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Immunological Examinations of Species Variability in Predator Gut Content Assays: Effect of Predator:Prey Protein Ratio on Immunoassay Sensitivity

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In qualitative predator gut content immunoassays, sensitivity of an immunoassay is important for determining whether a predator contains a targeted prey antigen. If the immunoassay employed is insensitive, the probability of obtaining a false-negative reaction is high. The sensitivity of an indirect enzyme-linked immunosorbent assay (ELISA) developed to detect pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), egg(s) in whole body homogenized predators varied in efficacy between species. Specifically, the indirect ELISA was more effective at detecting egg antigen in small predators than in large predators. In this study, we examined the effect that the predator:prey protein ratio has on the sensitivity of an indirect ELISA. Our results suggest that when assaying whole body homogenized predators, care must be taken not to overload an ELISA microplate with nontarget (predator) proteins. Predator samples should be diluted to less than 125 μg of total protein per sample. Any protein concentration above 125 μg per ELISA microplate well will likely result in an ELISA false-negative reaction. In another experiment, we compared the efficacy of an indirect ELISA with a dot blot immunoassay. Adults of *Hippodamia convergens* Guerin-Meneville (Coleoptera: Coccineliidae) that had eaten one pink bollworm egg were homogenized in variable dilutions of phosphate-buffered saline (PBS) and each sample was analyzed for pink bollworm egg antigen using both immunoassays. The dot blot immunoassay was more reliable than the indirect ELISA for detecting minute traces of egg antigen in the samples. Generally, the volume of PBS that the *H. convergens* were homogenized in had little effect on the qualitative outcome of the dot blot. However, the indirect ELISA was more effective when *H. convergens* was homogenized in a larger volume of PBS. This suggests that the efficacy of an indirect ELISA can be improved for large, protein-rich predators by grinding them in a larger volume of PBS, thus minimizing the total protein in a given sample. © 1997 Academic Press

KEY WORDS: predation; gut content analysis; ELISA; pink bollworm; monoclonal antibody.

INTRODUCTION

We have developed an indirect enzyme-linked immunosorbent assay (ELISA) for examining whole body homogenates of predators of pink bollworm, *Pectinophora gossypiella* (Saunders), eggs (Hagler *et al.*, 1994). This indirect ELISA has been useful for identifying the frequency with which predator species feed on pink bollworm eggs in the field (Hagler and Naranjo, 1994a,b; 1996). Recently, we conducted a series of controlled laboratory experiments that suggested that the indirect ELISA varies in sensitivity between predator species. Initially, we were not concerned with this variable sensitivity because predator gut content immunoassays are inherently qualitative in nature. It has been reported that differences between predator species in gut content immunoassays can be attributed to a combination of confounding abiotic and biotic factors. For instance, temperature variations, predator metabolic rate, quantity of prey consumed, and development stage of the prey consumed can all affect the quantitative outcome of a gut content immunoassay (McIver, 1981; Lovei *et al.*, 1985; Hagler and Cohen, 1990; Hagler *et al.*, 1992). While these variables explain some of the inherent variation common with gut content immunoassays, they do not explain why some species are more sensitive in the indirect ELISA than others. We determined in a standardized laboratory study (predators were fed a single, 1-day-old pink bollworm

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egg and assayed immediately after feeding) that the indirect ELISA was 100% effective at detecting egg antigen in whole body homogenates of the minute pirate bug, *Orius insidiosus* (Say) (Heteroptera: Anthocoridae). However, the indirect ELISA only detected egg antigen in 57% of big-eyed bug, *Geocoris punctipes* (Say) (Heteroptera: Lygaeidae), and 4.0% of convergent lady beetle, *Hippodamia convergens* Guerin-Meneville (Hagler and Naranjo, 1997). It appears that the efficacy of this standardized indirect ELISA (whole body insects were homogenized in 500 µl of buffer) was inversely proportional to the size (total protein content) of the predator under examination.

The indirect ELISA's variable sensitivity for detecting prey remains among different predator species prompted us to investigate this phenomenon further. In this study, we examine what effect total protein content has on the efficacy of a indirect gut content ELISA using *O. insidiosus, G. punctipes,* and *H. convergens.* We selected these three predator species because they are all known to feed on pink bollworm eggs, they represent a small (*O. insidiosus*), a medium (*G. punctipes*), and a large (*H. convergens*) predator species, and they have been shown to yield varying results between species with the indirect gut content ELISA. We also conducted additional experiments toward increasing the efficacy of the indirect ELISA for detecting prey remains in whole body homogenates of *H. convergens.* Specifically, we determined the optimal protein concentration that a whole body predator homogenate should contain for the indirect ELISA to minimize false-negative reactions. Finally, we compared the efficacy of the indirect ELISA with a gut content dot blot assay that we recently developed (Hagler *et al.,* 1995).

MATERIALS AND METHODS

Predators. Adult *O. insidiosus* and *G. punctipes* originated from laboratory cultures maintained at our facility. Adults of *H. convergens* were purchased from Nature's Control (Medford, OR). Predators were maintained at 27°C, 40% RH, and a photoperiod of 14:10 h (L:D). All predators were fed beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), eggs, cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), eggs, and green bean *ad lib.* for a minimum of 1 week prior to the feeding trials.

Voucher specimens of all predators used in this study were placed at the USDA-ARS Western Cotton Research Laboratory (Phoenix, AZ).

Indirect gut content ELISA. Frozen predators were assayed by the indirect ELISA described by Hagler and Naranjo (1994a,b). Samples were prepared for ELISA by homogenizing individual predators in 500 µl phosphate-buffered saline (PBS, pH 7.4) unless otherwise noted. Individual wells of the 96-well assay plate (Falcon Pro-Bind 3915) were coated separately with a 100-µl aliquot of each sample and incubated at 4°C overnight. The unbound antigen was discarded from the assay plate and 360 µl of 1.0% (10.0 mg/ml) nonfat dry milk in distilled H_2O was added for 30 min at room temperature to block any unoccupied protein binding sites in the wells. Wells were rinsed three times with PBS–Tween 20 (0.05%) and twice with PBS. Fifty microliters of pink bollworm monoclonal antibody (MAb) was then added to each well (Hagler *et al.,* 1994). Plates were incubated for 1 h at room temperature and then rinsed as above. Aliquots (50 µl) of goat anti-mouse IgG/IgM conjugated to alkaline phosphatase (TAGO Inc., Burlingame, CA) diluted 1:500 in 1.0% nonfat milk were added to the wells and incubated for 1 h at room temperature. Plates were again rinsed as above, and 50 µl of 1.0 mg/ml *p*-nitrophenyl phosphate substrate (Sigma Chemical, St. Louis, MO) in 1 *M* diethanolamine and 0.5 m*M* $MgCl_2$ (pH 9.8) was added to each well. After 2 h, the absorbance of each well was measured using a Cambridge Technologies Model 750 (Waterton, MA) microplate reader set at 405 nm. A sample was considered positive for pink bollworm egg antigen if an individual reading was three standard deviations above the value of the mean negative predator control reading.

Gut content dot blot immunoassay. A predator gut content dot blot immunoassay was conducted using the method described by Hagler *et al.* (1995). A 10-µl aliquot of predator homogenate was pipetted onto a 0.45-µm sheet of nitrocellulose membrane. After drying, the membrane was submerged in 100 ml of 1.0% nonfat dry milk in distilled water for 30 min to block any nonspecific binding sites. The nonfat milk was discarded and the membrane was rinsed three times in PBS–Tween 20 and twice in PBS. The membrane was then incubated for 1 h in 25 ml of pink bollworm MAb. After incubation, the MAb was discarded and rinsed as described above. The membrane was then soaked in 25 ml of goat anti-mouse IgG/IgM conjugated to alkaline phosphatase (TAGO Inc.) diluted to 1:500 in 1.0% nonfat milk for 1 h. The conjugated antibody was discarded and the membrane was rinsed as noted above. The membrane was then developed using an alkaline phosphatase conjugate substrate kit obtained from Bio-Rad Laboratories (Richmond, CA) for 3 min. The color of each insect sample was measured with a Minolta Chroma Meter Model CR-221 (Hagler *et al.,* 1995). The color values (hue) registered on a chroma meter range from 0 (black) to 100 (white); therefore, a positive response (purple) on a dot blot for the presence of pink bollworm egg antigen will yield a lower value than the value of a negative control. Individuals were scored positive for pink bollworm egg antigen if the color was three standard deviations below the value of the mean negative predator control reading (Hagler *et al.,* 1995).

Effect of predator's total protein content on indirect ELISA sensitivity. Predators devoid of pink bollworm egg antigen were frozen at −80°C for 72 h. Individuals were removed from the freezer, sorted by sex, and weighed. Most of these individuals (n = 25–35) were homogenized in 450 µl of PBS; the remaining predators were saved for negative controls. Separately, a stock solution was made by homogenizing 200 pink bollworm eggs in 10 ml of PBS. A 50-µl aliquot of this stock solution represented the equivalent of a single PBW egg. A 50-µl aliquot of stock egg antigen solution was added to each predator sample for a total volume of 500 µl. The remaining individuals (n = 7–21) were homogenized in 500 µl PBS (negative controls). The total protein content from each individual was determined by the Bradford method (1976) using the reagents supplied in a Bio-Rad protein determination kit (Bio-Rad Laboratories, Richmond, CA). Differences in the mean protein content between predator species were compared by an ANOVA (SAS Institute, 1982) and a Tukey's mean separation test to identify significant differences. Differences in the mean protein content within each predator species for each treatment (males with an egg added, male negative controls (no egg), females with an egg added, and female negative controls) also were compared by an ANOVA, and a Tukey's mean separation test was conducted to identify significant differences in protein content. The relationship of fresh predator weight (mg/predator) and total protein content (µg/predator) was calculated using the exponential rise to maximum option in SigmaStat (Jandel Scientific Software, Jandel Inc., San Rafael, CA).

Following protein determination, each sample was assayed by the indirect ELISA described above. The mean ELISA absorbance value was calculated for each treatment. In addition, each individual predator was scored positive for pink bollworm egg antigen if the ELISA absorbance value was three standard deviations above the value of the respective negative control mean (Schoof *et al.*, 1986; Sutula *et al.*, 1986).

In a separate experiment, we eliminated the predators from the samples and added 50 µl of pink bollworm egg stock solution (equivalent to one egg) to various concentrations of bovine serum albumin (BSA) (n = 8). BSA concentrations ranged from 0 (PBS control) to 10 mg/ml BSA (this is the nonfat milk protein concentration used to block the nonspecific binding sites of an ELISA microplate). Each sample was then assayed by the ELISA described above.

Data were plotted as quantity of BSA (µg) in a sample containing a single pink bollworm egg (x axis) versus the absorbance value yielded by the indirect ELISA (y axis). A double exponential decay equation was fitted to these data using SigmaStat (Jandel Scientific Software).

Optimizing gut content immunoassays. The previous experiments indicated that the sensitivity of our indirect gut content ELISA was inversely proportional to the total protein concentration present in the sample. Next, we tried optimizing the gut content ELISA by homogenizing individual *H. convergens* in various volumes of PBS. We separately homogenized individuals in PBS and added the equivalent of a single pink bollworm egg (50 µl from our stock egg antigen solution). Dilutions ranged from 250 to 1500 µl/beetle (in 250-µl increments, n = 20 per treatment). Each treatment was accompanied by an appropriate negative control dilution (*H. convergens* free of any pink bollworm egg antigen, n = 20). Each individual was assayed by the indirect ELISA described above. We also assayed each of these samples by the gut content dot blot described above.

In a second experiment, we fed *H. convergens* a single, 2-day-old pink bollworm egg and then immediately homogenized these individuals in a different volume of PBS (n = 20 per treatment). Again, each treatment was accompanied by the appropriate negative control (n = 20). Each individual was assayed by the indirect gut content ELISA and gut content dot blot assays described above.

RESULTS AND DISCUSSION

Effect of predator protein content on ELISA sensitivity. Protein content among the three predator species was significantly different (F = 391.7; df = 2; $P <$ 0.0001). The overall mean protein concentration (±SD) was 29.0 ± 15.0, 267.7 ± 62.3, and 717.4 ± 269.9 µg per insect for *O. insidiosus*, *G. punctipes*, and *H. convergens*, respectively. The mean total protein contents for the female treatments (egg and no egg treatments) were significantly higher than the mean total protein of the males of *O. insidiosus* and *G. punctipes*, but not *H. convergens* (Fig. 1).

After we determined total protein content, each predator was assayed by a standardized indirect ELISA. The standardized ELISA consisted of homogenizing each predator, regardless of its total protein content, in 500 µl of PBS. None of the no-egg negative controls reacted to the egg-specific ELISA (Fig. 1). Pink bollworm egg antigen was detected in every *O. insidiosus* sample that was spiked with a single egg, yielding a mean ELISA absorbance value greater than 1.0 (Fig. 1). The *G. punctipes* samples spiked with a single egg were not as immunoreactive as the *O. insidiosus* samples. However, 97% of the *G. punctipes* males and 73% of the females assayed scored positive for egg antigen. The ELISA was unreliable for detecting egg antigen in *H. convergens*. Only 27% of *H. convergens* males and 30% of the females containing a single egg reacted to the ELISA (Fig. 1).

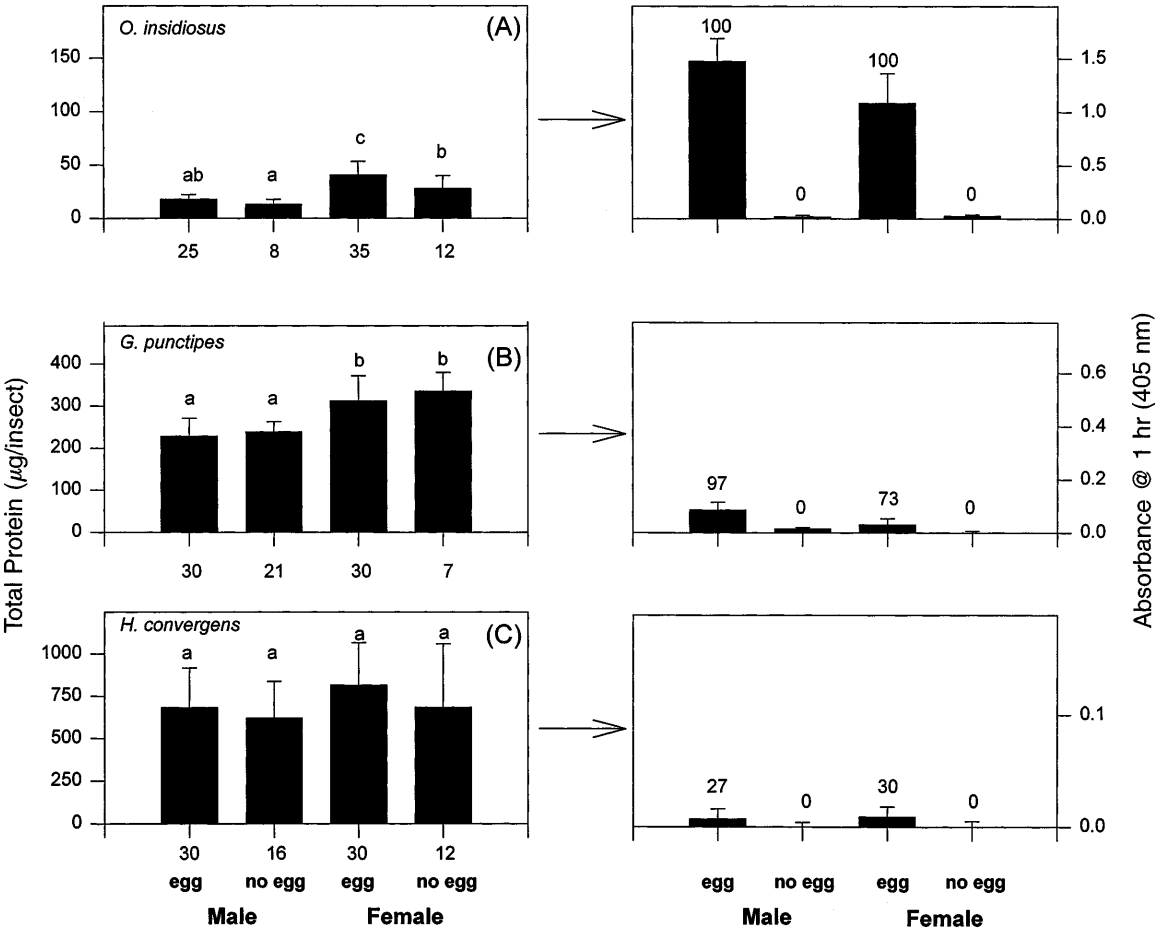

FIG. 1. Mean protein content of male and female (A) *Orius insidiosus*, (B) *Geocoris punctipes*, and (C) *Hippodamia convergens* that contain either one pink bollworm egg equivalent or no pink bollworm egg antigen (negative control). Letters above the error bars indicate significant differences between the treatment means as determined by a Tukey's mean separation test, $P < 0.01$. Numbers below the tick marks on the *x* axis are the sample size for each treatment. Graphs to the right are the mean ELISA absorbance values (±SD) for each predator species. Numbers above the error bars are the percentage of individuals scoring positive by ELISA for each treatment.

There was a strong nonlinear relationship between insect weight and total protein (Fig. 2). This suggests that weight alone can be used to estimate the total protein content of a predator. Therefore, the optimal quantity of PBS that a given predator species should be homogenized in can be determined simply by weighing the predator. Using the weight of a predator to predict its protein content would eliminate labor-intensive protein analyses and chemical waste.

Previous studies also have shown considerable species variation in predator gut content immunoassays (Sunderland *et al.*, 1987; Sopp and Sunderland, 1989). Most studies have attributed differences in immunoreactivity between and within predator species to variable metabolic rate as a function of time and/or temperature (e.g., Fichter and Stephen, 1984; Sopp *et al.*, 1992; Greenstone and Hunt, 1993). For example, Symondson and Liddell (1993) found that two closely related carabid beetles, *Abax parallelepipedus* Piller and Mitterpacher and *Pterostichus madidus* Fabricius, show considerable differences in immunoreactivity after feeding on slug prey. *P. madidus* was almost twice as immunoreactive and retained slug prey remains in its gut 2.5 times longer than *A. parallelepipedus*. Sopp and Sunderland (1989) found that temperature affected the outcome of their sandwich ELISA targeted at identifying aphid remains in the guts of predaceous arthropods. Specifically, they found that immunoreactivity decreased as temperature increases. Furthermore, prey retention time was less for staphyliniid (Insecta: Staphylinidae) predators than for carabid (Insecta: Carabidae) or linyphiid (Arachnida: Linyphiidae) predators (Sopp and Sunderland, 1989). We found considerable species variation in immunoreactivity among the three species examined in this study, as well as in another study in which we examined the retention of egg antigen in the guts of these three predator species in relation to time, temperature, and prey size. Our results concurred with another study in which a predator's prey retention time decreases as temperature increases

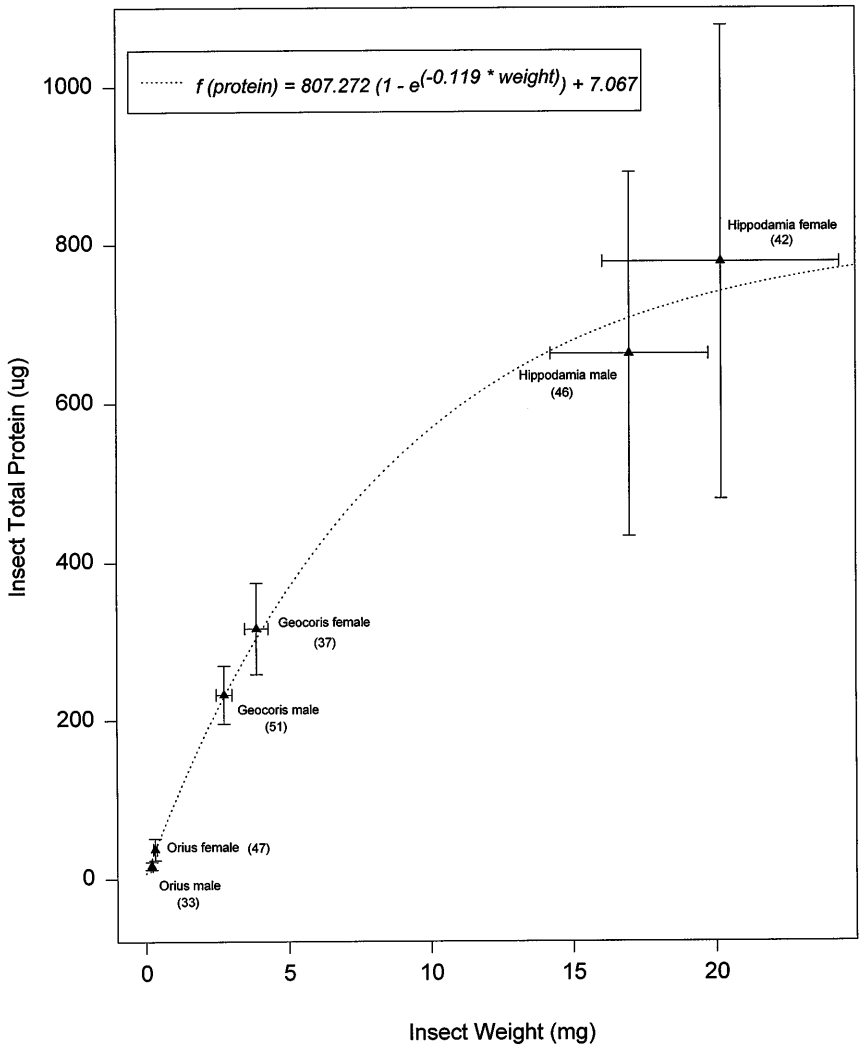

FIG. 2. Relationship between fresh insect weight and insect total protein. The six data points represent the mean weight ±SD (*x* axis) and the mean protein concentration ±SD (*y* axis) of *O. insidiosus* males, *O. insidiosus* females, *G. punctipes* males, *G. punctipes* females, *H. convergens* males, and *H. convergens* females, respectively. Numbers in parentheses near each data point represents the sample size for each insect species and sex examined.

(Hagler and Naranjo, 1997). Moreover, the retention time was greater for smaller predators than for larger predators (*O. insidiosus* > *G. punctipes* > *H. convergens*). These two studies suggest that predator:prey protein ratio affects the indirect ELISA sensitivity and ultimately the estimation of retention time in a predator's gut.

We also examined the effect of nontarget protein(s) on quantitative and qualitative outcomes of an indirect ELISA targeted for detecting minute traces of pink bollworm egg antigen in a given sample. In this study, we standardized the total protein concentration of the assay by eliminating predators from samples and substituting them with a known quantity of purified BSA. We then added the equivalent of a single pink bollworm egg to each sample and analyzed it using our indirect ELISA. The quantity of protein in the samples had a profound effect on the quantitative outcome of the ELISA. Samples containing a lower concentration of BSA yielded higher ELISA absorbance values than samples containing a higher BSA concentration (Fig. 3). The ELISA absorbance value declined exponentially with each serial increase in protein concentration. The qualitative outcome of the indirect ELISA (based on the percentage of positive responses) was not affected until the protein concentration exceeded 125 µg/sample (Fig. 3). Samples containing more than 125 µg of protein often yielded an ELISA false negative. Because of the rapid decline in ELISA absorbance value, we used a double exponential equation to model the sensitivity of the indirect ELISA relative to total protein content (Fig. 3). This relationship suggests that there is a rapid initial decay of ELISA sensitivity as protein content increases followed by a slower, more gradual decay.

It appears that samples containing a large quantity of nontarget protein(s) saturate the competitive bind-

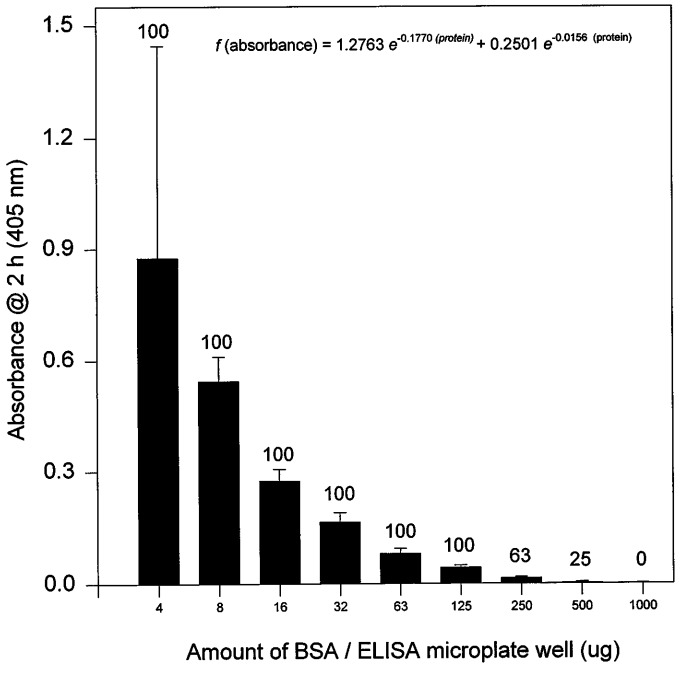

FIG. 3. Mean ELISA absorbance values for samples containing a single pink bollworm egg mixed in various concentrations of bovine serum albumin (BSA). Numbers above the error bars are the percentage of individuals scoring positive for each treatment ($n = 8$).

ing sites on an ELISA microplate matrix. Consequently, the probability of minute traces of targeted egg (prey) antigen reaching one or more of the binding sites on the ELISA microplate is greatly reduced. The net result of overloading an ELISA plate with extraneous predator proteins is an increased probability of obtaining an ELISA false negative. This suggests that the total protein present in a predator homogenate must be carefully calibrated if one is using an indirect ELISA. Previously, we used a standardized indirect gut content ELISA for all predator species because we were only interested in a qualitative evaluation of predator feeding behavior (Hagler and Naranjo, 1994a,b). Because uncontrollable factors such as variable predator digestive rate (Symondson and Liddell, 1993), predator prey size (Sopp and Sunderland, 1989), prior metabolic status (Lovei *et al.*, 1990), temperature (McIver, 1981), and the developmental stage of the prey (Hagler *et al.*, 1992) can all affect the quantitative outcome of gut content immunoassays, we standardized the first step (coating) in the indirect ELISA by grinding all predator species in 500 µl of PBS. However, it appears that a 500-µl dilution yields a solution too high in extraneous protein for large predators that consume a minute quantity of prey. The net result is an under estimation of the proportion of individuals feeding on the targeted prey. Most investigators employing gut content immunoassays have used whole body homogenates for their assays (Dempster, 1966; Ragsdale *et al.*, 1981; Fichter and Stephen, 1984; Hagler *et al.*, 1992); however, very few investigators have considered the total protein present in their samples as an important variable affecting the qualitative or quantitative outcome of indirect immunoassays. Lovei *et al.* (1985) adjusted their protein concentration in whole body homogenated predators to ensure that only 100 µg (1 µg/µl) of total protein was present in each sample for a sandwich ELISA. Ragsdale *et al.* (1981) and Greenstone and Hunt (1993) cut and analyzed portions of a predator to detect prey remains in its gut. Some investigators have dissected predator guts and assayed only the gut contents (Dempster, 1960; Sunderland *et al.*, 1987; Hagler and Cohen, 1990). Symondson and Liddell (1993) took gut content immunoassays a step further by dissecting and analyzing only the crop contents of carabid beetles. Carabids offer the advantage of being large and easy to dissect (Symondson, personal communication). Obviously, gut or crop dissections eliminate most of the extraneous nontarget predator proteins. However, for most pink bollworm egg predators, gut dissections are too tedious and laborious. Hence, gut dissections are not practical for qualitatively screening numerous predator species and hundreds of individuals on a daily basis (Hagler and Naranjo, 1994a,b).

Adding an egg to a standard protein, such as BSA, proved useful for estimating the outcome of an indirect ELISA based solely on the total protein present in a sample. An exponential decay relationship existed between the quantity of protein in the samples and the mean ELISA absorbance values (Fig. 3). If the total protein of a predator species is known, then the outcome of an ELISA can be predicted using an equation (Fig. 3) (assuming one egg has been eaten). For example, a female *O. insidiosus* contains an average of 32.9 µg of protein (Fig. 1). Since we assayed one-fifth of a predator (a 100-µl aliquot was assayed from a 500-µl homogenate) in our indirect ELISA, we have the equivalent of 6.58 µg of predator protein in the ELISA well. If 6.58 µg is substituted into the equation (Fig. 3), the predicted ELISA absorbance value is 0.623. This relatively high ELISA absorbance value suggests that the indirect ELISA will easily detect a single prey in a homogenized *O. insidiosus* sample.

Optimizing gut content immunoassays. We attempted to minimize the high frequency of the ELISA false-negative reactions that occur with the *H. convergens* samples containing a single pink bollworm egg. First, we added the equivalent of one egg (from a stock egg antigen solution) to *H. convergens* samples that were homogenized in 250 µl (286.8 µg/well) to 1500 µl (47.8 µg/well) PBS. We then analyzed each sample by an indirect ELISA. Additionally, each sample also was analyzed by the more sensitive dot blot immunoassay (Stuart and Greenstone, 1990; Hagler *et al.*, 1995). All of the negative control *H. convergens* analyzed by

TABLE 1

ELISA and Dot Blot Results Testing for Pink Bollworm (PBW) Egg Antigen When the Equivalent of One PBW Egg Was Added to *Hippodamia convergens* Samples Containing a Variable Volume of PBS

			Absorbance value for *H. convergens* assayed by ELISA					
			Negative control *H. convergens*			*H. convergens* with one PBW egg added		
Immunoassay	PBS vol. (µl)	*n*	$\overline{X} \pm$ SD	Critical[c] value	No. positive reactions[d]	*n*	$\overline{X} \pm$ SD	No. positive reactions
ELISA	250	20	0.029 ± 0.010[a]	0.059	0	20	0.069 ± 0.028	11
	500	20	0.019 ± 0.012	0.055	0	20	0.072 ± 0.028	16
	750	20	0.016 ± 0.010	0.046	0	20	0.067 ± 0.032	16
	1000	20	0.018 ± 0.008	0.042	0	20	0.115 ± 0.097	19
	1250	20	0.014 ± 0.006	0.032	0	20	0.240 ± 0.093	20
	1500	20	0.023 ± 0.006	0.041	0	20	0.452 ± 0.156	20
Dot blot	250	20	66.853 ± 4.958[b]	51.979	0	20	34.584 ± 6.181	20
	500	20	71.878 ± 5.750	54.628	0	20	35.867 ± 7.211	20
	750	20	76.223 ± 4.398	63.029	0	20	49.531 ± 7.614	20
	1000	20	77.561 ± 4.713	63.422	0	20	51.091 ± 8.941	20
	1250	20	75.602 ± 2.851	67.049	0	20	58.600 ± 4.072	20
	1500	20	77.141 ± 2.942	68.315	0	20	55.301 ± 5.764	20

[a] ELISA mean values are recorded from an ELISA microplate reader set at a 405-nm wavelength.
[b] Dot blot mean values are based on hue (color) values recorded from a Minolta Chroma Meter, Model 231.
[c] The critical value for the ELISA = $\overline{X}$ + 3 SD of the negative controls (Sutula *et al.*, 1986); the critical value for the dot blot = $\overline{X}$ − 3 SD of the negative controls (Hagler *et al.*, 1995).
[d] The number of positive reactions is based on the critical value of the negative control predators.

indirect ELISA and dot blot yielded negative absorbance values (Tables 1 and 2). The probability of obtaining an ELISA false negative decreased with each serial increase in PBS (Table 1). Nearly half (45%) of the *H. convergens* samples containing a single pink bollworm egg and mixed in 250 µl of PBS yielded an ELISA false negative. A single *H. convergens* contains approximately 717 µg of total protein (Fig. 1); therefore, a 100-µl aliquot from a 250-µl homogenate represents 286.8 µg of total protein. These results are similar

TABLE 2

ELISA and Dot Blot Results Testing for Pink Bollworm (PBW) Egg Antigen in the Guts of *H. convergens* after Eating a Single Egg

			Absorbance value for *H. convergens* assayed by ELISA					
			Negative control *H. convergens*			*H. convergens* fed one PBW egg		
Immunoassay	PBS vol. (µl)	*n*	$\overline{X} \pm$ SD	Critical[c] value	No. positive reactions[d]	*n*	$\overline{X} \pm$ SD	No. positive reactions
ELISA	250	20	0.024 ± 0.011[a]	0.057	0	20	0.040 ± 0.018	2
	500	20	0.026 ± 0.011	0.059	0	20	0.056 ± 0.032	9
	750	20	0.016 ± 0.008	0.040	0	20	0.040 ± 0.014	11
	1000	20	0.012 ± 0.007	0.033	0	20	0.047 ± 0.028	14
	1250	20	0.027 ± 0.014	0.069	0	20	0.067 ± 0.041	8
	1500	20	0.043 ± 0.022	0.109	0	20	0.085 ± 0.049	5
Dot blot	250	20	87.794 ± 3.959[b]	75.917	0	20	77.643 ± 5.620	8
	500	20	91.056 ± 4.069	78.849	0	20	77.302 ± 8.046	13
	750	20	102.330 ± 6.550	82.680	0	20	77.162 ± 9.413	15
	1000	20	82.738 ± 2.861	74.155	0	20	62.012 ± 10.270	17
	1250	20	79.648 ± 2.321	72.685	0	20	62.866 ± 13.081	15
	1500	20	91.933 ± 3.129	82.546	0	20	73.798 ± 10.848	14

[a] ELISA mean values are recorded from an ELISA microplate reader set at a 405-nm wavelength.
[b] Dot blot mean values are based on hue (color) values recorded from a Minolta Chroma Meter, Model 231.
[c] The critical value for the ELISA = $\overline{X}$ + 3 SD of the negative controls (Sutula *et al.*, 1986); the critical value for the dot blot = $\overline{X}$ − 3 SD of the negative controls (Hagler *et al.*, 1995).
[d] The number of positive reactions is based on the critical value of the negative control predators.

to the results obtained when an egg was added to 250 µg of BSA (Fig. 3). In that experiment, 37% of the samples assayed in 250 µg of BSA yielded false-negative ELISA reactions. The ELISA always detected egg antigen in the *H. convergens* samples homogenized in more than 1000 µl (or <71.7 µg of total protein per sample) of PBS. The dot blot immunoassay was more sensitive than ELISA for detecting egg antigen in the *H. convergens* samples. All of the samples to which we added an egg, regardless of the volume of PBS that the predators were homogenized in, scored positive by dot blot (Table 1). Furthermore, based on the lower Chroma Meter readings, the dot blot immunoreactivity was greater at the lower PBS dilutions (Table 1).

In the second experiment, we assayed individual *H. convergens* that had consumed a single, 2-day-old pink bollworm egg. The sensitivity and reliability of both immunoassays were less for detecting a single egg eaten by an individual *H. convergens* than for samples spiked with a single egg (Tables 1 and 2). Most of the *H. convergens* (90%) homogenized in 250 µl of PBS yielded ELISA false-negative reactions. While the ELISA absorbance value increased with each serial increase in PBS, the fewest false-negative reactions (30%) were associated with predators homogenized in 1000 µl of PBS. The predators homogenized in 1500 µl of PBS yielded absorbance values almost twice as high as the values of the 1000-µl treatment; however, some of the 1500-µl negative control samples yielded uncharacteristically high absorbance values. This resulted in a very high critical value for these treatments (Table 2). Subsequently, many of the samples that "looked" positive were statistically scored as negative reactions. Again, the dot blot immunoassay was more effective than the indirect ELISA for detecting egg antigen in *H. convergens* that had eaten a single pink bollworm egg. Over 70% of those individuals that had eaten an egg and then were homogenized in 750 µl or more of PBS responded to the dot blot immunoassay (Table 2). The maximum immunoreactivity (based on the lowest Chroma Meter readings and highest frequency of response) was achieved when predators were homogenized in 1000 µl of PBS (85% were positive). While none of the PBS dilutions were 100% effective at detecting pink bollworm antigen from a single egg, results suggest that the efficacy of gut content immunoassay can be improved by titering the total protein concentration in the sample and using a dot blot immunoassay.

Dot blots, although similar to indirect ELISAs, are more sensitive due to the greater number of binding sites on a nitrocellulose membrane than on an ELISA microplate matrix. Sensitivity appears to be crucial, particularly when analyzing the whole body macerates of large predators that consume a minute quantity of prey. Results from this experiment showed that the dot blot assay is more effective than the indirect ELISA at detecting pink bollworm egg antigen in *H. convergens*. The lack of 100% efficacy for *H. convergens* that ate a single pink bollworm egg may be attributed to differential reactivity of individual eggs (Hagler *et al.*, 1992), some enzymatic degradation of the antigen, a small degree of prey digestion, observer error during monitoring of predator feeding, or a combination of these factors.

It is well documented that the rarer a target antigen is in a complex mixture, the more difficult it is to detect by an indirect ELISA (Delves, 1995). The quantity of nontargeted (predator) protein in large predators is so overwhelming that prey antigens cannot effectively compete for the limited number of binding sites on an ELISA microplate matrix. The net outcome is a high incidence of false-negative responses. This problem, inherent to indirect ELISAs, can be overcome by developing a sandwich ELISA. A sandwich ELISA is designed to "pull out" a rare antigen from a complex mixture (see Greenstone, 1996, for a review of ELISA protocols). In a sandwich ELISA, each microplate well matrix is first coated with the pest-specific monoclonal antibody. After blocking, the homogenized predator sample is then added. Since the ELISA microplate was first coated with pest-specific antibody, the only antigen that can compete for a binding site on the matrix is the protein that the monoclonal antibody was developed to detect (the targeted prey remains). Thus, all extraneous predator proteins are eliminated as potential competitive binding sites on the ELISA microplate matrix. The "sandwich" is completed by adding a conjugated pest-specific antibody. Unfortunately, the development of a conjugated pest-specific antibody takes time, is an added expense, and requires technical expertise. However, if investigators are examining large predators that feed on minute quantity of prey, the benefits may outweigh the difficulty. Currently, we are developing a sandwich ELISA for future use in our predation studies.

In summary, the results from this study suggest that the predator:prey protein ratio will affect the quantitative and qualitative outcomes of a gut content immunoassay. The indirect ELISA is effective for detecting minute traces of prey in small predators, but is less effective at detecting small prey items in large predators. For large predator species that are easy to dissect, we recommend assaying only the dissected gut or crop contents. However, for mass screening many predator species, most of which are difficult to dissect (e.g., *O. insidiosus*, *G. punctipes*, and *H. convergens*), we recommend analyzing whole body homogenized predators (Boreham and Ohiagu, 1978). The total protein concentration present in the ELISA samples should not exceed 125 µg/sample to minimize the probability of an ELISA false-negative reaction. Since there is a strong exponential relationship between fresh insect weight

and protein, we suggest weighing the predator and using its weight (mg) to predict its protein content (μg) (Fig. 2). The volume of the PBS that a predator is homogenized in can then be adjusted so the protein concentration does not exceed 125 μg per ELISA microplate well. We also recommend, in some situations, a dot blot immunoassay over an indirect ELISA, despite some of the limitations of dot blots (for a review see Stuart and Greenstone, 1990, and Hagler *et al.*, 1995). Finally, we recommend studies such as these for each pest-specific gut content immunoassay. The sensitivity of pest-specific immunoassays will not only depend on the predator:prey protein concentration, but will also vary with the specificity and sensitivity of the pest-specific antibody employed and the sensitivity of the immunoassay used.

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