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Endocrine Control of Pheromone Production in Arthropods

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C. Abstract.

Heliothis armigera and Spodoptera littoralis females produce sex pheromone and call for mates during the scotophase. When females were ligated with string between the head and thorax, they failed to produce pheromone. Homogenates of brain injected from both species induced pheromone production by ligated females. Thus, neuroendocrine control in these species was found to be similar to mechanisms that function in H. zea. Isolated in vitro pheromone glands of H. armigera and H. zea were stimulated to incorporate ^{14}C -sodium acetate into pheromone when brain extracts were supplied to the physiological incubation mixture. Stimulation by brain hormone involves the terminal abdominal ganglion and tests show that stimulation is also dependent upon the cyclic adenosine monophosphate system as second messenger. A pheromone biosynthesis activating neuropeptide hormone controlling sex pheromone production (PBAN) in H. zea was identified and synthesized. The synthetic peptide, composed of 33 amino acids, triggered pheromone production in vivo in H. zea and a number of taxonomically unrelated species of moths.

D. Objectives of the original research proposal.

The main objective of the proposed research was to analyze the physiological and biochemical elements involved in neurohormonal control of sex pheromone production using *Heliothis* spp. and several other lepidopterans of agricultural importance as comparative models.

e. Report.

Materials and Methods:

Insect colonies: A colony of *Spodoptera littoralis* and *Heliothis armigera* was established from 100-200 field-collected larvae maintained on an artificial diet at 25°C and a 14:10 light:dark photoperiod. Male and female pupae were separated and cages of female pupae were kept in a separate incubator under the same conditions.

Calling behavior: Calling was observed during the scotophase in the presence of a dim red light and the number of females with extruded glands were recorded.

Pheromone extractions and separations: Pheromone produced by in vivo females was extracted by gently squeezing the abdomen and cutting the extruded 8th and 9th abdominal segments. These glands were subsequently extracted for 10 min in 100 µl hexane containing tridecyl acetate as internal standard. Separation was performed on a gas chromatograph as reported in Rafaeli & Soroker, 1989a.

Brain extracts: Brains (comprising of brains, subesophageal ganglia, corpora cardiaca and corpora allata) were dissected under ice into physiological medium or 80% methanol, homogenized, centrifuged and the supernatant was used as the hormone source. Partial purification of brain homogenates was performed on low-pressure C18 silica cartridge (Sep-pak).

In vivo bioassay for hormonal response: The ligation method (Raina and Klun, 1984) was employed in our in vivo analyses.

In vitro bioassay for hormone response: Abdominal tips were removed under sterile conditions from 2-3 day old beheaded virgin females during the first-third hour of the scotophase. These glands were washed twice in sterile TC-199 medium and then transferred individually to medium TC-199 containing 3-5 microCi 14C-sodium acetate (Soroker & Rafaeli, 1989). After the required incubation period, glands were extracted in the medium with 500 microliters hexane. Total incorporated radioactivity in an aliquot of the hexane extract was counted in a scintillation counter. Components from the extract were separated using TLC, HPLC and GC.

Isolation, identification, synthesis, and assay of synthetic

PBAN: PBAN was isolated from two sets of ca. 2,500 brain-subesophageal ganglion complexes dissected from adult male and female *H. zea*. A three-step isolation high-performance liquid chromatography procedure (Jaffe, Raina and Hayes 1986) was used for the purification of the peptide using the ligated-female assay (Raina and Klun 1984) to identify the biologically active fractions. Amino acid composition of the peptide was determined using an Applied Biosystems Model 420A derivatizer-analyzer and its amino acid sequence

was established by using an Applied Biosystems Model 477A pulsed liquid phase sequencer on line with a Model 120A phenylthiohydantoin analyzer. The mass spectrum of the peptide was acquired on a Califormium-252 time of flight plasma desorption mass spectrometer and the peptide was synthesized by solid phase synthesis. The synthetic peptide was assayed in a dose response test against *H. zea* females and against six other species of moths using the ligated-female assay (Raina et al., accepted for publication).

Results:

1. Effect of brain extracts on in vivo pheromone production by *Heliothis armigera* and *Spodoptera littoralis*.

1.1. Existence of a diel rhythm in calling behavior and pheromone production: Pheromone production by *Heliothis armigera* showed a diel periodicity with maximum levels occurring at 4.5 hours into the scotophase. This was consistent with calling behavior which also followed a diel periodicity (Rafaeli & Soroker, 1989a). Similar diel periodicity was also observed in *Spodoptera littoralis* females.

1.2. Effect of ligation and subsequent treatment with brain homogenates or extracts: On ligation of both species, pheromone production was stopped. This production, however, was restored after injections of brain homogenates or partially purified brain extracts. (Rafaeli & Soroker, 1989a). In addition, it was found that *H. armigera* ligated females injected with brain extracts from *S. littoralis* females were able to produce pheromone.

2. Hormonal stimulation of in vitro pheromone production by *H. armigera* pheromone glands:

2.1. Total incorporation of ^{14}C from sodium acetate by in vitro pheromone glands; time course: The integrity of the bioassay was tested by following a time course of incorporation of radioactivity by the glands under the control conditions. It was observed that the pheromone glands incorporated radioactivity at a linear rate for up to 24 hours.

2.2. Effect of brain extracts on total incorporation of ^{14}C from sodium acetate by in vitro pheromone glands; dose-response: Both male and female brain extracts significantly stimulated the control level of incorporation by the pheromone glands and a dose dependence was established where maximal response was obtained at 0.5 brain equivalent. This response was also observed to be light dependent where under light conditions the level of stimulation was lower than under dark conditions (Table 1; Soroker & Rafaeli, 1989).

2.3. Effect of brain extracts on incorporation of ^{14}C from sodium acetate into (Z)-11-hexadecenal:

2.3.1. TLC analysis: Pooled extracts from control and stimulated in vitro pheromone gland incubations were separated on normal-phase TLC. Control incubations revealed low levels of incorporation but incubations stimulated with brain extracts showed 4 distinct peaks of radioactivity (Fig. 1; Soroker & Rafaeli, 1989). Co-chromatography of non-radioactive compounds indicated that the peaks corresponded to incorporation into triglycerides, (Z)-11-hexadecenol, and (Z)-11-hexadecenal. The fourth peak was unidentified.

2.3.2. HPLC analysis: Similar separations of pooled hexane extracts from control and stimulated in vitro incubations were performed on reverse-phase HPLC columns. These revealed a significant increase in the level of radioactivity when pheromone glands were exposed to brain extracts in a peak coeluting with the marker component (Z)-11-hexadecenal (Figs. 2 & 3; Soroker & Rafaeli, 1989).

2.3.3. GC analysis: In the framework of our collaborative work (see section on collaboration) samples of pooled hexane extracts of both control and stimulated in vitro incubations of *H. armigera* were subjected to preparative radio-GC and the resulting incorporation was found to correspond to the marker (Z)-11 hexadecenal (Table II).

2.4. Effect of brain extracts on (Z)-11-hexadecenal production. Further verification of the presence of (Z)-11-hexadecenal in incubations stimulated by brain extracts was obtained by gas-chromatographic analysis. Hexane extracts of control incubations produced negligible quantities of (Z)-11-hexadecenal however, in the presence of 1.1 female equivalents of brain extract in vitro pheromone production reached 11.95 ng/gland (Table III, Soroker & Rafaeli, 1989). This level was somewhat lower than the normal in vivo pheromone production level in which pheromone production of up to 60ng/gland was reached (Rafaeli & Soroker, 1989).

3. Hormonal stimulation of in vitro pheromone biosynthesis by *H. zea* pheromone glands:

3.1. Total incorporation of ^{14}C from sodium acetate. The total in vitro incorporation of radioactivity from sodium acetate into hexane extractable products by in vitro pheromone glands of *H. zea* also showed an increase in the level of radioactivity when pheromone glands were stimulated by brain extracts (Table IV).

3.2. Preparative Radio-GC analysis: The total hexane extractable products of pooled control and stimulated incubations were separated using preparative GC. Effluent collection of the marker compounds revealed a significant stimulation of radiolabel incorporation into a peak co-eluting with (Z)-11-hexadecenal in the presence of brain extracts (Table V).

3.3. Effect of the presence of intact terminal abdominal ganglia in the in vitro bioassay: The level of incorporation from ^{14}C sodium acetate was tested in the presence of intact terminal abdominal ganglia (TAG). Both in control incubations and incubations containing brain extracts the level of incorporation was higher when TAG was present, however, the percentage stimulation was not higher than that obtained by isolated in vitro pheromone glands. Similar separations on preparative radio-GC were performed on pheromone gland incubations containing intact TAG in the presence and absence of brain extracts and a significant increase in incorporation of radioactivity occurred in the peak co-eluting with (Z)-11-hexadecenal (Tables II & IV.). This increase was significantly higher than that obtained in the absence of TAG.

4. Cyclic-AMP mediation of the hormonal stimulation of pheromone production by

in vitro pheromone glands.

4.1. Stimulation of intracellular cAMP levels by brain extracts: Stimulated pheromone glands showed a significant increase in the intracellular cAMP levels reaching a maximum level after 5-7 mins of incubation. This cellular stimulation was observed to follow a dose-dependence reaching a saturation level at 1.5 brain equivalents (Figs. 4 & 5; Rafaeli & Soroker 1989b).

4.2. Effect of cAMP analogues/mimetics on total ^{14}C incorporation: On addition of forskolin, IBMX and 8-Bromo c-AMP an increase in ^{14}C acetate incorporation by pheromone glands was observed (Table VI; Rafaeli & Soroker, 1989b).

4.3. Effect of 8-Bromo c-AMP on incorporation of radioactivity into (Z)-11-hexadecenal as separated by TLC: Hexane extracts of pooled incubation media after stimulation by 8-Bromo-c-AMP revealed a distinct radioactive peak, after TLC separation, which co-eluted with the marker (Z)-11-hexadecenal. Control incubations showed a low level of incorporation (Fig. 6; Rafaeli & Soroker, 1989b).

4.4. Effect of forskolin on incorporation of radioactivity into (Z)-11-hexadecenal as separated by HPLC: HPLC separations of pooled hexane extracts after forskolin stimulation also revealed radioactive peaks co-eluting with the pheromone component (Z)-11-hexadecenal, showing a 170% increase as compared with control incorporation levels (Fig. 7; Rafaeli & Soroker 1989b)

5. PBAN.

5.1 Structure of the peptide: Analyses indicated that the peptide was composed of 33 amino acids having the following structure. Leu-Ser-Asp-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Met-Tyr-Arg-Gln-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-NH₂. The peptide was prepared with an amide terminus because most known insect-derived peptide were amidated. The resulting peptide had a retention time on HPLC and a UV spectrum slightly different from that of the natural peptide. However, knowing that the natural peptide contained two methionine residues and that these were susceptible to oxidation in the purification process, the synthetic peptide was oxidized with dimethylsulfoxide and the resulting product's retention time and absorption spectrum proved to be identical to the isolated natural peptide.

5.2. When the unoxidized synthetic peptide in a sucrose-phosphate buffer solution was injected into ligated *H. zea* females at doses above 1 picomole, it stimulated pheromone production. At 2.0 picomoles, a pheromone titer approximately equivalent to that generated by the crude extracts of one BR-SOG was produced. A dose of 4 picomoles peptide produced maximal response, 8 and 16 picomoles stimulated a lesser titer, and at doses of 32 and 64 picomoles maximal titers were again produced (Table VII). Injection of 5 picomoles/10 microliters buffer/female into ligated females of six other species resulted in stimulation of pheromone production and cause production of titers that were greater to or equal to those produced by normal females in five of the six species (Table VIII).

Discussion

From our investigations on in vivo pheromone production, we conclude that hormonal control of pheromone production occurs. The diel periodicity of pheromone production in *S. littoralis* and *H. armigera* indicated that some control mechanism is involved in "switching on and off" of pheromone production. Ligation stopped production and this verified that the control exists in the head region. Restoration of pheromone production by injecting brain extracts substantiated the hypothesis that, like *H. zea*, a pheromone biosynthesis activating neuropeptide is also involved in the control mechanism of *S. littoralis* and *H. armigera*. Moreover, the fact that *S. littoralis* brain hormone was capable of inducing pheromone production in *H. armigera* (in which the pheromone components differ) supports a possible general role for PBAN in moth pheromone production. This notion is reinforced by the observation that synthetic PBAN stimulated pheromone production in ligated females of 6 species of moths.

Incorporation of radioactivity from acetate was stimulated in isolated pheromone glands by brain extracts. After separation by several analytical methodologies (TLC, HPLC, and Radio-GC), we conclude that brain extracts stimulate de novo biosynthesis of the pheromone component from acetate. Our findings with *H. armigera* were confirmed in studies of *H. zea* in which brain extracts also stimulated de novo biosynthesis of (Z)-11-hexadecenal. In addition, our studies showed that the presence of the terminal abdominal ganglia enhanced the stimulatory effects of brain extracts in the in vitro pheromone gland incubations. Thus, the terminal ganglion plays a complementary role in pheromone production.

The in vitro studies of pheromone production have also provided evidence that the physiological response of the pheromone gland to PBAN is mediated by cAMP. Determination of the transduction mechanism by which PBAN stimulates intracellular concentration of cAMP and mechanism by which pheromone biosynthesis is induced constitute the next fields of our research endeavors.

In the course of isolating PBAN from *H. zea*, the S-CH₃ moiety of the two methionine residues of the purified natural peptide were presumably oxidized to sulfoxide because, the UV absorption spectrum and chromatographic characteristics of the synthetic peptide matched those of the natural peptide only after it was oxidized. Yet, the oxidized natural-peptide isolate ostensibly retained its biological activity. At the same time, it was shown that unoxidized synthetic peptide also possessed hormonal activity. These facts introduce some uncertainty as to which oxidation state actually operates in vivo to stimulate pheromone production. Thus, although considerable progress has been made in understanding of PBAN, a number of fundamental questions remain unanswered.

f. Description of Cooperation.

Cooperation between the two laboratories (Israel & USA) included the exchange of samples for analysis during the initial phases of the research. This was followed by a trip to the Beltsville laboratory by Dr. Ada Rafaeli from Israel accompanied by her Ph.D. Student, Victoria Soroker, during the summer of 1988. During this visit, the in vitro pheromone gland bioassay established

in Israel, was set up in the Beltsville laboratory and was tested on H. zea (Section 3 Results). Having established the in vitro bioassay, collection of incubation media extracts led to analysis by Radio-GC. This analysis was unavailable in the Israeli laboratory and resulted in the final identification of the hexane extractable products by in vitro pheromone glands providing conclusive evidence for the de novo biosynthesis of pheromone in both Heliothis species as a direct result of stimulation by brain extracts.

g. Evaluation of Research Achievements.

Significant insight into endocrine control of sex pheromone production in pest moths has been achieved. The isolation, identification, and synthesis of PBAN from H. zea constitutes a major accomplishment. Its structure bears considerable similarity to the partially sequenced PBAN from Bombyx mori. This discovery and the fact that our synthetic PBAN has interspecific biological activity leads us to speculate that this peptide or ones structurally similar to it regulate pheromone production widely among moths. If a compound were developed to interfere with PBAN-neuroendocrine function and reproduction in H. zea, the same compound might also have utility as a control agent against other species as well. Thereby, a wide range of lepidopteran pests of agriculture might be controlled in the future by an entirely novel mechanism.

h. List of Publications.

Publications resulting from this research project are preceded by an asterisk.

*Rafaeli, A. & Soroker, V. 1989a. Influence of diel-rhythm and brain hormone on pheromone production in two Lepidopteran species. *J. Chem. Ecol.* 15:447-455

*Rafaeli, A. & Soroker, V. 1989b. c-AMP mediation during hormonal stimulation of ¹⁴C-acetate incorporation by Heliothis armigera in vitro pheromone glands. *Mol. & Cell Endocr.* (accepted for publication subject to revision).

*Soroker, V. & Rafaeli, A. 1989. In vitro hormonal stimulation of acetate incorporation by Heliothis armigera pheromone glands. *Insect Biochem.* (in press)

*Rafaeli, A., Raina, A. K. & Klun, J. A. 1985. Neuroendocrine control of sex pheromone production in Heliothis spp. *Phytoparasitica* 13:146-147.

*Raina, A. K., Jaffe, H., Kempe, T. G., Keim, P., Blacher, R. W., Fales, H. M., Riley, C. T., Klun, J. A., Ridgway, R. L. & Hayes, D. K. A neuropeptide that regulates sex pheromone production in female moths. *Science* (In press).

*Raina, A. K., Jaffe, H., Klun, J. A., Ridgway, R. L. & Hayes, D. 1987. Characteristics of a Neurohormone that controls sex pheromone production in Heliothis zea. *J. Insect Physiol.* 33:809-814.

Raina, A. K. & Klun, J. A. 1984. Brain factor control of sex pheromone production in the female corn earworm moth. *Science* 225:531-533.

TABLE I. EFFECT OF LIGHT INTENSITY ON THE INCORPORATION OF ^{14}C FROM SODIUM ACETATE BY IN VITRO PHEROMONE GLAND PREPARATIONS OF H. ARMIGERA.

TREATMENT	INCORPORATION LEVEL (cpm)		% STIMULATION
	CONTROL	STIMULATED	
DARK	2663 +/- 580 (7)b	7109 +/- 1516 (8)a	167 +/- 57 (8)
LIGHT	2806 +/- 547 (7)b	4491 +/- 1518 (7)b	72 +/- 50 (7)

Numbers represent mean +/- SEM, numbers in brackets indicate number of replicates. Numbers followed by the different letters indicates a significant difference according to Wilcoxon two sample test.

TABLE II. EFFECT OF BRAIN EXTRACTS ON ^{14}C INCORPORATION BY HELIOTHIS ARMIGERA PHEROMONE GLANDS INTO MARKER COMPOUNDS.

MARKER COMPOUND	RADIOACTIVITY (CPM)	
	CONTROL	+BRAIN
1. Hexadecenal	27	32
2. (Z)-11 Hexadecenal	31	140*
3. Hexadecenyl acetate	33	31
4. (Z)-11 Hexadecenyl acetate	34	26
5. Hexadecenol	32	28
6. (Z)-11 Hexadecenol	26	35

TABLE III. (Z)-11-HEXADECENAL CONCENTRATIONS IN THE HEXANE EXTRACTABLE PRODUCTS AFTER IN VITRO INCUBATIONS OF PHEROMONE GLANDS IN THE ABSENCE AND PRESENCE OF FEMALE BRAIN-COMPLEX METHANOL EXTRACTS AS QUANTIFIED BY GAS CHROMATOGRAPHIC ANALYSIS.

TREATMENT	(Z)-11-HEXADECENAL CONCENTRATION (ng/gland/incubation)
CONTROL (7 hr incubation)	0.62 +/- 0.49 a (8)
FEMALE BRAIN- COMPLEX EXTRACT (1.1 eq./gland, 7hr incubation)	11.95 +/- 4.7 b (14)

Numbers represent mean +/- SEM, figures in brackets indicate number of replicates. Means followed by a different letter are statistically different from one another according to the Wilcoxon two sample test.

TABLE IV. TOTAL INCORPORATION OF ^{14}C FROM SODIUM ACETATE BY IN VITRO PHEROMONE GLANDS OF HELIOTHIS ZEA.

TREATMENT	RADIOACTIVITY (cpm)	%STIMULATION
CONTROL PG	287.6 (50.16)	-
PG + BRAIN-COMPLEX EXTRACT (1.5FE)	933.7 (351.79)	224%
CONTROL PG+TAG	2285.2 (1004.4)	-
PG+TAG +BRAIN-COMPLEX EXTRACT (1.5FE)	5014.7 (699.90)	119%

Numbers represent mean (SEM), * indicates significant difference from control values.

PG = pheromone glands; TAG = intact terminal abdominal ganglion; FE = female equivalent.

TABLE V. EFFECT OF BRAIN EXTRACTS ON ¹⁴C INCORPORATION INTO MARKER COMPOUNDS.

MARKER COMPOUND	RADIOACTIVITY (cpm)	
	TAG ABSENT	TAG PRESENT
1. Hexadecenal	39 (7.9)	315 (153.5)*
2. (Z)-11 Hexadecenal	250 (192.9)*	1420 (275.9)*
3. Hexadecanyl acetate	30 (4.7)	43 (5.3)
4. (Z)-11 Hexadecenyl acetate	26 (2.8)	51 (7.2)
5. Hexadecanol	22 (0.9)	48 (14.3)
6. (Z)-11 Hexadecenol	37 (5.6)	123 (56.0)*

Numbers represent the mean (SEM) of the radioactivity separated from three separate preparative radio-GLC elutions using pooled hexane extracts of 10 Heliothis zea in vitro pheromone gland preparations in the presence of brain extracts (1.5 FE). TAG=terminal abdominal ganglion. * indicates significant incorporation when compared to control levels, [Average (n=18) control levels when TAG is absent = 24.9 +/- 1.01; when TAG is present = 32.5 +/- 3.30].

TABLE VI. STIMULATION OF ^{14}C -INCORPORATION DUE TO PHARMACOLOGICAL INCREASES IN INTRACELLULAR C-AMP LEVELS.

Treatment	^{14}C Incorporation levels		%Stimulation ^a	n
	Control	Stimulated		
Forskolin (53 M)	491 +/- 89	1572 +/- 312*	211 +/- 68	7
IBMX (0.2mg/ml)	1249 +/- 273 ^b	4873 +/- 893*	220 +/- 55	9
8-bromo C-AMP (10^{-3}M)	1249 +/- 273 ^b	4896 +/- 1928*	297 +/- 144	9
Brain-complex extracts (1.5FE) ^c	2411 +/- 437	8005 +/- 2724	239 +/- 110	7

Numbers represent mean +/- SEM, * = Statistical significance from control levels.

^aPercentage stimulation value relative to the mean control incorporation level.

^bControl and stimulated replicates were run concurrently, different treatments (except 8-bromo C-AMP and IBMX) were performed as separate experiments.

^cData from Soroker & Rafaeli, 1989 (in press).

TABLE VII. Sex pheromone titers generated by doses of synthetic peptide injected into ligated *H. zea* females. Standard error of the mean is in parentheses after the mean.

Peptide dose (picomoles)	Mean ng (n=5) (Z)-11-hexadecenal/female
0.0	2.1 (0.18)
0.25	2.7 (0.46)
0.5	3.9 (0.83)
1.0	6.3 (0.93)
2.0	68.1 (5.57)
4.0	185.2 (16.95)
8.0	97.8 (10.87)
16.0	121.4 (19.41)
32.0	216.6 (24.94)
64.0	224.6 (29.80)

TABLE VIII. Stimulation of sex pheromone production by synthetic peptide in females of six species of moths.

Test Species	Mean ng Major pheromone component in female (n=5)		
	Normal	Ligated	Peptide-injected
<u>Heliothis virescens</u>	177.9	5.7	189.2
<u>Spodoptera fugiperda</u>	6.9	1.5	42.9*
<u>Diaphania nitidalis</u>	17.5	2.3	49.2*
<u>Ostrinia nubilalis</u>	4.0	0.0	6.7
<u>Manduca sexta</u>	9.0	3.2	65.4*
<u>Lymantria dispar</u>	9.3	0.1	2.5*

Means followed by an asterisk are significantly different from mean titers produced by normal females.

Fig. 1. Effect of female brain-complex extracts on the incorporation of radioactivity from ^{14}C - sodium acetate into hexane extractable products as separated by TLC using hexane:ethyl acetate (1:1) as the solvent system. P.A. = palmitoleic acid, Z11HDAL = (Z)-11 hexadecenal, Z11HDOL = (Z)-11 hexadecenol, T. G. = triglycerides.

Fig. 2. Effect of male brain-complex extracts on the incorporation of radioactivity from ^{14}C - sodium acetate into (Z)-11 hexadecenal. The top graph shows the elution profile containing marker components (A), (Z)-9 hexadecenoic acid (50 μg) and (B), (Z)-11 hexadecenal (10 μg). The bottom graph depicts the corresponding radioactivity present in the collected 1 ml fractions.

Fig. 3. Effect of female brain-complex extracts on the incorporation of radioactivity from ^{14}C - sodium acetate into (Z)-11 hexadecenal. The elution profile shows the retention times of markers (a), (Z)-9 hexadecenoic acid (50 μg) and (b), (Z)-11 hexadecenal (10 μg). The cross-hatched histograms depict the level of radioactivity in the corresponding 1 ml fractions.

Fig. 4. Time course of response to brain-complex extracts (1.9 FE) by in vitro pheromone glands as compared to control pheromone gland incubations showing intracellular cAMP levels.

Fig. 5. Dose-response curve of methanolic brain-complex extracts by in vitro pheromone glands showing intracellular cAMP levels after 5 minutes of incubation.

Fig. 6. Effect of 8-bromo-cAMP on the incorporation of radioactivity from ¹⁴C- sodium acetate into hexane extractable products as separated by TLC (see Figure 1 for abbreviations and separation conditions).

Fig. 7. Effect of forskolin (75mM) on the incorporation of radioactivity from ^{14}C - sodium acetate into (Z)-11 hexadecenal. The top graph A shows the elution profile containing marker synthetic components. The bottom graph depicts the corresponding radioactivity present in the collected 1 ml fractions.