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Development and evaluation of an enzyme-linked immunosorbent assay to detect *Pieris rapae* remains in guts of arthropod predators

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Abstract

An enzyme-linked immunosorbent assay (ELISA) was developed to detect remains of *Pieris rapae* L. (Lepidoptera: Pieridae) immature stages in the guts of field collected arthropod predators. The assay can be used to help ascertain the relative importance of arthropod predator species in suppressing *P. rapae* in cabbage, *Brassica oleracea* var. *capitata* L. The ELISA is sensitive to all immature stages of *P. rapae*, although first and fifth instars can be detected more readily than eggs or pupae and third instars showed intermediate detectability. Assays on whole body homogenates of predators readily detected predation on *P. rapae* first instars by all seven of the predator species tested, although response generally declined with increasing predator size. Together the results show that the *P. rapae* ELISA possesses a sufficiently high level of sensitivity and specificity to be a useful tool in helping to elucidate the roles of arthropod predator species in reducing populations of *P. rapae* in cabbage.

Introduction

Immunological assays to detect prey remains in the guts of predators collected from the field are a useful tool to help researchers identify important trophic connections within an ecosystem (Powell et al., 1996). Once developed, these assays can serve as an efficient and sensitive means to test large numbers of field collected predators for evidence of feeding on a specific prey item, such as a key agricultural pest. However, immunoassays must be carefully assessed before they can be used in ecological studies (Boreham & Ohiagu, 1978; Sopp et al., 1992; Kidd & Jervis, 1996; Sunderland, 1996). Critical aspects that must be evaluated include sensitivity and specificity, and effectiveness in detecting predation by different species. This paper documents these attributes for an immunoassay that we developed for a key pest of cabbages (*Brassica oleracea* variety *capitata* L.) in New York State.

The target of our immunoassay, the imported cabbageworm, *Pieris rapae* L. (Lepidoptera: Pieridae), is a serious pest of cabbages and other crucifer crops in North America and many other parts of the world. While research elsewhere (including some using immunological assays) has shown that predatory arthropods can cause high levels of mortality in *P. rapae* populations (Dempster, 1967; Parker, 1970; Ashby, 1974; Hasui, 1977; Jones et al., 1987), until recently the impact of predators has not been evaluated in New York. The objective of this study was thus to develop and characterize an enzyme-linked immunosorbent assay (ELISA) to help identify important predators of *P. rapae* on cabbages in New York State. In particular, five aspects of the immunoassay were addressed: (1) the assay's sensitivity to first instar *P. rapae*, the stage which appears most susceptible to predation (Dempster, 1967; Ashby, 1974; Schmaedick & Shelton, 1999); (2) differences in sensitivity to the different immature stages of *P. rapae*; (3) specificity (i.e., the levels of cross-reactions to other species); (4) ability to detect predation by seven predator species; and (5)

the effect on the assay of different amounts of predator material in samples.

Materials and methods

Antiserum production and preparation. Immunogen was prepared by grinding *P. rapae* neonates in phosphate buffered saline (PBS, pH 7.4) containing 1.67 μM phenylthiourea to retard melanization. The homogenate was centrifuged 15 min at 10 000 *g* and filtered through glass wool to remove solids. Final protein concentration was 10.2 mg ml^{-1} as measured by the Lowry direct procedure (Sigma Diagnostics protein assay kit, Sigma Chemical Co., St. Louis, Missouri, USA). The immunogen was stored in aliquots at $-20\,^{\circ}$C until needed for immunization. A six month old rabbit (Flemish Giant $\times$ Chinchilla cross) was injected subcutaneously with a mixture of 0.5 ml immunogen solution and 0.5 ml Freund's complete adjuvant at 10 sites along the back. Additional injections were given 14, 34, 62, and 90 d after the first, using Freund's incomplete adjuvant. After the first immunization, each subsequent immunization was preceded by a test bleed and immunodiffusion (Johnson, 1986) against *P. rapae* egg and larval homogenates to assess titres and determine the need for additional immunization.

Immunodiffusion with antiserum from a test bleed taken after the fourth injection showed a higher antibody titre against *P. rapae* egg extract than against larval extracts. In an effort to increase titres of antibodies against larval antigens, 0.5 ml of second instar *P. rapae* extract (27.2 mg ml^{-1} protein) was used as the immunogen in the final injection. Antiserum was collected 106 d after the initial injection and purified by ammonium sulfate precipitation, followed by ion-exchange chromatography using procedures described by Harlow & Lane (1988).

The double antibody sandwich ELISA format that we used requires antibodies at two stages of the assay, the first to coat the well and capture antigen from the sample solution, and the second to bind specifically to the captured target antigen. In this assay the second antibody was conjugated to biotin so it could then be detected by addition of commercially available streptavidin-alkaline phosphatase conjugate followed by the enzyme substrate. This format takes advantage of the strong and highly specific binding reaction between biotin and streptavidin, the simplicity of the biotinylation procedure, and the availability of ready-made streptavidin-enzyme conjugates (Bayer & Wilchek, 1996). A portion of our purified IgG was biotinylated by first adjusting IgG concentration to 2 mg ml^{-1} and dialyzing against three changes of 50 mM sodium bicarbonate (pH 8.5). To 1.5 ml of the IgG solution we then added 37.5 μl of 13.3 mg ml^{-1} N-hydroxysuccinimidobiotin (EZ-Link^{TM} NHS-Biotin, Pierce Chemical Co., Rockford, Illinois, USA). The solution was incubated for 30 min at room temperature, then dialyzed against three changes of PBS and stored at 4 °C.

A preliminary ELISA showed a high level of cross-reaction with certain other Lepidoptera species, including two cabbage pests, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). The cross reacting antibodies were removed prior to subsequent assays by preadsorbing the biotinylated IgG with *T. ni* acetone powder (Harlow & Lane, 1988). The powder was produced by grinding batches of 50 g of fifth instar *T. ni* from a laboratory colony with 45 ml PBS and 5 ml of 0.05 mg ml^{-1} phenylthiourea. The mixture was extracted at 4 °C with 400 ml cold acetone and then centrifuged 10 min at 10 000 *g*. The pellet was extracted again in 400 ml cold acetone, centrifuged again and allowed to dry at room temperature, then ground with mortar and pestle, sifted through 32-mesh lumite screen, and stored in a desiccator at 4 °C. For each assay the IgG-biotin conjugate was preadsorbed by combining 1:400 with a 1% *T. ni* acetone powder mixture in PBS containing 0.05% Tween 20 (Sigma) and 1% bovine serum albumin (Sigma) (PBST-BSA). The preadsorbing mixture was incubated 1 h at 4 °C with end-over-end rotation. After centrifuging 15 min at 10 000 *g*, the supernatant was used in the ELISA immediately.

ELISA procedure. The double antibody sandwich ELISA was used to detect *P. rapae* antigens in whole predator samples. The ELISA procedures were modifications of those of Clark & Adams (1977). Optimal incubation times and temperatures and immunoreactant and sample concentrations were determined through a series of preliminary assays using standard test solutions extracted from homogenate of *Coleomegilla maculata lengi* Timberlake (Coleoptera: Coccinellidae) adults from a laboratory colony, either fed or not fed one *P. rapae* first instar (also from a laboratory colony) before testing. Assay conditions, incubation times, and immunoreactant and sample concentrations selected were those which most readily distin-

guished fed from unfed beetles (M. A. Schmaedick, unpubl.). The final ELISA protocol was as follows. Samples were prepared in advance by homogenizing 1:70 (w:v) in sample buffer (PBST-BSA) and storing at −20 °C. (Samples weighing less than 2.14 mg were prepared in 150 μl PBST-BSA.) At each step of the assay 100 μl of the appropriate immunoreactant was added to each of the central 60 wells of a polystyrene, 96-well, U-bottom immunoassay plate (Dynatech Laboratories, Chantilly, Virginia, USA). Between steps, the wells were emptied and washed four times by alternately filling with PBST, waiting 3 min, then emptying the wells and tapping the inverted plate dry on a paper towel. The initial step of the assay consisted of coating the wells with 10 μg/ml purified IgG in coating buffer (15 mM sodium carbonate, 34.8 mM sodium bicarbonate, 0.02% sodium azide, pH 9.6) and incubating for 4 h at 37 °C. After coating and washing with PBST, the samples were applied, and plates were incubated 16 h at 4 °C. After washing again, the preadsorbed biotinylated IgG was added and the plates were incubated at 37 °C for 4 h. Wells were again washed and streptavidin-alkaline phosphatase (Immunopure® , Pierce), diluted 1:70 000 in PBST-BSA, was added and plates were incubated for 2 h at 37 °C. After washing again, 1 mg ml^{-1} p-nitrophenyl phosphate substrate (Sigma) in 1 M diethanolamine (pH = 9.8) was added and, after 1 h at 20 °C, the reaction was stopped with 30 μl of 3 N NaOH per well. Absorbances at 405 nm were measured using a microplate reader (Spectra II, SLT Labinstruments, Grödig, Austria).

In addition to the sample wells, five wells each of sample buffer and three standards were included on each plate. The standards were extracted homogenates of *P. rapae* first instars, *C. maculata* adults starved 24 h before feeding on one *P. rapae* first instar each, and *C. maculata* adults that were not fed after starving 24 h. The *P. rapae* standard was produced by grinding 20 first instar *P. rapae* 1:25,000 in PBST-BSA, centrifuging, and filtering through glass wool. Test solutions of the *C. maculata* fed or not fed *P. rapae* were prepared by grinding 30 beetles 1:70 in sample buffer, centrifuging, and filtering through glass wool. Aliquots of the three standards were stored at −20 °C until needed.

Assay sensitivity. Sensitivity of the assay was evaluated by fitting curves to absorbances from dilution series of homogenized *P. rapae* first instars in solutions with and without a 1:70 concentration of *C. maculata* homogenate. Each dilution was replicated twice on each of two ELISA plates and each plate also had six wells each of sample buffer and *C. maculata* in sample buffer. The four curves (dilution series of *P. rapae* with and without *C. maculata* on each of two plates) were fitted simultaneously using 4-parameter logistic equations, $Y = (A - D)/[1 + (x/c)^b] + D$, and the program ALLFIT (De Lean et al., 1978). ALLFIT provides an algorithm for comparing parameters within families of sigmoidal curves by first fitting a full model consisting of a separate set of four parameters for each curve, then allowing the experimenter to sequentially set individual parameters for the different curves equal to each other until reduction in goodness of fit becomes significant. We used ALLFIT to combine the data from the two plates into a single model and to evaluate the effect of the presence or absence of predator protein on the *P. rapae* titration curve. A two-way ANOVA was used to check for any effect of plate or presence of *C. maculata* material on absorbances in the wells not containing *P. rapae* antigen.

ELISA sensitivity was determined by estimating the lowest concentration of *P. rapae* at which 99% of samples would be declared positive when using a positive-negative threshold that provides a 99% level of protection against false positives. Using the principles described by Fenlon & Sopp (1991), the detection limit of the ELISA can be expressed as the lowest *P. rapae* concentration at which 99% of samples would be declared significantly different from the population of negative controls. Assuming that variances of low concentration positive samples are approximately equal to those of negative controls and that errors are normally distributed, an estimate of the variance of the difference between a single sample well absorbance value and the mean of the negative controls is $s^2(1 + 1/n)$, where s^2 is the sample variance calculated from the negative controls and n is the number of control wells used to calculate mean absorbance of the negative control. A positive-negative threshold that provides a 99% level of protection against false positives can be calculated as

$$C = \bar{A}_0 + t\sqrt{\left(1 + \frac{1}{n}\right)s^2} \qquad (1)$$

where C is the threshold absorbance, $\bar{A}_0$ is mean absorbance of negative controls, and t is the 99th percentile of the Student's t distribution with degrees of freedom equal to those of s^2. The detection limit can be calculated as the mean absorbance of a population from which 99% of samples would give absorbance

above the threshold. Therefore a detection limit, D, that also provides for a 99% level of protection against false negatives is

$$D = \bar{A}_0 + 2t\sqrt{\left(1 + \frac{1}{n}\right)s^2} \quad (2)$$

Mean absorbances of the buffer wells with and without *C. maculata* and mean square error from the ANOVA (20 df) were used to calculate D for samples with and without *C. maculata*. The concentrations of *P. rapae* corresponding to D were then calculated using the 4-parameter models fitted to the *P. rapae* dilution series with and without *C. maculata*.

Effect of P. rapae *stage on assay sensitivity.* Because concentrations of proteins and other potential antigens change as insects develop (Chapman, 1982), the ELISA was expected to show differential sensitivity to the various immature stages of *P. rapae* (Boreham & Ohiagu, 1978). Dilution series of each stage were tested and sensitivities compared to characterize this important aspect of the assay. Five ELISA plates were used, and each plate contained two wells of each of five dilutions of *P. rapae* eggs, first instars, third instars, fifth instars, or pupae in sample buffer. In addition, 10 wells on each plate contained only sample buffer. Mean sample buffer absorbances were subtracted from the *P. rapae* sample absorbances and linear regressions fitted to logarithms of the adjusted absorbances versus logarithms of dilutions for each stage on each of the five plates. A positive-negative threshold absorbance for each plate was determined as the one-sided 99% prediction interval limit calculated from the ten buffer well absorbances as $m + ts$, where m is the sample mean, s the sample standard deviation, and t the 99th percentile of the Student's t distribution. The linear regression equations were then used to calculate the dilution at which mean absorbance equaled the positive-negative threshold. Assuming normally distributed errors, this dilution would be the point at which half the samples from the population would be declared positive and half negative. The threshold dilutions calculated for each stage were then compared by analysis of variance after transformation to logarithms with stage as treatment and plate as a blocking factor. Tukey's procedure (Steel & Torrie, 1980) was used to test for pairwise differences among the stage means.

Assay specificity and positive-negative threshold. The specificity of the ELISA was tested by assaying samples of potential predator or alternative prey species that occur in New York cabbage fields. No close relatives of *P. rapae* are known to occur on cabbages in our area, so we also assayed larvae of *Pieris napi oleracea* Harris from a laboratory colony to determine if there would be any cross-reaction with a more closely related species. The other species tested were also taken from laboratory colonies [*Delia radicum* (L.) and Lepidoptera] or were collected from areas in and around cabbage fields (all other species). For most species ten samples were tested, homogenate from each in a separate ELISA plate well. Predatory species collected from the field were starved 2–4 d before testing. Eggs, larvae, and pupae of several of the Lepidoptera were tested, because we anticipated the highest levels of cross-reaction in the Lepidoptera and expected to see differences between stages. All the samples were assayed at a concentration of 1:70 in the sample buffer. For very small species such as thrips and first instar Lepidoptera, many individuals were combined in each of the 10 samples so that the 1:70 solution would have sufficient volume for testing. Five wells each of sample buffer and positive control (first instar *P. rapae* diluted 1:25 000 in sample buffer) were included on each ELISA plate. Absorbances from these wells were used to adjust for any plate effects by subtracting the buffer mean absorbance from each sample absorbance and dividing this value by the difference between the positive controls mean and the buffer mean. All the specificity test results could then be expressed together as a percent of the positive control (de Savigny & Voller, 1980; Tijssen, 1985; Nielsen et al., 1996). Voucher specimens of the species tested were deposited in the Cornell University Insect Collection under lot number 1239.

We used percent of positive values of the species showing the highest level of cross-reaction to set a positive-negative threshold for the assay. The threshold was calculated as the mean of the species giving the highest cross-reaction plus its standard deviation times the 99th percentile of the Student's t distribution with 9 df. Assuming our samples were representative and errors normally distributed, this threshold would provide a 99% level of protection against declaring a sample that contained 100% of the highest cross-reacting species as positive for *P. rapae* antigens. An additional degree of protection is provided by the fact that test samples from field collected predators could contain at most only a fraction of the amount of prey material used in the specificity tests.

Assays with seven predator species. The ability of the ELISA to detect feeding by seven predator species was examined by assaying ten individuals fed one *P. rapae* first instar each after starving 1–7 d and comparing absorbances to those of ten unfed predators. The predators were taken from a laboratory colony (*C. maculata*) or collected from in and around cabbage fields (all other species). The experiment was conducted at room temperature (21 $\pm$ 2 °C), and the starved predators were presented with a larva and observed as they fed. They were homogenized in sample buffer and frozen immediately after feeding. Each ELISA plate contained five wells of positive control (1:25 000 *P. rapae* first instar) and five wells of sample buffer only. Sample absorbances were again adjusted by subtracting the mean buffer absorbance and expressing the difference as a percent of the mean positive control absorbance minus mean buffer absorbance.

Effect of predator homogenate on assay sensitivity. Hagler et al. (1997) found that increasing amounts of nontarget protein in samples decreased sensitivity of an indirect ELISA to detect *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) eggs. We tested for this possibility by assaying samples containing a constant concentration (1:30 000) of *P. rapae* first instar homogenate in a dilution series of *C. maculata* homogenate. A portion of each *C. maculuta* dilution was also tested without adding *P. rapae*. The test solutions were prepared by grinding the insects in sample buffer, centrifuging, and filtering through glass wool before mixing in the appropriate concentrations for each dilution series. Analysis of variance and orthogonal polynomial contrasts on logarithms of absorbances were used to test for any effect of *C. maculata* dilution on absorbance with or without *P. rapae* (Steel & Torrie, 1980).

Results

Assay sensitivity. Figure 1 shows observed values and fitted models for absorbance versus concentration of *P. rapae* with and without 1:70 *C. maculata*. The 4-parameter logistic models shown differ only in the parameter C, the locations of the curves' midpoints. Fitting the same model to both plates and using common *A*, *B*, and *D* parameters for the two treatments (with and without *C. maculata*) did not significantly reduce goodness of fit from that of the full model ($F = 1.31$; $df = 11, 80$; $P = 0.24$). When all four parameters were held the same, however, goodness of fit was significantly reduced ($F = 56.77$; $df = 1, 91$; $P < 0.0005$). Thus, the fitted model indicates that the effect of the *C. maculata* in the sample solution was to shift the curve to the right, without changing the upper and lower asymptotes (parameters *A* and *B*, respectively) or the steepness of the curve (parameter *D*); i.e., at midrange concentrations, the presence of *C. maculata* reduces the absorbance of samples containing *P. rapae* antigens.

Analysis of variance on absorbances of the wells containing sample buffer only, with or without *C. maculata*, found that plate had no significant effect on absorbances ($F = 0.05$; $df = 1, 20$; $P = 0.82$), but presence of *C. maculata* did ($F = 7.30$; $df = 1, 20$; $P = 0.0137$). The interaction between plate and *C. maculata* was not significant ($F = 0.02$; $df = 1, 20$; $P = 0.88$). The 95% confidence interval estimate for the difference between mean absorbance of sample buffer with *C. maculata* and mean absorbance without *C. maculata* was 0.0058 ± 0.0045. This difference, although significant, was apparently too small to show up as a significant shift in the lower asymptote of the curves fit to the *P. rapae* dilution series with and without *C. maculata* (Figure 1).

Data from the two plates were combined for calculating the limits of detection. The estimates for *P. rapae* concentrations at the limits of detection were 3.71 μg ml^{-1} with *C. maculata* and 1.66 μg ml^{-1} without *C. maculata* in the solution. The first instar *P. rapae* weigh approximately 100 μg each (M. A. Schmaedick, unpubl.), so these quantities represent approximately 0.00371 and 0.00166 of a first instar per 100 μl of sample solution in an ELISA plate well.

Effect of P. rapae *stage on assay sensitivity.* Figure 2 shows the estimated dilution for each *P. rapae* stage at which half the samples would be declared positive and half negative. We found substantial differences in this value for the different stages (overall ANOVA $F = 21.23$; $df = 4, 16$; $P = 0.0001$). The dilution of fifth instars at the positive-negative threshold was more than five-fold greater than the dilution of eggs at the same absorbance. In general, the assay is better able to detect material from *P. rapae* first and fifth instars than from eggs or pupae and shows intermediate sensitivity to third instars.

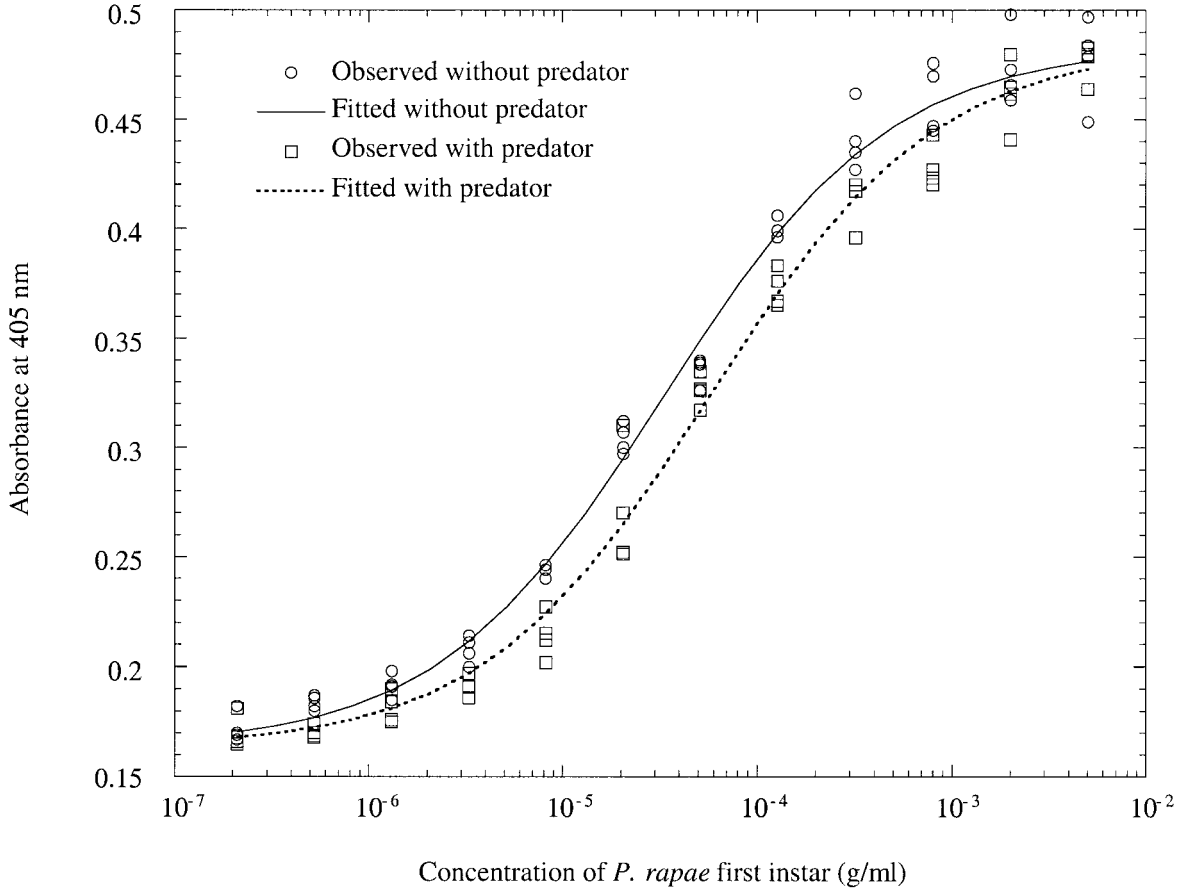

Figure 1. Observed and fitted absorbances for dilution series of *P. rapae* first instar without and with predator material (*C. maculata*) in sample solution. Fitted function: Absorbance $= (A - D)/[1 + (\text{Concentration}/C)^B] + D$. Parameters ($\pm$ approx. SE): $A = 0.484 \pm 0.00454$, $B = -0.745 \pm 0.0375$, $C = 3.34 \times 10^{-5} \pm 2.49 \times 10^{-6}$ (without predator) and $5.76 \times 10^{-5} \pm 4.28 \times 10^{-6}$ (with predator), $D = 0.163 \pm 0.00386$.

Assay specificity and positive-negative threshold. Results of *P. rapae* ELISA tests on various predators and potential alternative prey are shown in Tables 1 and 2. *Pieris napi* third instars had the highest level of cross-reaction of the species and stages tested. Still, the 1:70 dilution of *P. napi* gave a mean absorbance of only 120% that of the 1:25 000 concentration of *P. rapae* that was used as the standard in the specificity tests (Table 2). Aside from *P. napi*, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) first instars (Table 2) showed the highest cross-reaction. We therefore set the positive-negative threshold for the ELISA at $m + ts$, where m is the mean and s the standard deviation of the *O. nubilalis* samples and t is the 99th percentile of the Student's t distribution with 9 df. The calculation using *O. nubilalis* resulted in a positive-negative threshold of 47% of the positive standard.

Assays with seven predator species. With a few exceptions, predators that had fed on *P. rapae* first instars could readily be distinguished from those that had not (Figure 3). Except for the female *P. opilio*, all but two of the individual predators that had fed on *P. rapae* had absorbances above the 47% threshold. Six of the ten female *P. opilio* would have been declared negative, even though they had just fed on *P. rapae*, and one *P. rapae*-fed individual each of *S. comma* and *C. maculata* gave absorbances well within the range of the negative controls.

Effect of predator homogenate on assay sensitivity. Figure 4 shows that absorbance of a constant concentration of *P. rapae* increased with increasing dilution of *C. maculata* material in the sample. Both the linear and quadratic trends were significant for the dilution series with *P. rapae* ($F = 8.76$; df $= 1, 18$; $P = 0.0084$

Table 1. Mean absorbances of non-lepidopteran samples tested for cross-reaction with *P. rapae* ELISA expressed as percent of absorbance of the positive control after subtracting mean of buffer wells for each plate. For *D. insulare* $n = 9$. All others $n = 10$. Negative values indicate mean absorbances less than buffer mean. Adults tested unless otherwise noted. (Mean ± SD of average buffer absorbances across all plates was 0.213 ± 0.038)

Species/stage	Absorbance as % of pos. control ± SE
Cabbage (*Brassica oleracea* var. *capitata*)	6.67 ± 0.70
Lumbricidae (Annelida) (immatures)	15.55 ± 3.17
Derocerus sp. (Gastropoda)	18.99 ± 1.81
Julidae (Diplopoda)	15.72 ± 2.05
Henicopidae (Chilopoda)	9.14 ± 1.43
Meioneta unimaculata (Banks) (Araneae: Linyphiidae)	3.64 ± 1.81
Entomobryidae (Collembola)	5.16 ± 1.37
Thrips tabaci Lindeman (Thysanoptera: Thripidae) (adults and immatures)	3.94 ± 4.07
Brevicoryne brassicae (L.) (Homoptera: Aphididae) (adults and immatures)	−3.67 ± 1.26
Lygus lineolaris (Palisot de Beauvois) (Heteroptera: Miridae)	8.98 ± 0.95
Elaphropus anceps LeConte (Coleoptera: Carabidae)	3.48 ± 2.27
Bembidion quadrimaculatum oppositum Say (Coleoptera: Carabidae)	1.65 ± 2.08
Athetini (Coleoptera: Staphylinidae)	1.06 ± 1.27
Hippodamia variegata (Goeze) (Coleoptera: Coccinellidae)	8.75 ± 0.96
Anthicus cervinus LaFerté-Sénectère (Coleoptera: Anthicidae)	6.87 ± 1.50
Phyllotreta cruciferae (Goeze) (Coleoptera: Chrysomelidae)	4.65 ± 2.31
Delia radicum (L.) (Diptera: Anthomyiidae) (pupae)	1.20 ± 1.15
Prenolepis imparis (Say) (Hymenoptera: Formicidae)	6.06 ± 0.82
Diadegma insulare(Cresson) (Hymenoptera: Ichneumonidae) (pupae)	2.16 ± 3.36
Cotesia rubecula (Marshall) (Hymenoptera: Braconidae) (pupae)	5.45 ± 1.53

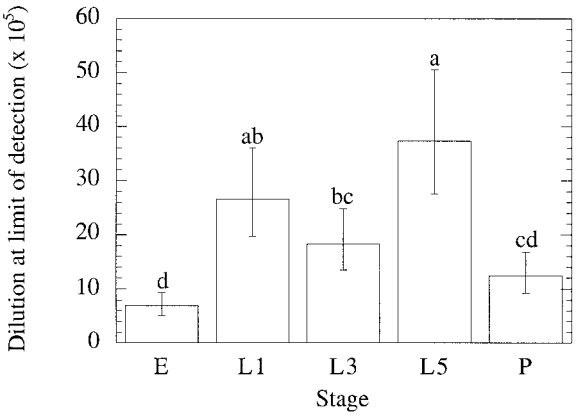

Figure 2. Dilution of each *P. rapae* stage (± 95% confidence interval) at which half the samples would be declared positive and half negative. (Values back-transformed from means and confidence limits calculated from $\log_{10}$ transformed data.) Values with same letter were not significantly different at 0.05 level by Tukey's mean separation procedure (Steel & Torrie, 1980).

and $F = 27.03$; $df = 1, 18$; $P = 0.0001$ respectively), but neither trend was significant for the same *C. maculata* dilutions without *P. rapae* ($F = 3.72$; $df = 1, 18$; $P = 0.070$ and $F = 4.31$; $df = 1, 18$; $P = 0.052$).

Discussion

ELISA sensitivity. Our ELISA was sufficiently sensitive for use in predation studies, although sensitivity was reduced slightly by the presence of predator material in the sample solution. The curve fits to the *P. rapae* dilution series (Figure 1) and the analysis of variance on the absorbances of wells without *P. rapae* showed that the presence of *C. maculata* material in the sample solution caused this decreased sensitivity in two ways: by decreasing the absorbance of positive samples and by increasing the absorbance of the negative controls used to calculate the positive-negative threshold. It remains to be seen whether other predator species differ from *C. maculata* in their effect on *P. rapae* ELISA sensitivity.

As expected, ELISA sensitivity was not equal across *P. rapae* growth stages. The pattern of differen-

Table 2. Mean absorbances of Lepidoptera tested for cross-reaction with *P. rapae* ELISA expressed as percent of absorbance of the positive control after subtracting mean of buffer wells for each plate. For all species/stages $n = 10$. Negative values indicate mean absorbances less than buffer mean. (Mean ± SD of average buffer absorbances across all plates was 0.213 ± 0.038)

Species/stage	Absorbance as % of pos. control ± SE
Plutella xylostella (L.) (Lepidoptera: Plutellidae)	
Eggs	−1.55 ± 1.52
First instars	1.32 ± 1.19
Fourth instars	2.63 ± 1.11
Pupae	9.40 ± 1.59
Ostrinia nubilalis (Hübner) (Lepidoptera: Crambidae)	
Eggs	1.80 ± 1.63
First instars	27.75 ± 2.14
Third instars	23.25 ± 1.45
Pupae	24.72 ± 3.42
Pieris napi oleracea Harris (Lepidoptera: Pieridae) (third instars)	122.0 ± 3.25
Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae)	
Eggs	3.09 ± 0.85
First instars	23.80 ± 2.05
Fourth instars	17.72 ± 2.07
Pupae	19.19 ± 2.24
Agrotis ipsilon (Hufnagel) (Lepidoptera: Noctuidae)	
Eggs	−1.64 ± 1.55
First instars	20.62 ± 1.62
Third instars	25.94 ± 1.55
Pupae	22.82 ± 1.76
Trichoplusia ni (Hübner) (Lepidoptera: Noctuidae)	
Eggs	−1.09 ± 1.03
First instars	20.95 ± 1.74
Third instars	15.48 ± 1.79
Pupae	25.02 ± 2.25

tial sensitivity between the stages likely results from a complex interaction between (1) the immunogenicities of antigens present in the larval extracts used for immunization and changes in titers of these antigens during *P. rapae* development and (2) the concentrations and affinities for these antigens of the various antibody clones present in the coating IgG solution and remaining in the IgG-biotin conjugate solution after preadsorbing with *T. ni* acetone powder. That the ELISA detected larvae more easily than other stages was not surprising, because the immunogen used to raise the original antiserum consisted of larval material. Nevertheless, the differential sensitivity will have to be considered in interpreting results from testing field collected predators.

ELISA specificity. Our ELISA was highly specific, showing only a low level of cross reaction, even with the closely related *P. napi*. Despite such a large difference in response, however, it would be impossible to set a positive-negative threshold that maintained high sensitivity to *P. rapae* while guarding against false positives from *P. napi*. We therefore decided to ignore this intrageneric cross-reaction as being of little relevance to the intended use of our assay. Although once considered a pest of crucifer crops in some parts of New York (Fitch, 1870; Riley, 1883), *P. napi* currently

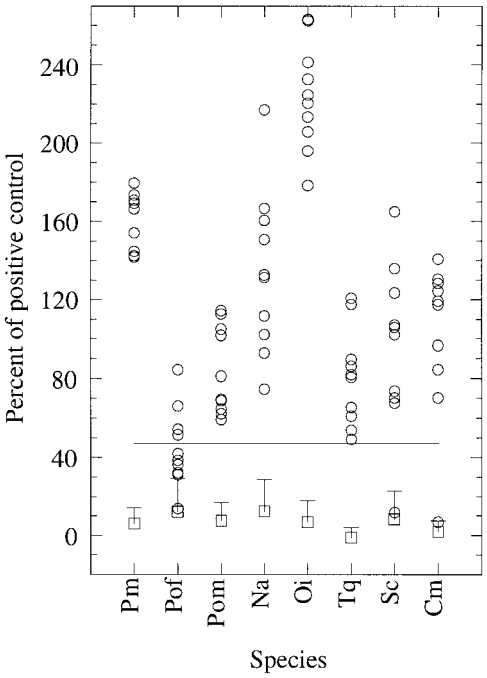

Figure 3. ELISA tests on predators fed one *P. rapae* first instar (circles) and unfed controls. Squares and error bars are means and 99%, one-tailed prediction limits for unfed controls. Horizontal line represents positive-negative threshold of 47% calculated from 99% prediction limit for species giving highest cross-reaction in specificity tests. All values calculated by first subtracting mean absorbance of buffer wells, then expressing difference as percent of positive control minus buffers mean. Overall average of buffer means ± SD was 0.201 ± 0.005. Pm = *Pardosa milvina* (Hentz) (Araneae: Lycosidae), Pof = female *Phalangium opilio* L. (Opiliones: Phalangiidae), Pom = male *P. opilio*, Na = *Nabis americoferus* (Carayon) (Heteroptera: Nabidae), Oi = *Orius insidiosus* (Say) (Heteroptera: Anthocoridae), Tq = *Trechus quadristriatus* (Schrank) (Coleoptera: Carabidae), Sc = *Stenolophus comma* (F.) (Coleoptera: Carabidae), Cm = *Coleomegilla maculata lengi* Timberlake (Coleoptera: Coccinellidae).

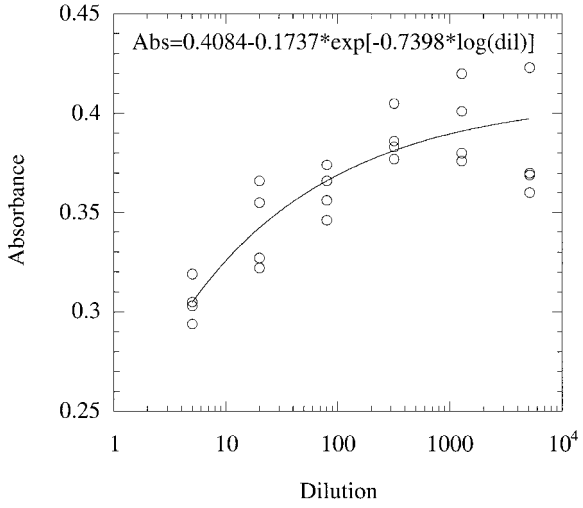

Figure 4. Absorbance of dilution series of *C. maculata* with constant concentration (1:30 000) of *P. rapae* first instar, observed values and fitted function.

appears to be confined mainly to wild crucifers growing in and near wooded areas, and *P. rapae* is now the only pierid known to occur in significant numbers on crucifer crops in the state (Hovanitz, 1962; Shapiro, 1968, 1974; Petzoldt et al., 1991).

Besides *P. napi*, many of the other species gave absorbances slightly above background levels; however, none of these cross-reactions was sufficiently high to compromise use of the ELISA to detect *P. rapae* predation in the field, provided a sufficiently conservative positive-negative threshold is established. The level of specificity of our ELISA is similar to that of other, similar ELISA tests to detect arthropod predation. In general, polyclonal antisera do not provide species-level specificity, and attain genus-level specificity only after pretreatment to remove cross reacting antibodies (Greenstone, 1996).

Assays with seven predator species. ELISA absorbances of predators fed *P. rapae* were highly variable, but in most cases fell well above absorbances of unfed controls and well above our positive-negative threshold. However, the assay did not effectively distinguish fed and unfed female *P. opilio*. Because the female *P. opilio* were the largest predators tested (mean weight 64.2 mg), the 100 μl assay samples for this species, which were taken from solutions prepared by homogenizing each individual at 1:70 concentration in sample buffer, would probably have contained the smallest amount of *P. rapae* material per 100 μl test volume. In contrast, *P. rapae*-fed individuals of the smallest species tested, *Orius insidiosus* (Say) (mean weight 0.31 mg), had the highest absorbances and probably contained the highest concentration of *P. rapae* in the test solution. Male *P. opilio* weighed about half as much as the females (mean weight 34.6 mg), perhaps accounting for the difference in assay response between sexes of this species (Figure 3). In future assays it may be advisable to homogenize only the guts of the larger predators to prevent excessive dilution of any *P. rapae* antigen present.

The large degree of variation within species in absorbances of fed predator samples (Figure 3) has also been observed in other immunological assays with arthropods (e.g., Fichter & Stephen, 1981, 1984; Lövei et al., 1985; Symondson & Liddell, 1993). This

variation can reflect many factors which may vary among the individuals tested, such as initial physiological condition and degree of satiation and the quantity of prey actually ingested in the experiment. The low absorbances of the one *P. rapae*-fed individual each of *S. comma* and *C. maculata* are puzzling however, since all the predators were observed to consume the proffered *P. rapae* first instars before being tested. Two possible explanations are incomplete homogenization of the samples or faster digestion of antigen in those individuals.

Effect of predator homogenate on assay sensitivity. Results of the tests on seven predator species fed *P. rapae* first instars showed a trend of decreasing assay response with increasing predator size. This resulted at least in part from a decrease in the amount of *P. rapae* material in the large predator samples. By assaying samples containing a constant concentration of *P. rapae* first instar homogenate in a dilution series of *C. maculata* homogenate (Figure 4), we demonstrated a second possibility: that increased ratio of predator material to *P. rapae* material in the sample solution may by itself contribute to reduced assay response. Hagler et al. (1997) found a similar result with increasing dilution of bovine serum albumin (BSA) in the sample solution, except in their case absorbance increased exponentially. In their ELISA the samples were placed directly into uncoated wells, so nontarget protein at high concentrations could potentially saturate the well's binding sites, reducing binding of the target protein. In the double antibody sandwich ELISA that we used, test solutions were placed in wells precoated with specific antibody, so even with nontarget antigens present at high concentrations, substantial binding of the target antigen can still occur. Thus, as Hagler et al. (1997) predicted, the effect of increasing nontarget protein appears less pronounced in the double antibody sandwich assay. The fact that it still occurs to some extent indicates that some cross-reacting antibodies are present in our coating IgG solution.

Kapuge et al. (1987) developed an indirect ELISA against immature stages of *P. rapae* using a format similar to that of Hagler et al. (1997). Although the assay was said to be both sensitive and specific, no data were presented documenting the sensitivity and specificity of their test, nor was it stated what threshold was used to distinguish positive from negative samples. Thus we have no basis by which to compare our assay to theirs.

Differences in assay conditions may cause diffences in binding capacity or reaction rates across multiple assays resulting in different absorbance measurements for a given quantity of antigen. ELISA standardization is therefore essential to ensure that results are consistent and reliable from one assay to the next. Numerous standardization protocols have been used in ELISA (reviewed by de Savigny & Voller, 1980; Tijssen, 1985), but standardization has often been neglected in arthropod predation assays. A method frequently used is to include a dilution series of antigen on each ELISA plate and fit a standard curve to absorbance versus concentration for the standards. This method has the advantage of allowing estimation of antigen concentration from the absorbance values of the test samples. The major disadvantage is the need to devote multiple wells on each plate to the dilution series, thus reducing the number of samples that can be tested on each plate. We standardized our assay and our positive-negative threshold by including a single dilution of a positive control on each plate and calculating absorbance of each sample as a percentage of this positive control value. This method allows one to use only a few wells on each plate for the positive standard and eliminates the need to fit a standard curve to the dilution series for each plate. The drawback in this case is that the method does not allow for estimates of antigen concentration, because the relationship between absorbance and antigen concentration tends to be curvilinear (Tijssen, 1985). The lack of a direct linear relationship between percent of positive and antigen concentration must be kept in mind when viewing the results of our specificity testing (Table 1) and the assays on predators fed *P. rapae* (Figure 3). Our objective, however, was not to estimate concentration of *P. rapae* antigens in predators, but rather to identify predators that have fed on *P. rapae*. Using a positive-negative threshold based on percentage of a positive control, while lacking the quantitative precision of the dilution series method, nevertheless helps increase assay consistency by using the positive controls on each plate to rescale the absorbances and thereby adjust for any differences between assays.

The development and characterization of our *P. rapae* ELISA illustrates several important issues in design and implementation of ELISAs for studying arthropod predation. First, by setting a conservative positive-negative threshold based on tests with possible alternative prey, we can ensure a low probability of false positives; however, the tradeoff for this benefit is that detection periods using such a threshold may

be reduced. The implications of this tradeoff will become clearer as we obtain data on detection periods for predator species of interest under various conditions likely to be experienced by the predators in the field. A second issue is raised by the significant differences we found in the assay's ability to detect different *P. rapae* stages. It seems likely that detection periods will also differ depending on the stage of *P. rapae* consumed, so this aspect should also be evaluated for those stages that are susceptible to predation. In addition, we have shown that the amount of predator material in samples affects the assay response. Even though the effect appears to be less pronounced than in an ELISA format using a single antibody to detect antigen bound directly to the plate wells (Hagler et al., 1997), it remained significant in our assay despite the use of a capture antibody in the double antibody sandwich ELISA format. In practice, the resulting reduced ability to detect prey in large predators could probably be remedied by assaying only their guts.

Our ELISA differed from most others developed for arthropod predation studies in that we used a biotinylated second antibody and streptavidin-alkaline phosphatase conjugate instead of the usual more direct antibody-alkaline phosphatase conjugate. Although it adds a step to the ELISA protocol, the streptavidin-biotin system has several advantages, including ease of conjugations and often greater sensitivity and lower backgrounds (Tijssen, 1985). In addition, it offers the opportunity for signal amplification via use of enzyme conjugated streptavidin-biotin complexes or complexes of streptavidin, biotinylated enzyme, and biotinylated-anti-enzyme IgG (Diamandis & Christopoulos, 1991; Wilson & Easterbrook-Smith, 1993; Kricka, 1994; Grumbach & Veh, 1995). Amplification was not used in our assay, although it remains an option if increased sensitivity is required.

Another aspect of our ELISA that differs from others used for arthropod predators is the use of an acetone powder to reduce cross-reactions by preadsorbing the antibody conjugate. Preadsorbing after – rather than before – conjugation may appear wasteful; however, in preliminary trials we found that preadsorbing before conjugation resulted in too great a reduction in sensitivity (M. A. Schmaedick, unpubl.). The success of our method could be due in part to the high ratio of acetone powder to IgG used and the fact that some soluble components of the acetone powder remaining in the solution when it was applied to the wells may have continued to compete with bound antigen for any cross-reacting biotinylated IgG. Whatever the cause, the preadsorption has proven to be an effective, simple procedure, and the acetone powder can be conveniently produced and stored in quantities sufficient for thousands of assays (Harlow & Lane, 1988).

In conclusion, use of the streptavidin-biotin system and preadsorption with an acetone powder have enabled us to produce a sensitive and specific ELISA from a polyclonal antiserum raised to crude extract of *P. rapae* larvae. Although additional experiments are required to measure detection periods, the results described here on other aspects of the *P. rapae* ELISA demonstrate that the test has sufficient sensitivity and specificity to become a useful tool in evaluating the effects of arthropod predators on *P. rapae* populations in cabbage fields.

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