STIMULATION OF SEX PHEROMONE PRODUCTION IN CORN EARWORM MOTHS BY INJECTION OF EXTRACTS OF HEADS OF MALES OF THE CARIBBEAN FRUIT FLY

PETER E. A. TEAL
Center for Medical, Agricultural and Veterinary Entomology, USDA, ARS
PO Box 14565, Gainesville, FL, USA, 32604

ABSTRACT

Females of the Corn earworm moth, *Helicoverpa zea* Boddie, were induced to produce sex pheromone during the photophase, when no pheromone is normally produced, by injection of aqueous extracts obtained from the heads of sexually mature males of the Caribbean fruit fly, *Anastrepha suspensa* (Loew). The amounts of sex pheromone present in extracts of pheromone glands from moths, obtained 1 h after injection of between 0.25-10 head equivalents of extracts, were greater than that present in extracts from females injected with only saline. Moths injected with 1 head equivalent of fly extract produced as much pheromone as was produced by moths injected with 5 pmol of synthetic pheromone biosynthesis activating neuropeptide (PBAN). However, the amount of pheromone was lower in extracts obtained from females injected with 10 head equivalents than in extracts from females injected with 1 head equivalent. ELISA studies, conducted using antisera which binds with PBAN, and the biologically active C-terminal decapeptide fragment of PBAN, indicated that material present in extracts from fly heads bound with the antibody in a dose dependent fashion.

Key Words: Sex pheromone biosynthesis, neuropeptide, Caribbean Fruit fly, Corn Earworm moth

RESUMEN

Palomillas hembra del gusano del maíz, *Helicoverpa zea* Boddie, fueron inducidas para producir feromona sexual durante la fotofase, cuando normalmente no producen feromona, con una inyección de extractos acuosos obtenidos de la cabeza de machos sexualmente maduros de moscas fruteras del Caribe, *Anastrepha suspensa* (Loew). Las cantidades de feromona sexual en los extractos de la glándula feromonal de las palomillas, extraída una hora después de inyectarlas con el equivalente del extracto de 0.25 a 10 cabezas de la mosca del Caribe, fueron mayores que las cantidades presentes en extractos de hembras inyectadas solamente con solución salina. Las palomillas inyectadas con 1 unidad del extracto produjeron una cantidad de feromona igual a la que produjeron palomillas inyectadas con 5 pmol del neuropéptido sintético que activa la biosíntesis de feromona (PBAN, "pheromone biosynthesis activating neuropeptide"). Sin embargo, la cantidad de feromona obtenida de palomillas hembra inyectadas con 10 unidades del extracto de la mosca fué menor que la cantidad obtenida de hembras inyectadas con 1 unidad. Las pruebas de ELISA, las que se realizaron usando antisueros que se enlazan al PBAN, al carbón terminal del fragmento decapéptido del PBAN biológicamente activo, indicaron que el material presente en los extractos de las cabezas de la mosca del Caribe se unió al anticuerpo de una manera dependiente del nivel de la dosis.

Adult females of Heliothine moths including *Helicoverpa* and *Heliothis* species exhibit a diel periodicity of sex pheromone production during which pheromone is produced only during discrete periods of the scotophase (Pope et al. 1982, 1984; Teal et al. 1993). However, females of these moths can be induced to produce pheromone during the photophase, when little or no pheromone is present in the sex pheromone gland, by injection with aqueous extracts of their cephalic ganglia or synthetic pheromone biosynthesis activating neuropeptide (PBAN) (Abernathy et al. 1995; Teal & Tumlinson 1989; Teal et al. 1993).

Decapitation of females of the Hessian fly, *Mayetiola destructor* (Say), inhibits pheromone production and injection of extracts of the heads into decapitated females restores the capacity to produce pheromone (Foster et al. 1991). Injection of PBAN from moths also induces production of pheromone in this fly (Foster et al. 1991). These results indicate that pheromonotropic peptides are used by insects other than Lepidoptera to regulate the diel periodic production of sex pheromone. Males of tephritid fruit flies, like the Caribbean fruit fly (*Anastrepha suspensa* (Loew)), produce and release sex pheromones in a diel periodic fashion (Nation 1990; Epsky & Heath 1993). In this regard, males of *A. suspensa* are similar to other insects, like moths and the Hessian fly, which regulate the diel periodic production of pheromones using pheromonotropic neuropeptides. However, studies on the endogenous regulation of production of pheromones by males of *A. suspensa* have not been conducted. I report here the results of studies conducted to determine if the heads of sexually mature males of *A. suspensa* contained factors that would induce pheromone production when injected into females of the Corn earworm moth, *Helicoverpa zea* (Boddie), and whether these factors had structural similarities with PBAN as indicated by ELISA studies.

Materials and Methods

Insect Cultures:

Females of *H. zea* were obtained as pupae from a colony maintained at our facility and were allowed to eclose in 4-L paper cartons with screen tops under a 14L:10D photoperiod at 26 ± 2°C and 60 ± 5% relative humidity. Newly eclosed females were transferred to different cages daily and were supplied with a 5% aqueous sugar solution soaked onto commercial cotton balls. All experiments with moths were conducted using 2-3 day old females during the 6th-8th h of the photophase, when pheromone is not normally produced (Teal & Tumlinson 1989). Pupae of *A. suspensa* were obtained from cultures maintained at the Division of Plant Industry, State of Florida in Gainesville Florida. Flies were housed in 30 cm^3 screen cages in environmental chambers maintained under the above conditions. Newly eclosed adult males were transferred to new cages daily and provided with a 3:1 mixture of torula yeast hydrolysate and sucrose as food and with water. Males were aged for 10 days prior to use to insure that they were reproductively mature (see Nation 1990). Only male flies were used because this sex produces the attractant sex pheromone.

Pheromonotropic Assays:

Heads of male flies were excised during mid scotophase, when no pheromone is produced (Nation 1990), and homogenized in cold (4°C) H_2O containing 0.1% trifluroacetic acid. The supernatant recovered after two separate homogenizations and centrifugations (18000 × g) was concentrated to dryness using a Speed Vac concentrator and reconstituted in physiological saline (Christensen et al. 1991) at doses of 0.1,

0.25, 0.5, 1.0, 5.0 or 10 head equivalents per 20 µl of saline. Pheromonotropic assays were conducted by injecting female moths with 20 µl of either saline or saline containing various doses of extracts of the heads of *A. suspensa* or 5 pmol of synthetic PBAN during the mid photophase (Teal & Tumlinson 1989). After a 1 h incubation the pheromone glands were excised and extracted in 10 µl of hexane containing 1 ng/µl each of heptadecane and nonadecane as internal standards. The extracts were analyzed by capillary gas chromatography to determine the amount of pheromone, indicated by (Z)-11-hexadecenal (Z11-16:AL), the component of the sex pheromone present in greatest amount (Teal & Tumlinson 1989). Data were analyzed using a Fisher's least significant difference test ($p = 0.05$) performed after an analysis of variance indicated differences among treatments.

ELISA Assays:

For these tests antisera formed against a synthetic PBAN-Keyhole limpet hemocyanin (KLH) complex were used. Five mg of peptide (L-S-D-D-M-P-A-T-P-A-D-E-M-Y-R-Q-D-P-E-Q-I-N-S-R-T-K-Y-F-S-P-R-L-NH_2) was mixed with 30 mg of KLH in 2 ml of 0.1 M sodium phosphate buffer (pH 7.25) in a 20 ml conical vial containing a teflon® coated vane magnetic stir bar. Linkage was accomplished by addition of 50µl of 25% glutaraldehyde in 5 µl aliquots while constantly stirring. One h later the sample was diluted by addition of 12 ml of 10 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.25). The sample was concentrated using an Amicon Centriprep® 30 concentrator, diluted to 5 ml with HPLC grade distilled H_2O and provided frozen to Kel Farms (Alachua, Fl.) for the production of antisera. Inoculation of each of 2 rabbits followed the protocol of Davis et al. (1989), except that booster inoculations were made at 3-week intervals and the final bleeds were obtained 12-weeks after the initial inoculation.

The immunoreactivity of the antisera to PBAN was determined by ELISA as described by Gazit et al. (1992) with minor modifications. Briefly, wells of Corning ELISA plates were coated with 200 µl of 0.1M Na_2CO_3 buffer (pH 9.6) containing amounts of PBAN ranging in concentration from 0.075-1.00 pmol/well and incubated at 4°C overnight. Wells were washed 3 times with PBS-Tween (0.15M NaCl in 50 mM Na_2HPO_4, pH 7.25, containing 0.05% Tween-20), filled with 200 µl of blocking solution (1% gelatin in PBS) and incubated at 35°C for 1.5 h. After blocking, the plates were washed with PBS-Tween and 200 µl of either preimmune sera or the antisera, diluted to 1:1500 in PBS, were added to each well and the plates incubated overnight at 4°C. After the final incubation all plates were washed as above and 200 µl of alkaline phosphatase conjugated goat anti-rabbit IgGs (Bio-Rad Laboratories) diluted 1:2000 in PBS were added to each well. The plates were incubated at 35°C for 1.5 h and washed. One hundred fifty µl of 1.0 mg/ml p-nitrophenyl-phosphate in diethanolamine buffer (Bio-Rad Laboratories) were added to each well, and incubated 1 h at room temperature. Enzymatic activity was stopped by adding 100 µl of 0.4M NaOH to each well. Plates were read at 405 nm using a Model 450 Bio-Rad Microplate Reader. Specificity of the antibody for other peptides was determined as above using up to 1 nmol of the peptides. Each plate included blank lanes, lanes containing different dilutions of the test peptides and lanes containing 0.75 pmol of PBAN as a positive control. After subtraction of blank values, binding of the antisera with test peptides was expressed as a percentage of binding of the antisera with 0.75 pmol of PBAN. All synthetic peptides were custom synthesized and purified by reversed phase liquid chromatography prior to use (see Abernathy et al. 1995).

For studies to determine if the extracts of the heads of males of *A. suspensa* reacted with the antibody five lanes of the plate were initially incubated with amounts of PBAN from between 0.05-0.75 pmol as above and six lanes were filled with 200 µl of

buffer containing 0.05-2.5 head equivalents of extract overnight at 4°C. Lane 12 contained only buffer and served as a blank control. After incubation the plates were treated with antibody and developed as above. ELISA results were calculated as a percentage of maximum binding to PBAN (0.75 pmol) after subtraction of the blank.

Results and Discussion

To determine if the heads of sexually mature males of *A. suspensa* contained compounds that were potentially pheromonotropic, extracts of heads were injected into females of *H. zea*. *H. zea* was chosen as a recipient for treatments because our laboratory has conducted a number of studies on the actions of pheromonotropic neuropeptides from different sources using this moth (Abernathy et al. 1995). Moths injected with 0.25-10.0 head equivalents of the head extract from male flies were induced to produce significantly more pheromone than that obtained from pheromone glands of females injected with only saline. In fact, females injected with 1.0 head equivalent produced as much pheromone as was present in extracts obtained from females injected with 5 pmol of synthetic PBAN, the optimal dose for stimulation of pheromone production in this moth (Abernathy et al. 1995). Although extracts obtained from moths injected with 10 head equivalents contained more pheromone than was present in extracts from females injected with only saline, the amount was lower than that present in extracts obtained when 5 head equivalents were injected. This appears to reflect the fact that moths injected with 10 head equivalents were immobile and were apparently suffering from a toxic reaction in response to injection of the crude extracts from the heads of the flies.

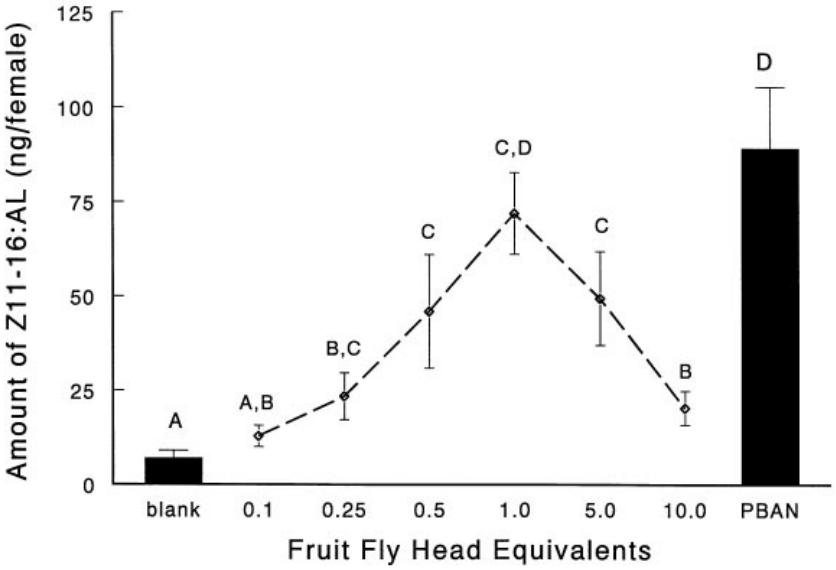

Fig. 1. Comparison of amounts of pheromone present in extracts obtained from females of *H. zea* 1 h after injection with different amounts of extracts of heads of male *A. suspensa* or 5 pmol of synthetic PBAN or only saline. Means (± SE, n = 5/treatment) superscribed with the same letter were not significantly different when compared in a Fisher's least significant difference test at p = 0.05.

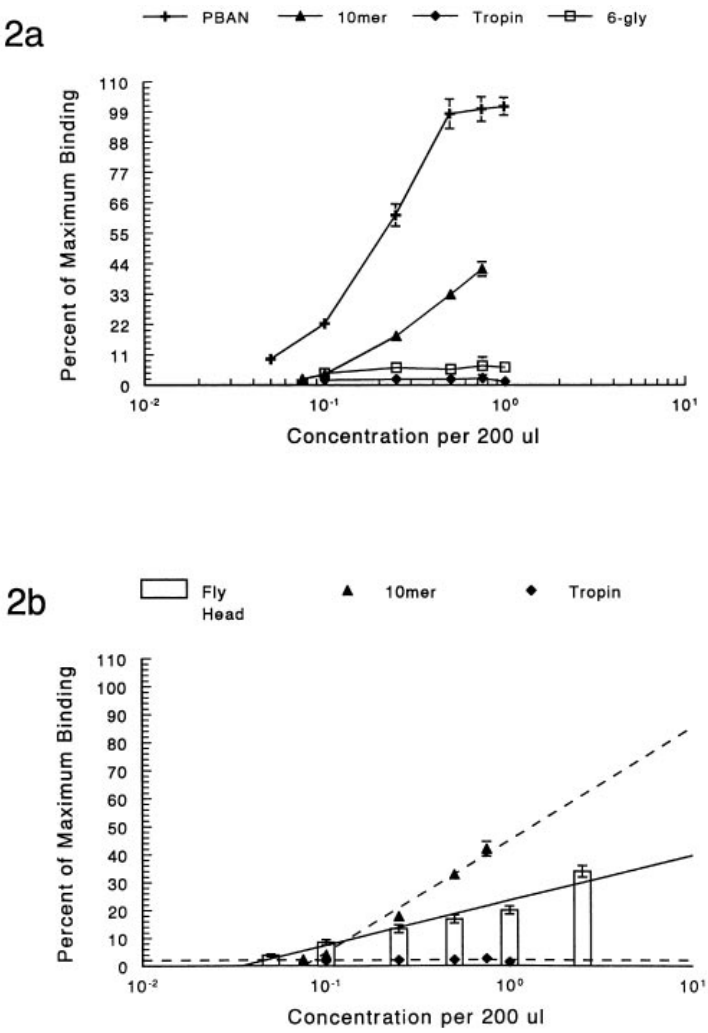

Fig. 2. Binding of antiserum to PBAN, the C-terminal 10 amino acid fragment of PBAN (10mer), allatotropin (Tropin), the pyroglutamated octapeptide (6-gly), and extracts of the heads of males of *A. suspensa* as indicated in ELISA studies. Data are presented as mean percentages of maximal binding (0.75 pmol PBAN) (± SEM, 8 replicates) after subtraction of the buffer blank. 2a. Relative binding of antiserum with synthetic peptides. T-tests and regression analysis indicated that significant binding occurred only when PBAN or the C-terminal 10 amino acid fragment of PBAN were used. 2b: Relative binding of fly head extracts compared with relative binding of the C-terminal 10 amino acid fragment and allatotropin (data from 2a). Regression analysis indicated that binding of from head extracts increased in a linear fashion with increasing dose ($R^2 = 0.886$). Mean absorption values for head extracts, calculated prior to subtraction of the blank, were significantly greater than that of the buffer blank lane for all concentrations (at 0.05 head equivalents T = 23.43, 14 df).

To determine if substances present in the head extracts from male *A. suspensa* had any structural similarity to PBAN we conducted ELISA studies. Studies on the specificity of the antiserum for PBAN and other neuropeptides including: allatotropin (A-K-S-Y-N-F-G-L-NH_2), a pyroglutamated octapeptide (pE-T-S-F-T-G-R-L-NH_2), the C-terminal 10 amino acid fragment of PBAN (S-R-T-K-Y-F-S-P-R-L-NH_2) and PBAN (L-S-D-D-M-P-A-T-P-A-D-Q-E-M-Y-R-Q-D-P-E-Q-I-D-S-R-T-K-Y-F-S-P-R-L-NH_2) indicated that significant binding occurred only when PBAN or the C-terminal decapeptide fragment of PBAN were used as substrates in the ELISA (Fig. 2). Additionally, immunochemical studies conducted by Davis et al. (1996) have shown that the antiserum complexes with PBAN and the pentapeptide F-T-P-R-L-NH_2, but not with other insect neuropeptides like FMRFamide and proctolin. Thus, the antiserum has reactivity for PBAN-like molecules and has antibodies directed against the C-terminal portion of the molecule. This C-terminal fragment is the active core of PBAN, which is necessary and sufficient for pheromonotropic activity (see Abernathy et al. 1996).

ELISA studies comparing the reactivity of head extracts of flies showed that the extracts bind the antiserum (Fig. 2). The binding increased in a linear fashion (percent maximum binding = $6.567 + 11.70 \times$ concentration of head extract, $r^2 = 0.886$) over the range of doses tested. Therefore, the extracts from sexually mature males of *A. suspensa* contained substances that had some structural similarity with PBAN.

I conclude from the results of pheromonotropic and ELISA studies, that pheromonotropic neuropeptides with structural similarity to moth PBAN are present in the heads of sexually mature males of *A. suspensa*. It is not known if these same peptides are present in the cephalic ganglia of females of *A. suspensa*, but it is likely that they are because PBAN is produced by males of the corn earworm moth (Raina & Klun 1984). Although males of *A. suspensa* exhibit a diel periodicity of pheromone production, which in insects like the Hessian fly is regulated by neuropeptides (Foster et al. 1991), studies to determine if these factors are responsible for induction of pheromone production by males of *A. suspensa* have not been conducted. It is possible that the peptides in question are responsible for regulation of other physiological functions, for example myotropic stimulation of the gut, because myotropic neuropeptides from roaches and the migratory locust will stimulate pheromone production when injected into females of H. zea (Abernathy et al. 1995). However, no myotropic neuropeptides having homology with the active core of PBAN have been identified from Diptera. Further research will be required to determine if males of *A. suspensa* regulate the induction of pheromone production with these or other neuropeptides.

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