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Source: Florida Entomologist, 90(2) : 321-326

Published By: Florida Entomological Society

URL: [https://doi.org/10.1653/0015-4040\(2007\)90\[321:IFITPO\]2.0.CO;2](https://doi.org/10.1653/0015-4040(2007)90[321:IFITPO]2.0.CO;2)

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# IMPROVED FECUNDITY IN THE PREDATOR *ORIOUS INSIDIOSUS* (HEMIPTERA: ANTHOCORIDAE) WITH A PARTIALLY PURIFIED NUTRITIONAL FACTOR FROM AN INSECT CELL LINE

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## ABSTRACT

A specific factor that stimulates egg production in the predator *Orius insidiosus* (Say) was earlier shown to be present in eggs of the Indian meal moth, *Plodia interpunctella* (Hübner). We investigated whether the embryonic cell line IPLB-PiE, derived from eggs of the Indian meal moth *P. interpunctella* also produces a specific factor that improves fecundity of the predator. We fractionated cells by preparative isoelectric focusing in a pH gradient of 3-10 and bioassayed the resultant fractions in test diets to determine their effects on egg production. Rates of oviposition were determined by placing adult predators on the test diets the third d after eclosion, allowing them to feed for 3 d, and then providing them with ovipositional substrates for 24 h on d 7. Six out of 20 fractions with isoelectric points between pH 5.2 and 7.3 had significant activity relative to the control diet. The nature of the factor(s) is unknown but corresponds to a partially purified fecundity factor from eggs of *Ephestia kuehniella* Zeller with an isoelectric point of pH 5 in an earlier study. The results indicate that the cell line, which was originally derived from embryos of *P. interpunctella*, has retained a differentiated function in culture by producing products similar to those produced in the *P. interpunctella* egg.

Key Words: artificial diet, factor, insidious flower bug, *Plodia interpunctella*, cell line, *Orius insidiosus*, *Ephestia kuehniella*, eggs

## RESUMEN

Un factor específico que estimula la producción de huevos del depredador *Orius insidiosus* (Say) anteriormente se mostro el estar presente en los huevos de la palomilla, *Plodia interpunctella* (Hübner). Nosotros investigamos si la línea celular IPLB-PiE de los embriones derivada de huevos de la palomilla, *P. interpunctella* también produce un factor específico que mejore la fecundidad del depredador. Desde entonces hemos distribuido las células por medio de un preparativo isoelectrónico enfocándose en un gradiente de pH de 3-10 y realizamos un bioensayo sobre las fracciones en pruebas de dieta para determinar sus efectos sobre la producción de huevos. Las tasas de oviposición fueron determinadas por medio de la alimentación de los depredadores adultos con las dietas de prueba al tercer día después de la eclosión, permitiendo que ellos se alimentaran por 3 días, y luego proveyendolos con sustratos oviposicionales por 24 h en el día 7. Seis de los 20 fracciones con puntos isoelectrónicos entre pH 5.2 y 7.3 tenían una actividad significativa mayor relacionada con la dieta de control. La naturaleza del factor(es) es desconocida pero se corresponde a un factor de fecundidad purificado parcialmente de los huevos de *Ephestia kuehniella* Zeller con un punto isoelectrónico de pH 5 en un estudio anterior. Estos resultados indican que la línea celular que fuera derivada originalmente de los embriones de *P. interpunctella* ha retenido una función diferenciada en el cultivo por la producción de productos similares a los producidos en el huevo de *P. interpunctella*.

Reduced fecundity is a general problem associated with the insidious flower bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (Ferkovich & Shapiro 2004a), and a number of other species of predators reared on artificial diets without insect components (Cohen 1985a, 1985b, 1992; 2000; De Clercq & Degheele 1992, 1993a, 1993b; Cohen & Staten 1994; De Clercq et al. 1998; Adams 2000a, 2000b; Rojas et al. 2000; Wittmeyer & Coudron 2001; Coudron et al. 2002). Insect hemolymph and tissue extracts have been used to improve artificial diets (Grenier et al. 1994). The use of established insect cell lines as replacements is a rela-

tively recent approach (Rotundo et al. 1988; Ferkovich & Oberlander 1991; Ferkovich et al. 1994; Hu et al. 1999; Ferkovich & Shapiro 2004b; Ferkovich & Lynn 2005; Heslin et al. 2005a, 2005b). The advantages of using cell lines in developing and/or improving artificial diets will be realized when the technology for large-scale cell production with cost-effective serum-free media is available. Cell lines could substitute for hemolymph or other insect materials (e.g., an embryonic cell line could substitute for insect egg homogenates), where those materials are critical for optimal development of insects. Also, the use of cell lines in

chemically defined media could simplify downstream purification and identification of growth-inducing factors, fecundity-inducing factors, and others found naturally in insect hosts and prey.

In an earlier work, we addressed the fecundity problem by supplementing artificial diet for *O. insidiosus* with 2 embryonic cell lines. One line, Ek-x4 was derived from eggs of *Ephestia kuehniella* Zeller (Lynn & Ferkovich 2004), which are generally used to rear *Orius* species by commercial insectaries, and 1 line, IPLB-PiE was derived from eggs of *Plodia interpunctella* (Hübner) (Tsang et al. 1985). Although the resultant fecundity was comparable with both cell lines, the growth characteristics of the IPLB-PiE cell line were conducive to larger scale production of those cells. In a recent study, we fractionated *P. interpunctella* egg proteins and bioassayed the fractions in diet, demonstrating the existence of a specific factor that stimulates egg production. Correspondingly, in this study we have examined the IPLB-PiE line derived from whole egg embryos to determine if a similar fecundity factor is also produced by the IPLB-PiE cells.

## MATERIALS AND METHODS

### *Orius* Rearing

A colony of *O. insidiosus*, originating from a Florida strain collected in Bronson, FL in 2002, was maintained on eggs of *E. kuehniella* Zeller (received frozen from Beneficial Insectary, Redding, CA). Briefly, freshly laid eggs of *O. insidiosus* (about 500 eggs in 1-3 green beans) were placed in 400-mL canning jars, each covered with a 15 × 15-cm square of nylon ripstop cloth. Each jar received 0.3 mL of *E. kuehniella* eggs, 1.25 mL of Hydrocapsules® (1-2 mm dia.; Analytical Research Systems, Gainesville, FL), and 2 granules of local pollen, (Buzzn Bee, Inc., West Palm Beach, FL). After the *O. insidiosus* eggs hatched (approximately 5 d), the green beans were replaced with fresh beans, *E. kuehniella* eggs, and additional pollen every other day. Adults started to emerge in approximately 3 weeks and, when adults began to oviposit in the green beans, new jars were set up with green beans containing eggs. The insects were held at 25.5 ± 1°C, with 70 ± 5% RH, and light: dark cycle of 16:8 h.

### Artificial Diet

Artificial diet was prepared under aseptic conditions in a clean room and encapsulated in stretched Parafilm® domes (25 µL) with a diet encapsulation apparatus (Analytical Research Systems, Gainesville, FL) described by Ferkovich et al. (1999). Diet ingredients were 330 mg brewers yeast, 30 mg sucrose, 180 mg soy protein acid hydrolysate, 3.8 mg of 99% palmitic acid (all from

Sigma, St. Louis, MO), 40 mg chicken egg yolk, and 80 mg honey in 1.2 mL of distilled water. Palmitic acid was mixed with the egg yolk component before adding it to the diet.

### Cells

The embryonic cell line (IPLB-PiE), originally derived from embryonated eggs of *P. interpunctella* by Tsang et al. (1985), was cultured in TNM-FH insect medium (Sigma, St. Louis, MO) in 25-cm<sup>2</sup> culture flasks for 7 d as described by Lynn (1996). For large-scale production of the cells, they were cultured in 250-mL magnetic spinner flasks (Bellco Glass, Vineland, NJ) at 24.9°C for 14 d. The cell suspension was centrifuged (1370 × g for 3 min) in a graduated conical centrifuge tube to obtain a soft pellet of cells. The pellet was re-suspended in 1.0 mL purified water, washed twice and homogenized with a hand-held homogenizer in 1.0 mL purified water. The homogenate was then sonicated for 60 s with a Polytron® unit (model W-375, Heat Systems-Ultrasonic, Inc., Plain View, NY). Twenty µL were removed and assayed for protein by the Lowry procedure (Protein Assay Kit, Sigma, St. Louis) and the remainder of the cell suspension was saved for the isoelectric procedure.

### Electrophoresis and Protein Assay

The Lowry procedure (Protein Assay Kit, Sigma, St. Louis, MO) was used to assay the soluble proteins in the egg protein solution and in the fractions after isoelectric focusing. Gradient SDS-PAGE (4-20%) was carried out in vertical minigels (Bio-Rad) as described by Shapiro et al. (2000).

### Preparative Isoelectric Focusing

The cell homogenate (approx 0.98 mL) and 3 mL of ampholyte solution (pH range 3-10, Bio-Rad, Hercules, CA) were mixed in 58 mL of distilled water. The protein solution was run in a Rotofor Cell® isoelectric focusing unit (Bio-Rad, Hercules, CA) for 2.5 h at 12 W constant power at 4°C. Twenty fractions were collected and their volumes (approx. 2 mL each) and pH values measured. Ampholytes were removed by bringing each fraction to 1 M NaCl for 15 min and then aliquots of 10-20 µL of each fraction were used for protein analysis. After the fractions were analyzed for protein, they were combined based on the protein profile. Fractions with low protein levels were combined, and ones with higher protein concentrations were kept as individual fractions. The fractions were combined as follows: 1-5, 12-16 and 17-20. Fractions 6-11, which were cloudy and noted to contain minor precipitates, were kept as individual fractions. The combined and individual fractions

were then concentrated to 0.5 mL in Centriprep® concentrators (10k molecular weight (MW) cutoff; Millipore, Bedford, MA) and 10–20 µL of each fraction were used to analyze for soluble protein.

#### Proteinase K Digestion of Cells

One mL of pelleted PiE cells was homogenized in 1 mL of PBS (0.15 M sodium chloride/0.1 M sodium phosphate, pH 7.0), centrifuged for 2 min, and the supernatant was applied to 5 mL Zebra® desalt spin columns (Pierce, Rockford, IL). The resultant desalted protein solution was incubated with 20 mg proteinase K (immobilized on agarose beads) per 30 mg cell protein and held on a shaker-bath at 37°C for 18 h. A second 1-ml sample without proteinase K was incubated at the same temperature. The proteinase K sample was centrifuged to remove the beads and 40 µL were removed for the protein assay and PAGE analysis. Dry diet ingredients were then added to both of the samples for the diet bioassay.

#### Diet Bioassay of IEF Fractions

Newly emerged (24 h after eclosion) adults of mixed sexes were collected with a camel hair brush and maintained on *E. kuehniella* eggs for 3 d before they were placed on the diet capsules containing the IEF fractions. Each replicate consisted of 6 females and 4 males in a 100-mL plant tissue culture jar (Sigma, St Louis, MO) with 4 jars per treatment. Each jar contained 0.6 mL of Hydrocapsules®, 2 capsules of treatment diet (each 25 µL), and 3 crumpled strips of wax paper (5 × 80 mm) as substrates. *Ephestia kuehniella* eggs and beads of water and diet were replaced daily and mortality was recorded. At the end of d 6, one 7-cm section of green bean pod, used as a substrate for oviposition, was placed in each jar for 24 h. Eggs deposited in the green beans were then counted under a microscope. The insects were held in a growth chamber at 25.5 ± 1°C, with 75 ± 5% RH, and a photoperiod of 15:9 (L:D) h. Diet treatments consisted of the following: (1) Eggs (standard)—jars each contained whole *E. kuehniella* eggs (3 mg, approx. 150 eggs), which were used as a standard in the bioassay because they are widely used by commercial insectaries for rearing predators; (2) Diet (control)—jars contained artificial diet with no additional substances; and (3) Diet (amended)—jars contained artificial diet supplemented with combined fractions 1–5, 12–16, or 17–20, or individual fractions 6 through 11 as separate treatments.

#### Data Analysis

Each treatment was replicated 4 times. Egg counts (eggs/female) were adjusted for female mortality within each treatment. Data were ana-

lyzed by ANOVA with StatMost software (Dataxiom Software, Inc.). Dunnett's test was used to determine if the number of eggs laid per female on each of the diet treatments supplemented with the isoelectric focusing fractions was significantly greater than the number of eggs laid per female on the Diet (control).

## RESULTS

Figure 1 shows the protein profile versus pH of the IPLB-PiE proteins separated in a pH gradient of 3–10. The average rate of eggs oviposited per female was highly significant relative to the Diet (control) ( $P < 0.01$ ) in fractions 7 through 9 with isoelectric points ranging from pH 5.2 to 6.1 and significant ( $P < 0.05$ ) in fractions 10 and 11, pH 6.8 and 7.3 (Fig. 2). The active fractions contained 12.6 mg or 40.3% of the total protein (31.4 mg) recovered in all the fractions; the remaining fractions contained 18.8 mg of the total protein and no associated activity. Recovery of the total protein applied to the gradient was 82.6% (38 mg applied; 31.4 mg recovered); light precipitates in fractions 7–11 after isoelectric focusing resulted in a loss of protein in these fractions when the samples were dialyzed and concentrated. SDS-PAGE analysis of the fractions is shown in Fig. 3. Of the active fractions (fractions 7–11), fractions 10 and 11 had fewest bands and displayed 4 easily discernible bands in a relative molecular weight range of 34–133k. Treatment of the cell homogenate with proteinase K revealed a loss of the cellular proteins on an SDS-PAGE gel (Fig. 4); however, females fed proteinase K-treated cells showed oviposition rates similar to those fed untreated cells (Fig. 5).

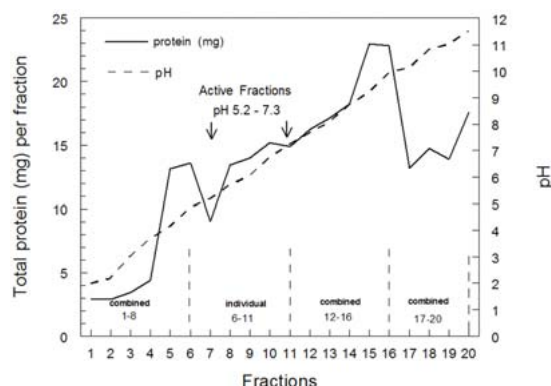


Fig. 1. Protein profile of IPLB-PiE cellular homogenate separated by isoelectric focusing on a pH gradient of 3–10. Fractions that were combined for bioassay in artificial diet are shown by vertical dotted lines. Arrows indicate range of fractions with ovipositional stimulating activity and pH values indicate isoelectric points.

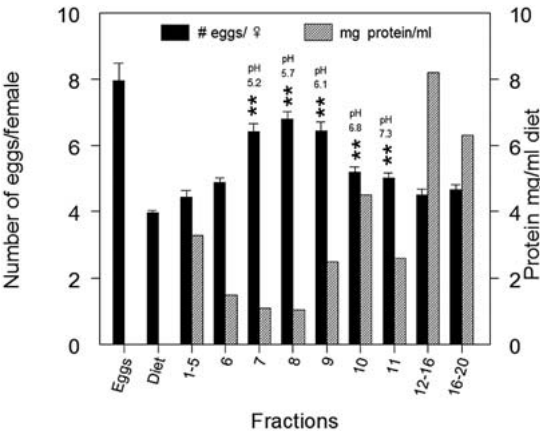


Fig. 2. Average number of eggs oviposited by females of *O. insidiosus* after being fed artificial diet supplemented with fractions from isoelectric focusing separation of IPLB-PiE cellular homogenate shown in Fig. 1. Eggs (standard), whole eggs of *E. kuehniella*; Diet (control); Diet (amended), combined and individual fractions were each bioassayed in separate diet treatments; error bars refer to standard error; \*\* indicates  $P < 0.01$ .

DISCUSSION

The fecundity of *O. insidiosus* females was improved when fed diet supplemented with 6 of the 20 fractions from isoelectric focusing separation of the homogenate of IPLB-PiE cells. This indicated that the effect was not due to a protein deficiency, since all of the fractions contained protein. Moreover, the activity was limited to fractions in a pH range of 5.2-7.3 and was associated with 40% of the total protein recovered. Since multiple bands were present in all the active fractions, the fecundity-promoting activity could not be attributed to a specific polypeptide. However, active

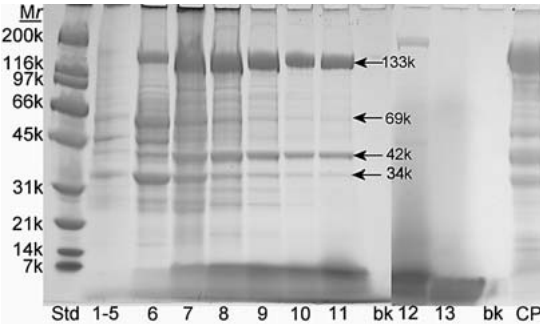


Fig. 3. SDS-PAGE analysis of fractions separated as shown in Fig. 1. MW standards (Std); combined fractions 1-5; individual fractions 6-11; blank (bk); combined fractions 12-16; blank (bk); combined fractions 17-20; blank; crude protein (CP). Twenty micrograms of protein applied per lane.

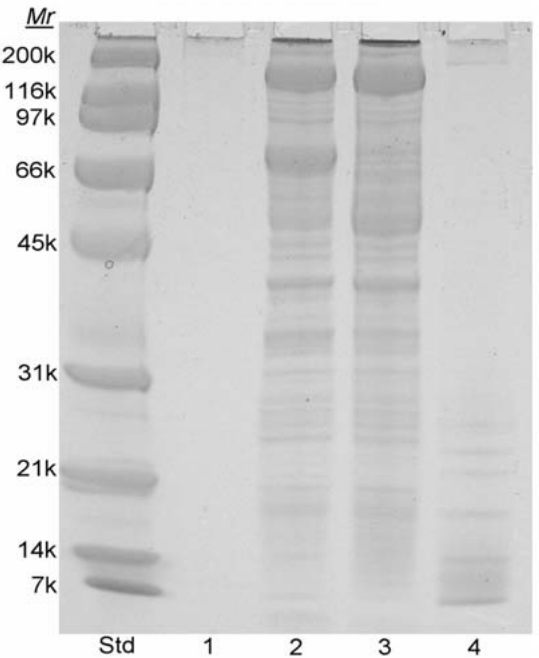


Fig. 4. SDS-PAGE analysis of fractions separated as shown in Fig. 1. MW standards (Std); lanes: 1) blank, 2) untreated, 3) without proteinase K but incubated at 37° for 18 h, and 4) with proteinase K at 37°C for 18 h. Ten micrograms of protein applied per lane.

fractions 10 and 11 contained fewer bands, with 2 densely staining bands at MW 156k, 69k, 42, and 34k. A recent study that used whole eggs of *E. kuehniella* and the same procedure employed in this study found only 1 active fraction associated with 16% of the total recovered protein at a pH of 5 (Ferkovich & Shapiro 2005). In addition, SDS-PAGE analysis of the whole egg fractions revealed the presence of 1 major protein band with MW 47k and other faint bands at 163k, 51k, 39k, 31k, and 27k. Other egg components such as extracts of egg lipids and nucleic acids (DNA and RNA) had no effect on the ovipositional rate nor did other non-insect proteins such bovine serum albumin and hen egg albumin tested at similar concentrations.

The results of this study suggest that the IPLB-PiE cell line, originally derived from embryos of *P. interpunctella*, has retained a differentiated function in culture and produces products similar to those synthesized in the *P. interpunctella* egg. We now know that the active material is at least associated with cellular proteins. Digestion of the proteins did not result in a loss of activity, indicating that none of the basic nutritional components of the cell homogenate were destroyed by the enzyme. The activity could be associated with a peptide fragment resulting from the

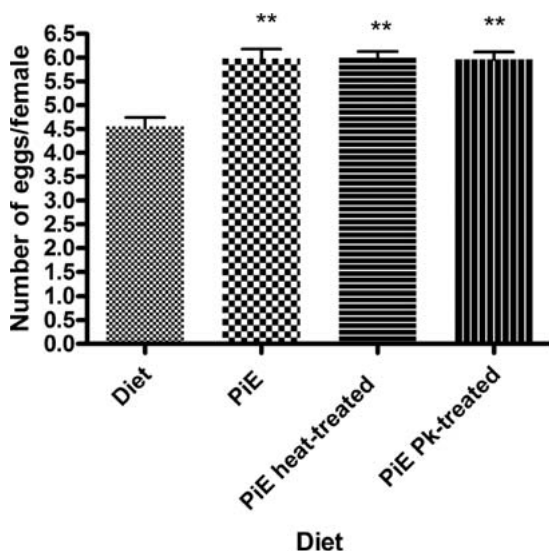


Fig. 5. Average number of eggs oviposited by females of *O. insidiosus* after being fed artificial diet alone (Diet), and diet supplemented with fresh PiE cells (PiE), PiE cells incubated at 37°C for 18 h (PiE heat-treated), and PiE cells incubated with proteinase K at 37°C for 18 h (PiE Pk-treated). Error bars refer to standard error; \*\* indicates  $P < 0.01$ .

proteolytic digestion or a ligand carried by one of the proteins. Proteins in diets not only provide amino acids as nutrients, but also serve to functionally bind lipids, ions, enzyme co-factors, and flavors, act as emulsifiers and film-formers between diet components, and have buffering and stabilizing effects on diet components (Cohen 2004), none of which was addressed in this study. An alternative possibility for the enhanced fecundity that was observed is that proteins in insect diets may provide other needed nutrients or factors such as the “token stimuli” described by Cohen (2004), which stimulate predators to feed on diets. Although the nature of active material is unknown, this information provides a new avenue for isolation and identification of the fecundity-enhancing substance.

#### ACKNOWLEDGMENTS

We appreciate the excellent technical assistance of Delaine Miller in this study.

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