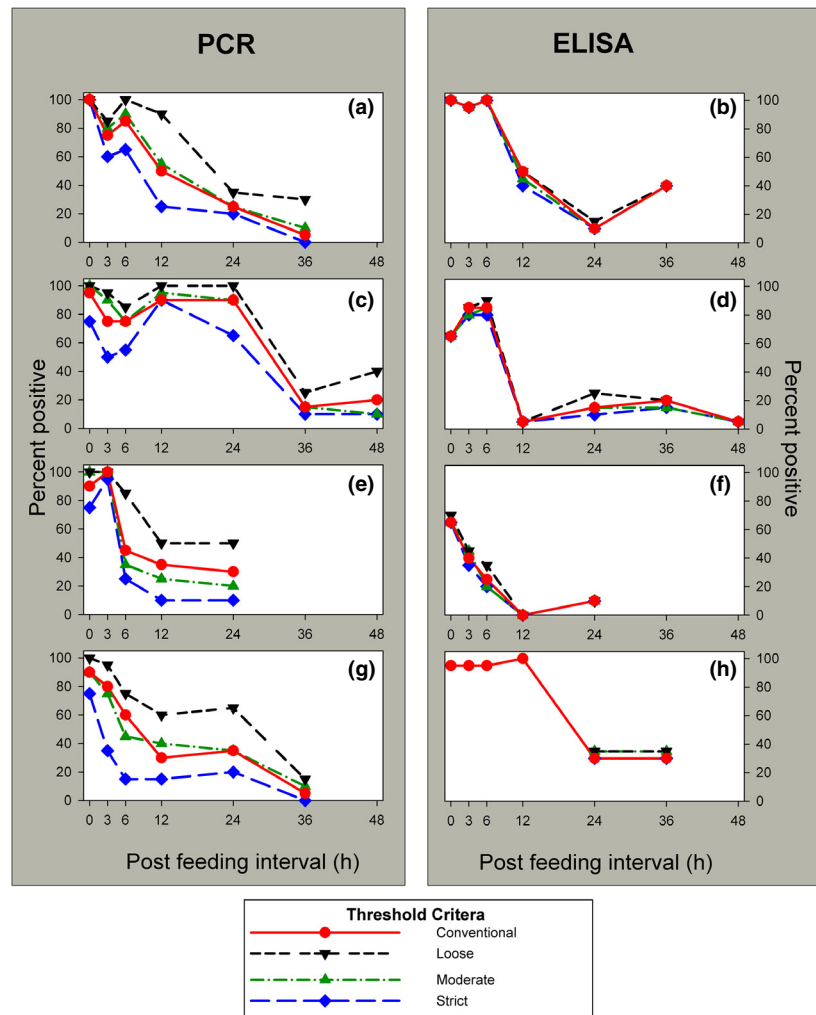


Fig. 1. Percentage of *Collops vittatus* (a, b), *Hippodamia convergens* (c, d), *Geocoris punctipes* (e, f) and *Zelus renardii* (g, h) yielding a positive PCR for *Chrysoperla carnea* DNA (figures in left column) and a positive ELISA reaction for protein-marked *C. carnea* (figures in right column), respectively, at various post-feeding time intervals ($n = 20$ for each post-feeding interval treatment).

COMPARISON OF THE PCR ASSAY AND ELISA WITHIN PREDATOR SPECIES

Collops vittatus

The reproducibility ratios of the PCR assay for detecting prey in *C. vittatus* ranged from 0.35 at 12 h after feeding to a perfect 1.0 immediately after feeding (Table 1). Prey DNA was readily detectable in $\geq 75\%$ of the *C. vittatus* samples for up to 6 h after feeding if the moderate and conventional TLCs were used to judge the specimens and for up to 12 h if the loose TLC was used (Fig. 1a). Overall (e.g. when all the individuals assayed at each time interval were combined), the PCR detected prey DNA in 73, 60, 57 and 45% of the *C. vittatus* using the loose, moderate, conventional and strict TLCs, respectively.

The reproducibility ratios for ELISA were ≥ 0.90 for each prey retention interval (Table 1). The ELISA detected protein-marked prey in $\geq 95\%$ of the *C. vittatus* for up to 6 h (Fig. 1b), regardless of which TLC was used to judge the specimens. Most (57) of the 60 *C. vittatus* assayed in triplicate by ELISA beyond the 6-h prey retention interval yielded all negative (39 individuals) or all positive (18 individuals) ELISA reactions.

Overall, the ELISA detected prey in 67, 66, 65 and 64% of the *C. vittatus* using the loose, conventional, moderate and strict TLCs, respectively.

Contingency table analysis of results yielded by the conventional TLC (i.e. only a single random sample was used to judge for predation) indicated a decline in the numbers of detected predation events with increased time since predation ($\chi^2 = 32.0$, d.f. = 5, $P < 0.01$; Fig. 1a, b), but no overall difference in the numbers of predation events detected by the two methods ($\chi^2 = 0.0$, d.f. = 1, $P = 0.99$). Examination of the three-way interaction (time \times method \times status) was not significant ($\chi^2 = 8.34$, d.f. = 5, $P = 0.14$). Examination of the interaction slices confirmed that the decline in detection of predation by *C. vittatus* over time was similar for the two methods (Fig. 1a, b).

Hippodamia convergens

The reproducibility of the PCR for detecting prey in *H. convergens* was highly variable between the various prey retention intervals. Reproducibility ratios ranged from 0.55 at 3 h to 0.9 at 12 h after feeding (Table 1). Generally, prey was detectable by PCR in $\geq 75\%$ of the beetles for up to 24 h after a meal when

Table 1. The results of the predator gut assays comparing the efficacy of the *Chrysoperla carnea*-specific PCR assay and the protein-specific marking ELISA on four predator species

Predator	Post-feeding interval (hours)	# of samples assayed in triplicate	Number of triplicated subsamples scoring positive by PCR				PCR* reproducibility ratio	Number of triplicated subsamples scoring positive by ELISA				ELISA* reproducibility ratio
			None	1	2	3		None	1	2	3	
<i>Collops vittatus</i>	0	20	0	0	0	20	1.00	0	0	0	20	1.00
	3	20	3	1	4	12	0.75	1	0	0	19	1.00
	6	20	0	2	5	13	0.65	0	0	0	20	1.00
	12	20	2	7	6	5	0.35	10	1	1	8	0.90
	24	20	13	2	1	4	0.85	17	1	0	2	0.95
	36	20	14	4	2	0	0.70	12	0	0	8	1.00
<i>Hippodamia convergens</i>	0	20	0	0	5	15	0.75	7	0	0	13	0.95
	3	20	1	1	8	10	0.55	3	1	0	16	0.95
	6	20	3	2	4	11	0.70	2	1	1	16	0.90
	12	20	0	1	1	18	0.90	19	0	0	1	1.00
	24	20	0	2	5	13	0.65	15	2	1	2	0.85
	36	20	15	2	1	2	0.85	16	1	0	3	0.95
<i>Geocoris punctipes</i>	0	20	12	6	0	2	0.70	19	0	0	1	1.00
	3	20	0	0	5	15	0.75	6	1	0	13	0.95
	6	20	0	0	1	19	0.95	11	0	2	7	0.90
	12	20	3	10	2	5	0.40	13	3	0	4	0.85
	24	20	10	5	3	2	0.60	20	0	0	0	1.00
	36	20	10	6	2	2	0.60	18	0	0	2	1.00
<i>Zelus renardii</i>	0	20	0	2	3	15	0.75	1	19	missing	missing	
	3	20	1	4	8	7	0.40	1	19	missing	missing	
	6	20	5	6	6	3	0.40	1	19	missing	missing	
	12	20	8	4	5	3	0.55	0	20	missing	missing	
	24	20	7	6	3	4	0.55	13	0	1	6	0.95
	36	20	17	1	2	0	0.85	13	0	1	6	0.95

*Reproducibility ratio = number of triplicated samples that were all negative + number of triplicated samples that were all positive/# of samples assayed.

†Due to an assay error, only one *Z. renardii* sample was assayed by ELISA for the 0-, 3-, 6- and 12-h post-feeding interval treatments.

the loose, moderate and conventional TLCs were used to judge the samples (Fig. 1c). Overall, the PCR detected prey in 78, 68, 66 and 51% of the *H. convergens* using the loose, moderate, conventional and strict thresholds, respectively.

The reproducibility ratios yielded by ELISA were ≥ 0.85 for each prey retention interval (Table 1). Protein-marked prey was accurately detected by ELISA in $\geq 75\%$ of the beetles for 3 and 6 h after a meal, regardless of the TLC used (Fig. 1d). Beyond 6 h, most *H. convergens* samples (76 out of 80 predators) contained 3 subsamples that were either all negative ($n = 69$) or all positive ($n = 7$) for marked prey. Overall, the ELISA detected prey in 42, 40, 39 and 37% of the *H. convergens* using the loose, conventional, moderate and strict TLC, respectively.

The contingency tables for *H. convergens* using the conventional TLC revealed a significant time \times method \times status interaction ($\chi^2 = 32.0$, d.f. = 6, $P < 0.01$), indicating the pattern of change in the numbers of positive assays for predation over time differed between the two methods. Examination of the interaction slices showed the PCR method detected a high incidence of predation at 12 and 24 h after the predation event, whereas the ELISA method did not (Fig. 1c, d).

Geocoris punctipes

The reproducibility of the PCR for detecting prey in *G. punctipes* was highly variable. Reproducibility ratios ranged from 0.40 at 6 h to 0.95 at 3 h (Table 1). The PCR accurately detected prey in *G. punctipes* for up to 3 h after feeding, regardless of the TLC used, and for up to 6 h using the loose TLC (Fig. 1e). Overall, the PCR detected prey in 77, 60, 56 and 43% of the *G. punctipes* using the loose, conventional, moderate and strict TLC, respectively.

The reproducibility ratios yielded by ELISA were ≥ 0.85 for the various prey retention intervals (Table 1). However, the high ratios were due, in large part, to failure of the ELISA to detect the protein-marked prey (Fig. 1f). In other words, there was a high incidence of false-negative reactions at all of the time intervals tested. Overall, the ELISA detected prey in only 32, 28, 28 and 26% of the *G. punctipes* using the loose, conventional, moderate and strict thresholds, respectively.

Contingency table analysis of the data yielded by the conventional TLC indicated the frequency with which predation was detected declined with time since predation ($\chi^2 = 23.6$, d.f. = 4, $P < 0.01$). Also, no overall difference in the numbers of positive assays was shown for the two methods ($\chi^2 = 0.0$,

d.f. = 1, $P = 0.97$). The non-significant time \times method \times status interaction ($\chi^2 = 0.41$, d.f. = 4, $P = 0.98$) indicated changes in the numbers of assays positive for predation with increased time were not different for the two methods (Fig. 1e, f).

Zelus renardii

The reproducibility ratios yielded by PCR for detecting prey in *Z. renardii* ranged from 0.40 at 3 and 6 h to 0.85 (e.g. numerous false-negative reactions) at 36 h after feeding (Table 1). The PCR accurately detected prey remains in $\geq 75\%$ of the specimens for up to 3 h after feeding using the loose, conventional and moderate TLCs (Fig. 1g). Overall, the PCR detected prey in 74, 50, 49 and 27% of the *Z. renardii* using the loose, conventional, moderate and strict TLC, respectively.

Due to an ELISA assay error during the first attempt to assay the three samples of each individual, there was only enough sample buffer remaining to conduct a single ELISA for those *Z. renardii* held for 0 to 12 h after a meal. As a result, these prey retention interval treatments could only be assessed by the conventional TLC. The reproducibility ratios yielded by the ELISA for *Z. renardii* examined 24 and 36 h after feeding was 0.95 (Table 1). Of these, 13 contained 3 subsamples that lacked the targeted prey item and 6 contained 3 subsamples that contained protein-marked prey (Table 1). The conventional ELISA TLC accurately detected marked prey in *Z. renardii* in $\geq 95\%$ of the individuals that were examined within 12 h after feeding (Fig. 1h).

Contingency tables of data yielded by the conventional TLC for *Z. renardii* yielded results similar to those for *C. vittatus* and *G. punctipes*. Only the time \times status interaction was significant ($\chi^2 = 53.3$, d.f. = 5, $P < 0.01$), which reflected the decline in numbers of positive assays for predation with increased time. The non-significant time \times method \times status interaction ($\chi^2 = 6.4$, d.f. = 5, $P = 0.27$) provided no evidence that the temporal patterns of positive assays differed between methods as time since predation increased (Fig. 1g, h).

Discussion

A unique aspect of this study was that each predator sample was examined in triplicate for the presence of prey remains by both a prey-specific PCR assay and a protein-specific ELISA. The data yielded highlight the importance of knowing the reproducibility (precision) and accuracy of any given gut content ELISA or PCR assay. Reproducibility is important, especially if one is relying on the outcome of a single assay per predator sample. But reproducibility is high when the assay is very accurate and again when the assay is very inaccurate (e.g. high incidences of false negative reactions). This is why for most of the predators assayed by PCR, the lowest estimates of reproducibility were observed for the intermediate time intervals as the assay results transitioned from fairly accurate to inaccurate (false negatives). The higher level of reproducibility for the ELISA assay reflects a sharper time-dependent transition in accuracy than for the PCR assay. The importance of

having a highly reproducible gut assay with a short prey detection interval cannot be stressed enough. Some have implied that long prey detection intervals are advantageous (Agustí, Unruh & Welter 2003b; Harper *et al.* 2005). Although a long prey retention interval increases the chance of obtaining a positive assay reaction from a field-collected specimen, it confounds the interpretation of the assay result because it cannot distinguish between an old and a recent predation event (Hagler & Naranjo 1996; Naranjo & Hagler 1998; Holland, Perry & Winder 1999; Hagler 2006; Read *et al.* 2006). For instance, suppose an assay with low reproducibility (one out of three subsamples often yields a positive reaction) can detect the prey in a predator's gut for 36 h and that the predator feeds every few hours. Then, every field-collected predator should yield a positive assay reaction (assuming each predator specimen is assayed in triplicate). However, if an assay with high reproducibility can detect prey in a predator for only a few hours after a meal (e.g. 6 h) then a more intense sampling schedule (e.g. every 12 h) could be employed to more accurately estimate the predation rate. As such, we maintain highly reproducible assays with short detection limits are the best assay for gut analysis research.

Only a fraction of studies to date using PCR or ELISA gut content analysis have also used multiple subsamples from an individual predator to identify predation events. A few PCR studies examined each predator homogenate in triplicate, but the authors did not elaborate on how conflicting subsample results were interpreted (Agustí, De Vicente & Gabarra 1999, 2000; Agustí *et al.* 2003a; Zhang *et al.* 2007; Schmidt *et al.* 2009). Two PCR gut assay studies conducted duplicate samples for each predator specimen and then scored the predator positive for prey if 'at least one' yielded a positive response (McMillan *et al.* 2007; Kuusk *et al.* 2008). However, neither study reported on the reproducibility of their PCR assay. For the few ELISA studies, the investigators examined their predators in duplicate or triplicate. Again, in most of these studies, there was no mention of the criteria used to resolve information provided by conflicting subsample outcomes. For a few studies, the investigators judged a predator as positive for prey remain if the average of the ELISA readings was above the calculated TLC (e.g. Sopp & Sunderland 1989; Georgianne *et al.* 2008; Monzó *et al.* 2012). Again, none of these studies reported on the reproducibility of their ELISA.

To simulate the single sample method used for almost every prey-specific gut assay study conducted to date, we randomly selected a single subsample for each predator (the 'conventional' TLC) to judge it for the presence of prey. These results were then compared to the outcomes obtained using a loose, moderate and strict TLC. This allowed us to assess both the accuracy and reproducibility of each assay type for each predator taxon. We subjectively define an accurate assay as one that detects the targeted prey item in the predator shortly after it was consumed (e.g. $T \leq 6$ h after feeding). In other words, a negative assay response for a predator that recently fed was deemed a true type II error (false positive) because it failed to detect the targeted prey item. However, this study and many others have shown that as time passes after a meal, the

likelihood of detecting the prey diminishes because the prey is degraded or digested by the predator (Sopp & Sunderland 1989; Hagler & Naranjo 1997; Hagler *et al.* 1997; Greenstone *et al.* 2007; Gagnon *et al.* 2011; Hagler & Blackmer 2013). Therefore, as time after the feeding event increases, the likelihood of detecting the prey decreases because there is little if anything left to detect.

The prey detection capability of both assays examined here were similar to the results yielded in many previous studies (Hagler 2006, 2011; Mansfield, Hagler & Whitehouse 2008; Hagler & Blackmer 2013; Zilnik & Hagler 2013). In general, both assays detected the prey in the various taxa for at least 6 h. The PCR assay was accurate at detecting prey in the guts of the various predators if the loose TLC was used to judge the samples. However, the low reproducibility ratios (Table 1) and the lack of overlapping TLC curves (Fig. 1) of the PCR assay show that the moderate, strict and conventional TLCs are insufficient for judging the predators. In short, the PCR was accurate when the loose TLC was employed, but it was not reproducible. Obviously, the reproducibility of the PCR assay might be improved with further PCR optimization tests. One such test would be to determine whether different sized molecular markers might increase the reproducibility of the PCR. For instance, Agustí, Unruh & Welter (2003b) compared molecular markers of 271 and 188 bp for detecting *Cacopsylla pyricola* (Förster) remain in predators. They showed that prey detectability was always longer when the short primers were used. Perhaps a smaller molecular marker than the one used here (e.g. <207 bp) would also increase the reproducibility of this PCR assay. The ELISA reliably (e.g. most of the samples yielded a positive reaction within 6 h after a meal) detected protein-marked prey in *C. vittatus*, *H. convergens* and *Z. renardii* (note that *Z. renardii* was only judged using the conventional TLC). Moreover, the high reproducibility ratios (Table 1) and overlapping TLC prey retention curves (Fig. 1) indicate that predators can be assessed accurately by ELISA using the conventional TLC. In short, the ELISA accurately detected prey remains in three of the four taxa and it was reproducible for all four. These results suggest that predators should be examined in triplicate by PCR in order to obtain more accurate assay results. Conversely, the loose TLC is sufficient for the ELISA. These findings have serious consequences for using the PCR-based gut content analysis approach to assess field predation. Specifically, it was reported that the cost of analysing a single predator sample by PCR was 15 times more than by ELISA (Fournier *et al.* 2008).

The ELISA was not reliable at detecting protein-marked prey remains in *G. punctipes*. However, the ELISA was reproducible as, for the most part, each subsample yielded the same outcome for the presence or absence of prey. This previous research (Hagler 2011) strongly indicates that the high occurrence of false-negative reactions was not due to the ELISAs inability to detect the protein-marked prey in *G. punctipes*. Instead, it indicates that the *G. punctipes* never ingested any rabbit IgG during the feeding event. These spurious results were not totally unexpected. For example, even though *G. punctipes* and *Z. renardii* both have piercing – sucking type

mouthparts, they handle their prey in completely different manners. Specifically, *Z. renardii* simultaneously spears its prey and grasps it with its forelegs and feeds for an extended period of time (e.g. >1 h; pers. obs.), whereas *G. punctipes* spears its prey, but does not handle it with its forelegs (Cohen & Byrne 1992; pers. obs.). In short, *Z. renardii* has more contact with its prey than *G. punctipes*. The poor ELISA results yielded for *G. punctipes*, most likely due to less physical contact with its prey, might be remedied in several ways. First, the prey marking procedure can be modified by providing a bigger ‘target’ for the ELISA. For instance, a greater amount (volume and/or concentration) of protein could be administered to the prey. In this study, we topically (externally) applied a much lower concentration and volume of rabbit IgG than has been used for previous studies (Hagler 2006, 2011; Zilnik & Hagler 2013). Secondly, the prey detection by ELISA would likely be enhanced if the piercing – sucking predator consumed a prey item that was also marked internally with protein. For example, a study showed that $\geq 90\%$ of *G. punctipes* and *H. convergens* (a chewing predator) scored positive by ELISA 24 h after consuming an internally and externally marked *L. hesperus* nymph (Hagler 2011). The prey can be internally marked by simply feeding them protein-enriched foodstuffs (Hagler & Jackson 1998; Hagler 2011). Finally, the amount of predator sample used in the ELISA could be increased. In this study, due to the methodological constraints of conducting both a PCR and ELISA on each predator sample, it was essential that the samples were prioritized (biased) for the PCR. Specifically, each predator was initially macerated in 180 μL of buffer. From that, a 160- μL aliquot was then used to analyse each predator by a PCR protocol optimized by Hagler & Blackmer (2013). The remaining sample buffer (20 μL) was then diluted with 180 μL of TBS buffer to produce enough to assay the sample in triplicate (at 50 μL per subsample) by ELISA. In short, the amount of predator sample buffer used for the ELISA was only a fraction of the amount used in previous studies (Hagler & Durand 1994; Hagler 2006, 2011). Given these factors, it seems likely that the standard immunomarking ELISA would yield better results than the ones obtained in this study.

The prey-specific ELISA and PCR gut assay approaches will undoubtedly continue to be valuable tools for predator assessment. The costs of developing and conducting the assays will continue to decrease as technology improves. However, the prey immunomarking procedure offers a viable alternative for researchers to conduct unique types of biological control research without the burden of developing prey-specific assays. To date, the method has been used to study various aspects of field predation, some of which are impossible using the prey-specific PCR assay and ELISA approaches. Hagler (2006) identified the diel feeding patterns of members of the cotton predator assemblage on chicken IgG- and rabbit IgG-marked prey items. The procedure was used to qualitatively identify ant predation on rabbit IgG-marked termites (Buczowski & Bennett 2007). Prey immunomarking was used to quantify predation on a key cotton pest by the cotton predator assemblage in field cages (Hagler 2011). Neither the prey-specific PCR

assay nor ELISA approach has this capability (Naranjo & Hagler 1998; Sheppard & Harwood 2005). Immunomarkers have been used to tag immobile foodstuffs such as sentinel egg masses in the open field (Mansfield, Hagler & Whitehouse 2008). Also, Lundgren, Saska & Nonék (2013) tracked granivory exhibited by an arthropod complex in the field by protein marking dandelion seeds with rabbit IgG. Zilnik & Hagler (2013) showed that the technique has enormous potential to study predator scavenging activity and can be adapted to assess cannibalism. Differentiating between predation, carrion feeding and cannibalism is not possible with prey-specific gut assays (Hagler & Naranjo 1994; Calder, Harwood & Symondson 2005; Foltan *et al.* 2005; Juen & Traugott 2005; Sheppard & Harwood 2005). Most recently, Kelly, Hagler & Kaplan (2012) used prey-specific ELISA to determine the frequency of open field predation incurred on *Manduca sexta* L. caterpillars by released predatory stink bugs, *Podisus maculiventris* Say. While the prey immunomarking technique has many advantages over the prey-specific ELISA and PCR approaches, it has one major limitation. Specifically, field cage studies (as opposed to open field studies) are almost always necessary for a prey immunomarking experiment [but see Mansfield, Hagler and Whitehouse (2008) and Lundgren, Saska and Nonék (2013)]. Unfortunately, field cage experiments might have limited appeal because they do not exactly replicate what occurs in nature.

In summary, the two assay types were about equally accurate at detecting prey remains except in the case of *H. convergens* at time intervals between 12 and 24 h after predation, where the PCR method yielded a higher number of positive assays. This was somewhat surprising given that the predator sample preparation was heavily biased for the PCR assay, and the prey marking protocol was not as stringent as those employed in previous studies. Moreover, the ELISA proved more reproducible than PCR. The relatively low reproducibility rates yielded by the PCR should serve as a warning for future research using this procedure. The data presented here, coupled with recently published research, indicate that the prey immunomarking gut assay procedure is a viable and cost-effective method to assess predation.

Acknowledgements

Funding was provided by USDA CRIS 5347-22620-021-00D. We are grateful for the technical support of Scott Machtley. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Data accessibility

Data deposited in the Dryad repository: <http://datadryad.org/resource/doi:10.5061/dryad.f52s2>

References

Agustí, N., De Vicente, C. & Gabarra, R. (1999) Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new

- polymerase chain reaction-based technique for predator gut analysis. *Molecular Ecology*, **8**, 1467–1474.
- Agustí, N., De Vicente, C. & Gabarra, R. (2000) Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology*, **9**, 263–268.
- Agustí, N., Unruh, T.R. & Welter, S.C. (2003b) Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of Entomological Research*, **93**, 179–185.
- Agustí, N., Shaylor, S.P., Harwood, J.D., Vaughn, I.P., Sunderland, K.D. & Symondson, W.O.C. (2003a) Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. *Molecular Ecology*, **12**, 3467–3475.
- Buczowski, G. & Bennett, G. (2007) Protein marking reveals predation of termites by the woodland ant, *Aphaenogaster rudis*. *Insectes Sociaux*, **54**, 219–224.
- Calder, C.R., Harwood, J.D. & Symondson, W.O.C. (2005) Detection of scavenged material in the guts of predators using monoclonal antibodies: a significant source of error in measurement of predation? *Bulletin of Entomological Research*, **95**, 57–62.
- Cohen, A.C. & Byrne, D.N. (1992) *Geocoris punctipes* as a predator of *Bemisia tabaci*: a laboratory evaluation. *Entomologia Experimentalis et Applicata*, **64**, 195–202.
- Foltan, P., Sheppard, S., Konvicka, M. & Symondson, W.O.C. (2005) The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. *Molecular Ecology*, **14**, 4147–4158.
- Fournier, V., Hagler, J.R., Daane, K., de León, J. & Groves, R. (2008) Identifying the predator complex of *Homalodisca vitripennis* (Hemiptera: Cicadellidae): a comparative study of the efficacy of an ELISA and PCR gut content assay. *Oecologia*, **157**, 629–640.
- Gagnon, A.-È., Doyon, J., Heimpel, G.E. & Brodeur, J. (2011) Prey DNA detection success following digestion by intraguild predators: influence of prey and predator species. *Molecular Ecology*, **16**, 4390–4400.
- Georgianne, J., Griffiths, J.K., Alexander, C.J., Holland, J.M., Kennedy, P.J., Perry, J.N., Symondson, W.O.C. & Winder, L. (2008) Monoclonal antibodies reveal changes in predator efficiency with prey spatial pattern. *Molecular Ecology*, **17**, 1828–1839.
- Greenstone, M.H. (1996) Serological analysis of arthropod predation: past, present and future. *The Ecology of Agricultural Pests, Biochemical Approaches* (eds W.O.C. Symondson & J.E. Liddell), pp. 265–300. Chapman & Hall, London.
- Greenstone, M.H. & Shufrin, K.A. (2003) Spider predation: species-specific identification of gut contents by polymerase chain reaction. *Journal of Arachnology*, **31**, 131–134.
- Greenstone, M.H., Rowley, D.L., Weber, D.C., Payton, M.E. & Hawthorne, D.J. (2007) Feeding mode and prey detectability half-lives in molecular gut-content analysis: an example with two predators of the Colorado potato beetle. *Bulletin of Entomological Research*, **97**, 201–209.
- Hagler, J.R. (1997) Field retention of a novel mark-release-recapture method. *Environmental Entomology*, **26**, 1079–1086.
- Hagler, J.R. (2006) Development of an immunological technique for identifying multiple predator-prey interactions in a complex arthropod assemblage. *Annals of Applied Biology*, **149**, 153–165.
- Hagler, J.R. (2011) An immunological approach to quantify consumption of protein-tagged *Lygus hesperus* by the entire cotton predator assemblage. *Biological Control*, **58**, 337–345.
- Hagler, J.R. & Blackmer, F. (2013) Identifying inter- and intra-guild feeding activity of an arthropod assemblage. *Ecological Entomology*, **38**, 258–271.
- Hagler, J.R. & Durand, C.M. (1994) A new method for immunologically marking prey and its use in predation studies. *Entomophaga*, **39**, 257–265.
- Hagler, J.R. & Jackson, C.G. (1998) An immunomarking technique for labeling minute parasitoids. *Biological Control*, **27**, 1010–1016.
- Hagler, J.R. & Naranjo, S.E. (1994) Determining the frequency of heteropteran predation on sweet potato whitefly and pink bollworm using multiple ELISAs. *Entomologia Experimentalis et Applicata*, **72**, 59–66.
- Hagler, J.R. & Naranjo, S.E. (1996) Using gut content immunoassays to evaluate predeceous biological control agents: a case study. *The Ecology of Agricultural Pests, Biochemical Approaches* (eds W.O.C. Symondson & J.E. Liddell), pp. 383–399. Chapman & Hall, London.
- Hagler, J.R. & Naranjo, S.E. (1997) Measuring the sensitivity of an indirect predator gut content ELISA: detectability of prey remains in relation to predator species, temperature, time, and meal size. *Biological Control*, **9**, 112–119.
- Hagler, J.R., Naranjo, S.E., Erickson, M.L., Machtley, S.A. & Wright, S.F. (1997) Immunological examinations of species variability in predator gut

- content assays: effect of predator:prey protein ratio on immunoassay sensitivity. *Biological Control*, **9**, 120–128.
- Hagler, J.R., Jackson, G.C., Henneberry, T.J. & Gould, J.R. (2002) Parasitoid mark-release-recapture techniques—II. Development and application of a protein marking technique for *Eretmocerus* spp., parasitoids of *Bemisia argentifolii*. *BioControl Science and Technology*, **12**, 661–675.
- Harper, G.L., King, R.A., Dodd, C., Harwood, J.D., Glen, D.M., Bruford, M.W. & Symondson, W.O.C. (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology*, **14**, 819–827.
- Holland, J.M., Perry, J.N. & Winder, L. (1999) The within-field spatial and temporal distribution of arthropods in winter wheat. *Bulletin of Entomological Research*, **89**, 499–513.
- Juen, A. & Traugott, M. (2005) Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. *Oecologia*, **142**, 244–252.
- Kelly, J.L., Hagler, J.R. & Kaplan, I. (2012) Employing immunomarkers to track dispersal and trophic relationships of a piercing-sucking predator, *Podisus maculiventris* (Hemiptera: Pentatomidae). *Environmental Entomology*, **41**, 1527–1533.
- King, R.A., Read, D.S., Traugott, M. & Symondson, W.O.C. (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology*, **17**, 947–963.
- Kuusk, A.-K., Cassel-Lundhagen, A., Kvarnheden, A. & Ekblom, B. (2008) Tracking aphid predation by lycosid spiders in spring-sown cereals using PCR-based gut-content analysis. *Basic and Applied Ecology*, **9**, 718–725.
- Lundgren, J.G., Saska, P. & Nonék, A. (2013) Molecular approach to describing a seed-based food web: the post-dispersal granivore community of an invasive plant. *Ecology and Evolution*, **3**, 1642–1652.
- Mansfield, S., Hagler, J.R. & Whitehouse, M.E.A. (2008) A comparative study on the efficacy of a pest-specific and a prey-marking enzyme-linked immunosorbent assay for detection of predation. *Entomologia Experimentalis et Applicata*, **127**, 199–206.
- McMillan, S., Kuusk, A.-K., Cassel-Lundhagen, A. & Ekblom, B. (2007) The influence of time and temperature on molecular gut content analysis: *Adalia bipunctata* fed with *Rhopalosiphum padi*. *Insect Science*, **14**, 353–358.
- Monzó, C., Sabater-Muñoz, B., Urbaneja, A. & Castañera, P. (2010) Tracking medfly predation by the wolf spider, *Pardosa cribata* Simon, in citrus orchards using PCR-based gut-content analysis. *Bulletin of Entomological Research*, **100**, 145–152.
- Monzó, C., Urbaneja, A., Ximénez-Embún, M., García-Fernández, J., García, J.L. & Castañera, P. (2012) Selection of *Ceratitis capitata* (Diptera: Tephritidae) specific recombinant monoclonal phage display antibodies for prey detection analysis. *PLoS ONE*, **7**, e51440.
- Naranjo, S.E. & Hagler, J.R. (1998) Characterizing and estimating the effect of Heteropteran predation. *Predatory Heteroptera: Their Ecology and Use in Biological Control* (eds M. Coll & J. Ruberson), pp. 171–197. Proceedings Thomas Say Publications in Entomology. Entomological Society of America, Lanham, MD.
- Read, D.S., Sheppard, S.K., Bruford, M.W., Glen, D.M. & Symondson, W.O.C. (2006) Molecular detection of predation by soil micro-arthropods on nematodes. *Molecular Ecology*, **15**, 1963–1972.
- Ridgway, R.L., Morrison, R.K. & Badgley, M. (1970) Mass rearing a green lacewing. *Journal of Economic Entomology*, **63**, 834–836.
- SAS Institute (2012) *SAS Release, ed. 9.4*. SAS Institute, Cary, NC.
- Schmidt, J.E.U., Almeida, J.R.M., Rosati, C. & Arpaia, S. (2009) Identification of trophic interactions between *Macrolophus caliginosus* (Heteroptera: Miridae) and *Myzus persicae* (Homoptera: Aphididae) using real time PCR. *BioControl*, **54**, 383–391.
- Sheppard, S.K. & Harwood, J.D. (2005) Advances in molecular ecology: tracking trophic links through complex predator-prey food webs. *Functional Ecology*, **19**, 751–762.
- Sopp, P.I. & Sunderland, K.D. (1989) Some factors affecting the detection period of aphid remains in predators using ELISA. *Entomologia Experimentalis et Applicata*, **51**, 111–120.
- Stroup, W.W. (2013) *Generalized Linear Mixed Models: Modern Concepts, Methods and Applications*. CRC Press, Boca Raton, FL.
- Zhang, G.-F., Lü, Z.-C., Wan, F.-H. & Lövei, G. (2007) Real-time PCR quantification of *Bemisia tabaci* (Homoptera: Aleyrodidae) B-biotype remains in predator guts. *Molecular Ecology*, **7**, 947–954.
- Zilnik, G. & Hagler, J.R. (2013) An immunological approach to distinguish arthropod viviphagy from necrophagy. *BioControl*, **58**, 807–814.

Received 19 May 2015; accepted 27 June 2015

Handling Editor: Sean Rands