Yolk Protein Immunoassays (YP-ELISA) to Assess Diet and Reproductive Quality of Mass-Reared *Orius insidiosus* (Heteroptera: Anthocoridae)

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ABSTRACT A yolk protein enzyme-linked immunosorbent assay (YP-ELISA) was developed for the predator *Orius insidiosus* (Say). The YP-ELISA is intended to assess reproductive response to dietary and other rearing conditions, and to assist in quality control and diet development for mass rearing. Hybridomas and monoclonal antibodies were produced against homogenates of eggs dissected from females. Hybridomas were selected for secretion of IgG that reacted with extracts of both females and their eggs, and that did not react with male extracts. Each cloned hybridoma produced a monoclonal antibody that specifically reacted on western blots against one of the two major yolk polypeptides, apoVn-I (180,000 molecular weight) or apoVn-II (40,000). Yolk protein ELISAs were developed with these antibodies to assess yolk protein content of female *O. insidiosus* as a measure of reproductive fitness and as a potential predictor of fecundity. Protocols for an indirect antigen ELISA and double antibody sandwich ELISA were developed to assess yolk protein contents of eggs and total contents in whole body homogenates. ELISA standards consisted of homogenates of eggs collected 0–24 h following oviposition. As determined with the sandwich ELISA, yolk protein contents of eggs declined with age before hatch, with a half-life of 32–34 h. Results were similar whether the detecting antibody-enzyme conjugate was anti-apoVn-I or anti-apoVn-II. Optimal conditions and sampling parameters were developed for the sandwich ELISA, which demonstrated minimal nonspecific interference in whole-insect extracts. In an initial application of the YP-ELISA, oviposition rates over a 10-d period were compared with yolk protein contents at the end of that period, dependent on diets of differing nutritional composition and quality. High and low yolk protein contents correlated with oviposition rates on respective diets, though oviposition showed more graded response to diets than did yolk protein. Improvements in sampling methods are discussed.

EFFICIENT MASS REARING of beneficial insects is limited by technical issues affecting quality control, efficiency of production, standardization, and adaptation of systems to new species. Existing basic knowledge of insect reproduction can help to resolve some of these issues by providing the tools needed to assess the efficiency, reliability, and durability of insect mass rearing systems. We recently proposed one such tool, the yolk protein enzyme-linked immunosorbent assay (YP-ELISA) (Shapiro et al. 2000). Its development is based on determination of the yolk protein vitellin (Vn) in eggs or whole insects, and of its precursor, vitellogenin (Vg), in hemolymph. The YP-ELISA can be used to directly determine the total content of yolk proteins in the whole body or the concentrations of their precursors in the hemolymph. Assuming that nutritional and environmental conditions affect rates of yolk protein synthesis, cumulative yolk protein content in an insect before oviposition should indicate the strength of her reproductive response. By quantifying reproductive responses and comparing them to response under standard conditions, the YP-ELISA may by used to develop and improve artificial insect diets by rapidly testing the fitness and reproductive potential of adult females in a colony.

Several ELISAs incorporating monoclonal antibodies (MAbs) against Vn have been used to differentiate closely related species of herbivores and their eggs (Greenstone et al. 1991, Goodman et al. 1997, Agusti et al. 1999) and mosquitoes (Ma et al. 1990), and to identify prey with the predators or parasites that ingested them (Hagler et al. 1992, Hagler et al. 1993, Greenstone and Hunt 1993, Greenstone and Trowell 1994, Stuart and Greenstone 1997, Zeng et al. 1998, Sansone and Smith 2001). Anti-Vn MAbs and quantitative ELISA have been used to assess reproductive response in pharaoh ant queens (*Monomorium pharaonis* L.) in relation to presence or absence of workers (Jensen and Borgesen 1995), and to assess Vn titers in whiteflies (Tu et al. 1997). Vg titers in hemolymph were recently measured by ELISA, and correlations were drawn with levels of mRNA and juvenile hor-

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mone in the grasshopper *Romalea microptera* (Borst et al. 2000). Use of the ELISA in nutritional studies was suggested. Cremonez et al. (1998) used a rocket immunoassay to measure Vg levels in response to different diets in honey bee workers. ELISAs have also been used to determine response of Vn or Vg levels to parasitism of *Tenebrio* (Webb and Hurd 1995), age and mating status in tortricid moths (*Choristoneura* spp.), and pupal development in the southwestern corn borer, *Diatraea grandiosella* Dyar (Zeng et al. 2000).

None of these studies have addressed the need to improve mass rearing systems. To assess the potential of YP-ELISA in predicting the reproductive fitness of mass-reared predators or parasitoids, we recently developed MAbs against egg polypeptides of the predatory pentatomid *P. maculiventris* (Shapiro et al. 2000). Here we report the development of MAbs against the predator *Orius insidiosus* Say (Hemiptera: Anthocoridae), and an ELISA that employs them to quantitatively assess whole-body content of yolk proteins. *O. insidiosus* feeds on a wide variety of hosts such as aphids, mites, thrips, whiteflies, and eggs of Lepidoptera (Kiman and Yeargan 1985). In an initial application of the YP-ELISA, we compared the effects of diets of differing nutritional composition and quality on yolk protein contents with the goal of increased speed of artificial diet development and improvement.

Materials and Methods

Insects and Diet. Adult *O. insidiosus* were purchased from Entomos, Gainesville, FL, or provided by Syngenta Bioline, Oxnard, CA, and had eclosed less than 24 h before receipt. Eggs from *O. insidiosus* for use as ELISA standards were collected off the Parafilm- carrageenan substrate 0–24 h after oviposition. Parafilm was excised, and oviposited eggs were vigorously rinsed from the underside of the Parafilm and from the carrageenan with distilled water from a wash bottle onto black filter paper (S&S, Keene, NH), removed under a dissecting microscope, and stored at −80°C.

Diet Quality Experiment. Adults were obtained from Entomos and placed on the diets on the third day after eclosion. Each diet treatment consisted of six females and four males in a 100-ml plant tissue culture jar (Sigma, St. Louis, MO) with four jars per treatment. Each jar contained the following components: one Parafilm-encapsulated water dome (50 μl), bee pollen-type III (3 grains, Sigma), one 7-cm section of green bean pod for oviposition, and three crumpled strips of waxed paper (5 by 80 mm) as substrate. Green beans, water domes, and diets were replaced every other day and eggs within the bean were counted under a microscope. *Plodia interpunctella* (Hübner) eggs were obtained from a laboratory colony reared on a standard diet as described by Silhacek and Miller (1972), held at 4°C for 24 h, and frozen and stored at −80°C until fed to *O. insidiosus*. Jars were held in a growth chamber at 25.5 ± 1°C, with 75 ± 5% RH and a photoperiod of 15:9 (L:D) h. The experiment was continued for 10 d after setup.

In addition to the above components, each jar contained two 50-μl artificial diet domes or eggs of *P. interpunctella* or *Ephestia kuehniella* Zellar. Control diet jars contained domes of artificial diet (Weiru and Ren 1989) that consisted of 4.0 g Brewer's yeast, 0.4 g sucrose, 2.1 g soy protein acid hydrolysate, 45 mg of 99% palmitic acid (all from Sigma), 0.5 g chicken egg yolk, and 1.0 g honey in 12.0 ml of distilled water. Palmitic acid was mixed with the egg yolk component before adding it to the diet. Treatment jars contained control diet or one of seven treatments: (1) *P. interpunctella* eggs (≈3 mg); (2) *E. kuehniella* eggs (≈3 mg); domes with artificial diet including one of the following: (3) bovine serum albumin (BSA; fraction V powder, Sigma) at 2.0 g/12.0 ml of diet; (4) ground chicken liver at 2.5 g/12 ml; (5) ground beef liver at 1.5 g/12 ml; (6) pasteurized chicken egg white (Deb-EL Foods, Elizabeth, NJ) at 3.0 g/12 ml; (7) *P. interpunctella* egg protein extract (see below). Diets (except for intact *P. interpunctella* or *E. kuehniella* eggs) were prepared under aseptic conditions in a clean room and encapsulated in Parafilm using a diet encapsulation apparatus (Ferkovich et al. 1999).

Plodia interpunctella egg protein extract was prepared by homogenizing 5 g of *P. interpunctella* eggs on ice using a homogenizer (1-cm-diameter generator, Brinkmann Polytron, Westbury, NY) for 2 min at full speed in 20 ml of ammonium acetate buffer (1.16 g/liter, titrated to pH 7.5 using ammonium hydroxide). The homogenates were centrifuged at 20,800 × *g* for 5 min. The supernatant contained three layers following centrifugation. Soluble protein in the middle layer (beneath the upper lipid layer) was collected and filtered through a 0.45-μm/29-mm-diameter filter (Millex-HV, Millipore, Bedford, MA). The supernatant was applied in 1.25-ml aliquots to a D-Salt Excellulose desalting column (Pierce, Rockford, IL) equilibrated in ammonium acetate buffer, eluted in 1-ml fractions, and proteins were monitored at 280 nm and collected in the void volume. These fractions were combined (37 ml), frozen in a dry ice/methanol bath and freeze-dried, resulting in a fluffy white powder. The freeze-dried desalted powder was added to diet by first adding 1.0 ml of water to 2.0-g equation and mixing with diet. Protein in extracts was determined using the Lowry procedure for soluble proteins (Protein Assay Kit, Sigma).

Monoclonal Antibodies. Hybridomas and MAbs were produced by the University of Florida Hybridoma Core Lab (HCl) as described (Shapiro et al. 2000). Mature eggs for use as immunogen were dissected from gravid females and stored at −80°C. Eggs were removed from −80°C storage, homogenized, centrifuged to remove chorions and insoluble proteins, and kept on ice until inoculation of mice. Mice were inoculated and boosted with immunogen (homogenized eggs, 30–60 μl of a 1 mg/ml solution) that was freshly prepared and kept on ice before inoculations. For primary screening of whole mouse sera following booster injections, ELISAs were plated with egg homogenate that was also freshly prepared. Secondary screenings of mass cell cultures were done by

96-well immuno-dot blots (Shapiro et al. 2000), except that cell supernatants were reacted on nitrocellulose against egg homogenate at 4.7 egg-eqs/well (140 eggs homogenized in 3.0 ml, 100 μl/well added to the blot) and fresh homogenate of whole female or male insects at 0.33 insect-eqs/well (10 insects homogenized in 3.0 ml, 100 μl/well). All MAbs were purified as IgG on a protein G HiTrap column (Amersham, Piscataway, NJ), aliquoted, and stored at −80°C at ≈1 mg/ml (Shapiro et al. 2000).

Reagents and Instrumentation. Phosphate-buffered saline (PBS; 0.15 M NaCl (Sigma), 50 mM sodium phosphate (Sigma), pH 7.25) was prepared with 0.05% Tween-20 as PBST. Tris-buffered saline (TBS; 50 mM Tris base [Bio-Rad, Hercules, CA], 0.15 M NaCl, pH 8.5) was used to bind antigen or antibody directly to microtiter plates in the initial step of the assay. MAbs were diluted immediately before use in PBST or TBS (DAS-ELISA) with 1% BSA to 0.5 μg/ml. Secondary antibody conjugate (rabbit anti-mouse HRP-conjugated IgG; Pierce #31450) for the indirect antigen ELISA was partially diluted, aliquoted in PBS with 50% glycerol, and stored at −80°C until use, or stored at 5°C partially diluted in a peroxidase conjugate stabilizer/diluent (Pierce Guardian). For use, the commercial conjugate was further diluted, for a total 1:14,000 dilution. Conjugated MAb for the DAS-ELISA was produced using an HRP peroxidase labeling kit (Pierce #31497), according to manufacturer's instructions. Plates were developed with addition of TMB substrate combined from a two-part kit (Pierce Immunopure) immediately before use. Plates were washed with an automatic 8-well strip plate washer (EL_X50, Bio-Tek Instruments, Winooski, VT) and read at 450 nm using a spectrophotometric plate reader (μQuant, Bio-Tek; KCjunior software, version 1.17, Bio-Tek). Electrophoresis, western blots, dot-blots, and densitometry of SDS-PAGE gels were conducted as described (Shapiro et al. 2000).

Standard and Sample Preparation. Standards were prepared from eggs that had been collected from an artificial substrate (Castane and Zalom 1994) within 24 h after oviposition and stored at −80°C until use. Standards were prepared by homogenizing 50 eggs in a 1.5-ml microcentrifuge tube with a plastic pestle in 50 μl TBS, centrifuging at 20,800 × g for 2 min, and twice resuspending the pellet in 50 μl TBS and centrifuging. Supernatants were combined and the volume determined with an adjustable pipet. Protein concentrations were determined by reading the A_{280} of samples in a UV-transparent microtiter plate (Costar) with the plate reader. Standard solutions were diluted to final volumes at the desired initial concentration (2.5 μg/ml). Final volumes were calculated by converting the pathlength-corrected A_{280} to protein concentration using a specific absorbance of 4.22 $mg^{-1}ml^{-1}$. Specific absorbances were determined by comparing the A_{280} of multiple egg homogenates with their protein concentrations determined relative to BSA standards by bicinchoninic acid protein assay (Pierce).

Table 1. Procedure for indirect antigen ELISA

1. Pipet 200 μl/well of antigen[a] in TBS (pH 8.5) into microtiter plate and incubate overnight at 5°C.
2. Wash 4 times with PBS (no Tween-20).
3. Pipet 200 μl/well of blocking solution (1% BSA dissolved in PBS/0.05% Tween-20 [PBST]) and incubate for 2 h at 27 or 30°C.
4. Wash 4 times with PBST.
5. Pipet 200 μl/well of primary Ab (mouse MAb) in 1% BSA/PBST at the predetermined protein concentration.[b] Incubate 2 h at 27 or 30°C.
6. Wash 4 times with PBST.
7. Pipet 100 μl/well of rabbit anti-mouse IgG-HRP conjugate (RAM-HRP) in 1% BSA/PBST and incubate 2h at 27 or 30°C.
8. Wash 4 times with PBST.
9. Apply 100 μl TMB substrate and develop 20 min.
10. Stop the reaction with 100 μl of 2N H_2SO_4.
11. Read at 450 nm in a microtiter plate reader.

[a] Antigen solution consists of the egg homogenate (standard solution), or whole insect extract, prepared and diluted as per protocol.
[b] Primary MAb and secondary antibody (RAM-HRP conjugate) concentrations were determined as described in Methods.

Insect homogenates were prepared in 15-ml conical polypropylene tubes. One or five insects were placed on ice into 1.9 ml TBS and 0.1 ml of a protease inhibitor (PI) cocktail (Sigma P-2714) and homogenized with a Polytron homogenizer (Brinkmann, PTA5EC 5-mm generator) at high speed for ≈30 s. Homogenate was centrifuged for 5 min at 20,800 × g, supernatant was collected below the floating lipid layer into a fresh tube, and 1.0 μl was diluted to 1.0 ml in TBS to concentrations of 0.05 or 0.25 insect-eqs/100 μl (one- and five-insect samples, respectively). Five-insect samples were diluted to a final concentration of 0.025 insect-eqs/100 μl.

ELISA Protocol. In the indirect antigen ELISA, the antigen (Vn or Vg in standards or samples) is bound directly to the microtiter plate overnight and detected by first binding the primary MAb and overlaying with a secondary antibody-enzyme (HRP) conjugate. Standards (egg homogenates) and samples (whole insect homogenate) in TBS were pipetted into 96-well microtiter plates (Nunc Maxisorp) and diluted in 1:1 (homogenate:TBS) serial dilutions down 8-well columns, for standard concentration ranges of 2.0–250 ng/well. All primary and secondary antibody solutions included 1% BSA to minimize background. For DAS-ELISA, capture antibodies in TBS were added to a plate overnight, and antigen was bound in BSA followed by a detecting MAb-HRP conjugate (Table 2).

ELISAs were developed by first determining optimal concentrations of reagents through sequential checkerboard titration of primary and secondary antibodies, followed by titration of primary antibody versus standard antigen (egg homogenate) dilutions (Crowther 2000). Egg homogenates were then used to generate standard curves in triplicate serial dilutions. Plots of absorbance versus yolk protein-equivalent concentrations were fitted with a 4-parameter curve and routinely yielded $r^2 > 0.99$.

Data Analysis. Results were produced using a KCjunior software protocol with a 4-parameter curve fit and regression, which interpolated from optical

Table 2. Procedure for double antibody sandwich ELISA

1. Pipet 200 μl/well of capture MAb 2F3 (5 μg/ml) in TBS (pH 8.5) into microtiter plate and incubate overnight at 5°C.
2. Wash 4× in PBS.
3. Pipet 200 μl/well of blocking solution (1% BSA dissolved in PBS/0.05% Tween-20 [PBST]) and incubate for 2 h at 30°C.
4. Wash 4× with PBST.
5. Pipet 100 μl/well of antigen[a] in PBS (pH 7.25)/BSA (0.05%). Incubate 2 h at 30°C.
6. Wash 4 times with PBST.
7. Pipet 100 μl/well of 1F3-HRP (or 3F11-HRP) conjugate in 0.05% BSA/PBST and incubate 2 h at 30°C.
8. Wash 4 times with PBST.
9. Apply 100 μl TMB substrate and develop 20 min.
10. Stop the reaction with 100 μl of 2N H_2SO_4.
11. Read at 450 nm in a microtiter plate reader.

[a] Antigen solution consists of the egg homogenate (standard solution), or whole insect extract, prepared and diluted as per protocol.

densities to egg yolk protein equivalents in ng/well (ng/100 μl of diluted insect homogenate). Final results were tabulated in a macro-based spreadsheet, accounting for the number of insects homogenized and the final volume of homogenate. Following an analysis of variance (ANOVA), Tukey's test was used to determine groupings of means.

Results

Selection and Reactivity of MAbs Versus *O. insidiosus* Proteins. Supernatants from hybridoma mass cell cultures were screened for immunoreactivity on dot blots against an egg extract and homogenates of females and males. Of 25 cell cultures screened, 20 showed moderate to high reactivities against eggs and 23 against females, while only one reacted significantly with males. Two cell cultures (5C2 and 5C4) that reacted most strongly against eggs and females were selected and cloned. When supernatants from 24 clones were screened, all reacted strongly against eggs, 23 reacted moderately against female extracts, and none against males. Two clones (5C2–1F3 and 5C4–2F3) that reacted most strongly with both egg and female extracts, and not with male extracts, were selected, cultured, and MAbs (IgG) were purified by affinity chromatography on a protein G column.

MAb 5C2–1F3 and 5C4–2F3 were tested on western blots against proteins from egg, female, and male extracts blotted from SDS gels (Fig. 1A). The reactive egg polypeptide in *O. insidiosus* eggs was a 40k M_r (relative molecular weight) polypeptide, termed vitellin apoprotein II (apoVn-II). Both antibodies reacted with apoVn-II and with a second slightly larger (57k M_r) polypeptide in females. Males were unreactive.

The 24 stored mass-cultured hybridomas were recultured and selected for reactivity to the predominant 180k M_r egg polypeptide (apoVn-I) using western blots. Only seven of 24 hybridomas cell cultures showed significant reactivity against apoVn-I, and only two of these reacted more strongly against apoVn-I than apoVn-II. These cultures were selected, one clone (3C3–3 F11) was isolated and cultured, and its MAb was harvested and IgG purified. This MAb reacted against apoVn-I and not apoVn-II (Fig. 1B). One slightly smaller band reacted lightly against egg and female extracts, and was likely a product of proteolysis. Male extracts were unreactive.

From densitometric analysis Den of digital images of coomassie-stained SDS-PAGE gels, apoVn-I was estimated to represent ≈52% of total yolk protein, apoVn-II ≈14% of total yolk protein. Assuming similar mass-specific densities of stain among bands, this represents an approximate 1:1 molar ratio between the two bands.

ELISA development. Indirect and double antibody sandwich (DAS) ELISAs were developed using the yolk protein MAbs. In the indirect ELISA, antigen was bound to the microtiter plate and detected with MAb 2F3 and a commercial HRP-conjugated rabbit anti-mouse antibody. In the DAS-ELISA, MAb 2F3 was bound to the microtiter plate as a capture antibody, followed by antigen and HRP-conjugated MAb 1F3 (anti-apoVn-II), or HRP-conjugated MAb 3F11 (anti-apoVn-I), as the detecting antibody. Standard curves (Fig. 2) were developed based on the total protein content in egg homogenates, as determined spectrophotometrically from absorbance at 280 nm. Standard concentrations were measured by reading the absorbance of whole egg homogenate at 280 nm and converting to mg/ml using specific absorbances determined as in Materials and Methods.

Optimal concentrations of reagents were determined through sequential checkerboard titration of primary and secondary antibodies, followed by titration of primary antibody versus standard antigen (egg homogenate) dilutions (Crowther 2000). Standard curves, generated in an indirect ELISA (Fig. 2A) or DAS ELISA (Fig. 2 B and C), were plotted as absorbance versus yolk protein concentration. Yolk protein concentrations of homogenates are represented as mass-equivalents of total yolk protein. Standard dilution series routinely yielded $r^2 > 0.99$. Both the indirect (Fig. 2A) and DAS-ELISA (Fig. 2B) using two MAbs against apoVn-II showed optimal response (slope of absorbance versus log concentration) to dilutions of 20–100 ng/well. A DAS-ELISA using MAb 2F3 (anti-apoVn-II) as the capture antibody and MAb 3F11 (anti-apoVn-I) as the detecting antibody gave a standard curve that was about one-third as sensitive at midpoint as the same ELISA using MAb 1F3 (anti-apoVn-II) as the detecting antibody (Fig. 2C).

Extracts of eggs collected and frozen 0–24 h after oviposition produced standard curves that were replicable in slope, with some shift in sensitivity. This was not fully corrected by normalizing extract concentrations by their absorbance at 280 nm. To test whether variation in sensitivity could be due to differences in the age of eggs and degradation of yolk polypeptides or their epitopes during aging, changes in yolk protein content in eggs of increasing age were determined by DAS-ELISA. Results from two DAS-ELISAs were compared, using detecting MAbs against either apoVn-II (MAb 2F3) or apoVn-I (MAb 3F11). Eggs of increasing age showed comparably reduced activity in

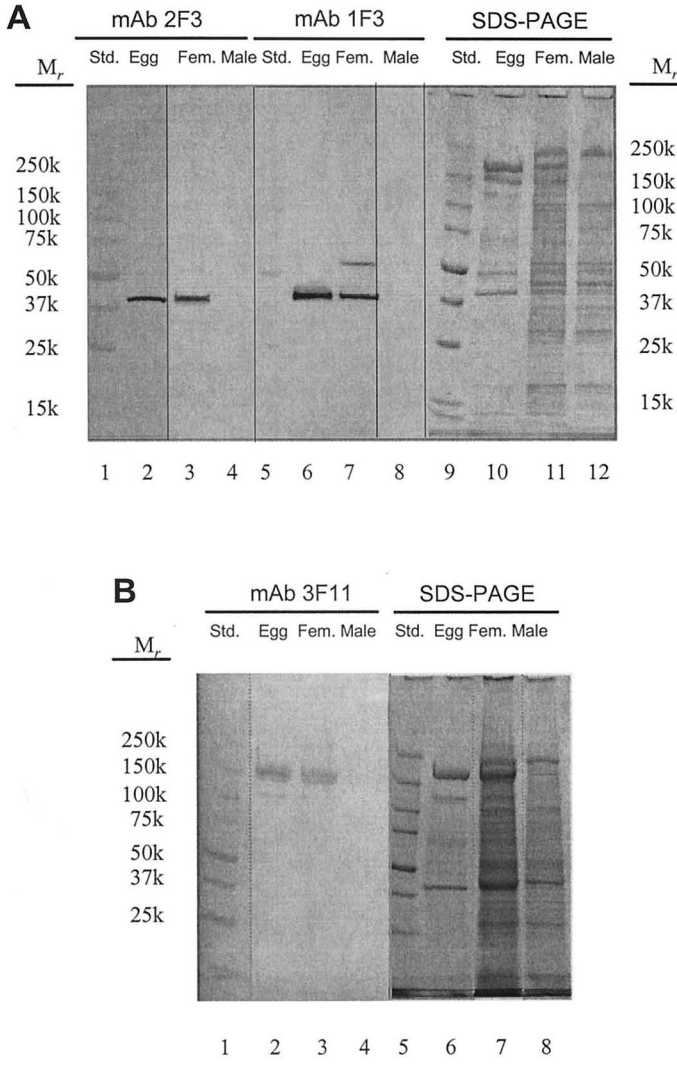

Fig. 1. Western blot (1–8) of MAbs against the yolk proteins of *O. insidiosus* and corresponding SDS-PAGE (lanes 9–12). (A) Reactivity of MAbs 2F3 and 1F3 against apoVn-II in extracts of eggs (lanes 2, 6, 10), females (3, 7, 11), or males (4, 8, 12). (B) Reactivity of MAb 3F11 against apoVn-I in extracts of eggs (lanes 2 and 6), females (3 and 7), and males (4 and 8). M_r, molecular weight of protein standards (Std.).

both ELISAs (Fig. 3), with mean half-lives of 32 h for MAb 3F11 (Fig. 3A) and 34 h for MAb 1F3 (Fig. 3B), estimated from fitted exponential decay curves (r^2 = 0.87 and 0.89, respectively; n = 11).

Sample Preparation. To determine whether proteases released from whole insect extracts might result in proteolysis of antigen, a protease inhibitor cocktail was added to samples immediately following homogenization of female *O. insidiosus*. At the concentration used, the inhibitors did not interfere with indirect antigen or DAS-ELISAs (unreported results). When triplicate samples of five females each were homogenized and quickly treated with the inhibitor cocktail or control buffer (0.5 mg/ml BSA in PBS), the result showed no significant difference between the two treatments (6.14 ± 1.30 μg/insect without inhibitors versus 6.14 ± 1.34 μg/insect with inhibitors; mean ± SD). The cocktail was nonetheless used in all experiments to ensure against proteolytic degradation of samples. To test whether protein could nonspecifically interfere with the DAS-ELISA, BSA was added in increasing concentrations to homogenates of female *O. insidiosus*, and no significant change in response occurred at concentrations as high as 10 mg/ml (Table 3). BSA (0.5 mg/ml) was used thereafter to reduce nonspecific interactions and loss of sample during homogenization. As in dot blots, extracts of male *O. insidiosus* showed no significant activity in the YP-ELISAs.

Response to Diet Quality. Fecundity was compared with yolk protein content in response to a variety of diet compositions. Six female and four male *O. insid-*

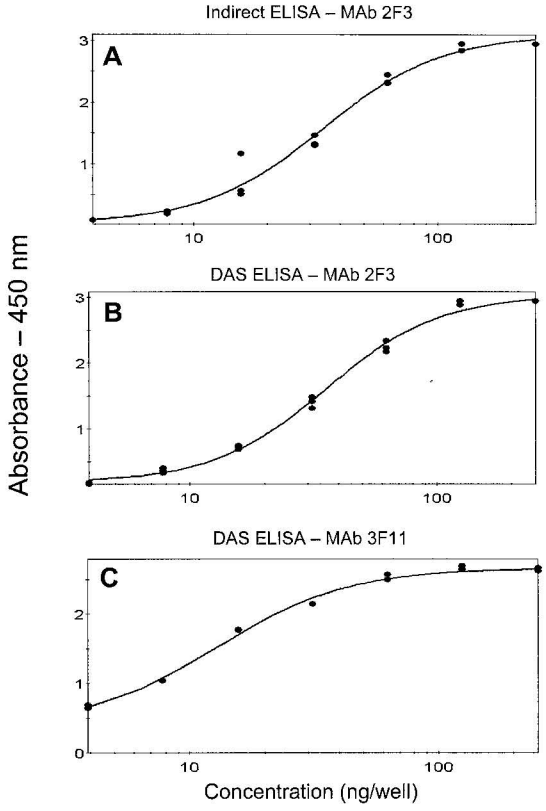

Fig. 2. YP-ELISA standard curves ($n = 3$) with 4-parameter fit (equation: $y = (A - D)/(1 + (x/C)\hat{}B) + D$) for (A) indirect ELISA using MAb 2F3 against apoVn-II (A = 3.1004 B = −1.7944 C = 33.9288 d = 0.0353, R^2 = 0.9865), (B) DAS-ELISA using MAb 2F3 (A = 3.0368 B = −1.8844 C = 35.5264 d = 0.1729, R^2 = 0.9955), and (C) DAS-ELISA using MAb 3F11 against apoVn-I (A = 2.6616 B = −1.6455 C = 12.7492 d = 0.3633, R^2 = 0.9943).

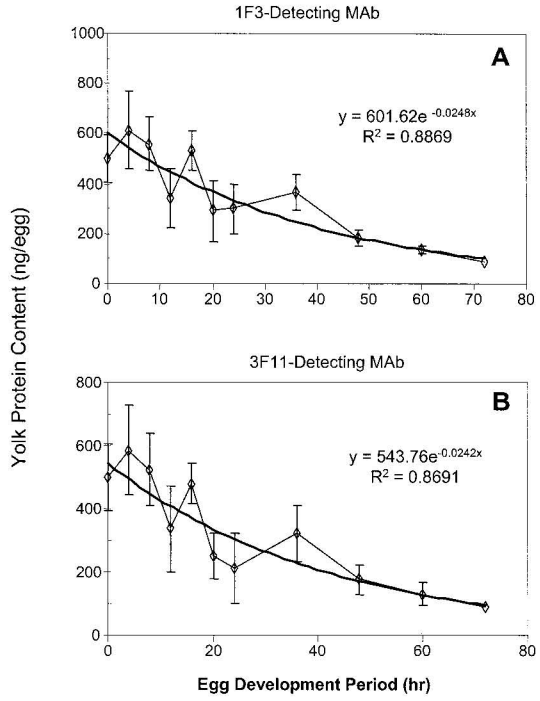

Fig. 3. Yolk protein content of eggs aged for increasing times following oviposition. (A) MAb 1F3 against apoVn-II. (B) MAb 3F11 against apoVn-I.

iosus per replicate were fed a basal diet (Weiru and Ren 1989) or the same diet supplemented with a variety of protein sources, beginning about four days after adult emergence and continuing for 10 d. A wide range of oviposition and yolk protein contents resulted in two clear response groups (Fig. 4). The diet itself or the diet supplemented with egg white, chicken liver, or beef liver resulted in significantly lower oviposition than a diet of *P. interpunctella* or *E. kuehniella* eggs ($P < 0.05$). Diet supplemented with BSA or *P. interpunctella* egg extract resulted in intermediate oviposition rates. When yolk protein content of *O. insidiosus* females was determined at the end of the experiment, a similar pattern was evident, though only two groups were identified following ANOVA (Fig. 4). Supplementation with BSA or *P. interpunctella* egg extract resulted in no significant difference from the diet alone. Though a lack of intermediate response to diet limited its utility, a regression of the correlation between fecundity and yolk protein content yielded a good fit ($r = 0.87$ [$n = 8$]).

Discussion

We have isolated MAbs against female-specific proteins from *O. insidiosus*, identified the proteins as major components of egg extracts, and developed monoclonal antibodies and immunoassays to quantify their contents in *O. insidiosus*. Of the two types of ELISA described here, the indirect antigen ELISA proved easiest to implement. Only one MAb was required, and the HRP-conjugated detecting antibody was readily available from commercial sources. However, inconsistent comparison of whole insect homogenates with standard curves led to adoption of the DAS-ELISA, which is unaffected by potentially interfering protein in extracts. This ELISA requires conjugation of HRP with the detecting MAb. It does allow flexible use of the capture antibody with a choice of detecting conjugates, such as those against either apoVn-I or apoVn-II.

Table 3. Yolk protein contents of females homogenized and diluted in increasing concentrations of bovine serum albumin in PBS

BSA (mg/ml)	Yolk Protein (μg/insect)
0	14.9 ± 4.5
1	18.5 ± 4.7
2	22.4 ± 7.6
5	18.3 ± 3.6
10	18.3 ± 6.1

$n = 4$; five insects/sample; mean ± SD.

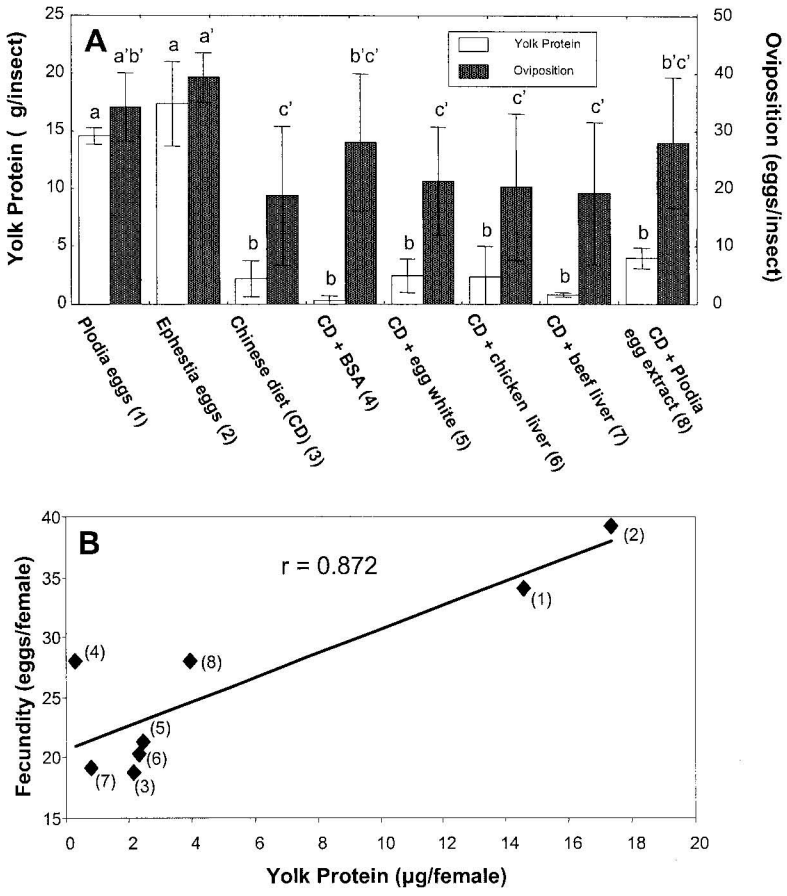

Fig. 4. (A) Oviposition and yolk protein contents of females, 14 d old at conclusion of study, in response to feeding for 10 d on a basal control diet (CD) alone or with various additives. Six females per treatment were sampled every 3 d for oviposition into a fresh green bean. Four females per treatment were collected, extracted, and analyzed by ELISA at conclusion. Mean ± SD, mean yolk protein contents (open bars) not differing significantly ($P < 0.05$) by Tukey's multiple range test are marked by the same letter; mean oviposition (filled bars) not differing significantly are marked by the same primed letter. (B) Correlation of oviposition with yolk protein contents by linear regression. Numbers in parentheses correspond to diets in A.

Standard curves were routinely developed with samples of 50 eggs collected from an artificial substrate after a 0- to 24-h oviposition period. However, within the 24-h period, the average developmental state of a 50-egg sample could vary. As indicated by the 34-h half-life, or a 40% reduction of detectable epitope at 24 h after oviposition, variation in developmental state could significantly affect results. Ideally, standards will consist of rigorously preserved egg extracts prepared from a large batch of eggs from a well-defined age class.

MAbs specific to yolk proteins proved relatively quick to develop and screen, and the quantity of immunogen required was minimal. Interestingly, though *P. maculiventris* and *O. insidiosus* eggs contain yolk polypeptides of similar sizes, egg homogenates as immunogens in mice seem to stimulate primarily antibodies against apoVn-I in *P. maculiventris* and against apoVn-II in *O. insidiosus*. In contrast, *O. insidiosus* MAb 1F2 and 1F3 were reacted against apoVn-II (40k M_r), not apoVn-I (180k M_r). A minor band on western blots of whole *O. insidiosus* homogenates at 57k M_r may represent a precursor to apoVn-II from fat body and/or hemolymph. The reactivity of the small Vn subunit versus that of the large subunit probably correlates with either the exposure of the epitope or the relative strength of its antigenicity, or both. The differences in immunogenicity between *P. maculiventris* and *O. insidiosus*, members of different families of Heteroptera, may relate to differing modes of storage of Vn in eggs, or differences in egg physiology. *P. maculiventris* eggs are relatively resistant to desiccation, as they are oviposited in clusters on the undersides of leaves, while *O. insidiosus* eggs are oviposited within plant structures, and are thus protected from desiccation by their environment.

ELISAs using a detecting MAb against apoVn-I or apoVn-II showed nearly identical results in the egg development experiment. This suggests that either apoVn detects the same native protein, a Vn that

contains both apoVn-I and apoVn-II. Although the MAbs detect specific epitopes on either of the two apoVns, the units of measurement were given in equivalent masses of total yolk protein (e.g., μg-eqs/female). Assuming a constant or average yolk protein content per egg, units of micrograms per female of yolk protein can easily translate to egg-equivalents per female. Alternatively, if each apoVn represents a constant percentage of the total protein in egg extracts, the same units can readily be translated to mass-equivalents of apoVn-I or apoVn-II per female. Regardless, with MAbs in hand for any selected species, an ELISA can be readily developed that will yield a clear numerical measure of an insect's reproductive state.

Beyond the basics of developing and verifying the mechanics of the YP-ELISA, we conducted an initial experiment to test reproductive response to a wide range of diets. Results (Fig. 4) indicated significant quantitative differences in response to the different diets, when response was tested as yolk protein content or cumulative oviposition. With a multiple range test, means separated into only two distinct groups when yolk protein content was measured, though oviposition yielded intermediate values when fed diet mixed with BSA or *P. interpunctella* egg extract, or fed intact *P. interpunctella* eggs. *E. kuehniella* eggs are the most common mass-rearing medium. Since yolk protein content and oviposition are presumed to be continuously variable, simple linear regression was also employed. This yielded a significant correlation (r = 0.872), but it is limited by the bimodal nature of the data.

Several aspects of the experiment may account for the incomplete correlation. First, insects were initially kept on *E. kuehniella* eggs, then placed on diet approximately 4 d after adult emergence. Second, three sets of egg counts were accumulated over 10 d, measured at 3–4 d intervals, while YP contents were measured once at the end of the period. Third, since YP contents were measured after insects had oviposited for several days, YP levels may have been in various states, depending upon how recently insects had oviposited. Presumably, the sample size limited the variability from this effect, and larger samples or more replicates would have further reduced it. Clearly, more testing and improved protocols will be required for reliable comparisons of yolk protein content and fecundity in females responding to multiple dietary regimes.

The quantitative YP-ELISA may prove especially useful in studies where predators or parasites are of small size and their reproductive development cannot easily be described morphologically, or where collection and counting of eggs for oviposition studies is very difficult. Methods described here can be applied to insects such as *P. maculiventris* or *O. insidiosus*, allowing the rapid production and use of monoclonal antibodies and ELISA. By assessing the vitellogenic response in predators that require a proteinaceous meal before egg development, we believe that we can predict effects of nutritional and environmental conditions on long-term fecundity. In other insects, e.g., parasitic wasps, the adult may eclose with a full complement (and perhaps her only clutch) of eggs. For these insects, a YP-ELISA should predict fecundity even more accurately, assuming that populations are sampled before oviposition. As demonstrated with *O. insidiosus*, size of the insect is of little consequence, due to the inherent sensitivity of ELISA.

This ability to assess fitness by YP-ELISA will lead to a three-fold advantage in the rearing of predators. First, it will provide a tool for rapid development of new artificial diets based on reproductive response and projected fecundity rates. Second, it will expedite and simplify quality control for insect rearing, whereby reproductive state can be rapidly tested before shipment of adult insects, or in colonies of ovipositing insects. Finally, it can be used to predict at least one dimension of female reproductive success in the field, fecundity, joining species and prey identification methods.

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