

Regulation of pheromone biosynthesis by a brain hormone in two moth species

(neuroendocrine regulation/pheromone biosynthesis/fatty acid synthesis/*Argyrotaenia velutinana*/*Trichoplusia ni*)

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ABSTRACT Experiments were performed to characterize the action of a brain hormone on pheromone biosynthesis in female redbanded leafroller and cabbage looper moths. Results showed that the two species differed in their respective control mechanisms. In the cabbage looper, pheromone titer from decapitated females that received either saline or brain extract injections was not significantly different from control females, suggesting that pheromone biosynthesis was not dependent on the presence of the brain hormone. In contrast, with redbanded leafroller females, studies using radiolabeled acetate incorporation as well as incorporation of deuterium-labeled hexadecanoic acid showed that (i) the brain hormone was required for pheromone biosynthesis, (ii) the brain hormone regulated pheromone biosynthesis by activating synthesis of octadecanoyl and hexadecanoyl intermediates, and (iii) the brain hormone did not control other enzymes in the pathway. Regulation of fatty acid synthetase was unlikely since assays of the enzyme from decapitated and normal females showed no differences in the amount or distribution of the 18- and 16-carbon acyl end products. These results in conjunction with those from organ cultures of the pheromone gland suggest that the brain hormone acts by increasing the substrate supply for fatty acid synthesis.

Recent research in moths has greatly advanced our understanding of the endogenous control mechanisms responsible for sex pheromone production and release. Female moths emit pheromone only when they call, a behavior that requires input from the brain or other higher nervous centers (1–4). Production of pheromone, on the other hand, is under neuroendocrine control by a peptide localized in the subesophageal ganglion portion of the brain complex (5–7).

Several lines of evidence support the existence of a brain hormone that controls pheromone production. After neckligation or decapitation, females exhibited a decrease in pheromone titer (5, 6). A subsequent increase in titer was induced by injection of brain extract. Since hemolymph showed pheromonotropic activity only during periods when a female was producing pheromone, it was concluded that control of pheromone production was mediated by release of the brain hormone into the hemolymph at specific times of the day (5). The presence of functionally similar pheromonotropic factors in several families of moths has been demonstrated (5, 7) as well as from brains of insects from other orders—e.g., a cockroach, *Periplaneta americana*, and a cricket, *Gryllus bimaculatus* (L.S. and W.L.R., unpublished results).

The biosynthetic pathways of pheromone production for the two species examined in the present study, the redbanded leafroller moth (RBLR), *Argyrotaenia velutinana*, and the cabbage looper moth (CL), *Trichoplusia ni*, are depicted in

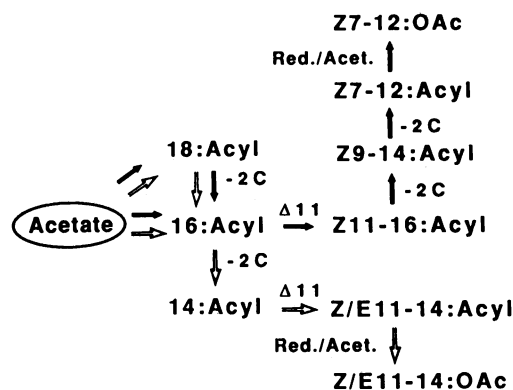


FIG. 1. Key steps in the biosynthesis of the RBLR and CL pheromones (8–10). Solid arrows describe the pathway found for the CL and open arrows for the RBLR. $-2C$, chain shortening; $\Delta 11$, $\Delta 11$ desaturation; Red./Acet., reduction/acetylation; 18-, 16-, and 14:Acyl, octadecanoate, hexadecanoate, and tetradecanoate esterified to a complex lipid, respectively; Z7-12:OAc, (Z)-7-dodecanyl acetate; Z7-12:Acyl, Z9-14:Acyl, Z11-16:Acyl, and Z/E11-14:Acyl, (Z)-7-dodecenoate, (Z)-9-tetradecenoate, (Z)-11-hexadecenoate, (Z)- and (E)-11-tetradecenoate, respectively, esterified to a naturally occurring lipid; Z/E11-14:OAc, (Z)- and (E)-11-tetradecanyl acetate.

Fig. 1 (8–10). The enzymatic steps involved (fatty acid synthesis, chain shortening, $\Delta 11$ desaturation, reduction, and acetylation) are common to a variety of moth species (11, 12). The influence of the brain hormone on these pathway steps, however, is yet to be reported. In this paper, decapitation and brain extract injection were used to evaluate the effect of the presence and absence of brain hormone. Where appropriate, the indices used to monitor effects were pheromone titer, titer of biosynthetic intermediates in the pathway, incorporation of [$1-^{14}C$]acetate, and incorporation of [$16,16,16-^3H$]hexadecanoic acid. In addition, glands from decapitated and normal RBLR females were compared for *in vitro* fatty acid synthetase activity and for [$1-^{14}C$]acetate incorporation into pheromone and biosynthetic intermediates in organ culture.

MATERIALS AND METHODS

Insects. A pinto bean diet was used to rear both species (13). CL were housed at $28 \pm 2^\circ C$ with a light/dark cycle of

Abbreviations: RBLR, redbanded leafroller moth; CL, cabbage looper moth; MAI, most abundant isotopomer; Examples of compound abbreviations are: Z11-14:Acyl, (Z)-11-tetradecenoate esterified to a complex lipid; Z11-14:OAc, (Z)-11-tetradecanyl acetate; and Z11-14:ME, methyl (Z)-11-tetradecenoate.

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16 hr/8 hr; RBLR were housed at $26 \pm 2^\circ\text{C}$ with a light/dark cycle of 16 hr/8 hr. Sexes were separated as pupae and adult females were segregated daily.

Brain Extract Preparation. Heads from 4- to 5-day-old RBLR and 2- to 3-day-old CL females were collected 2 hr before lights-off and homogenized in cold methanol/water, 1:1 (vol/vol). After repeated centrifugation ($10,000 \times g$ for 3 min at 4°C) and pellet resuspension, the supernatant was lyophilized and stored at -15°C . Before use the extract was resuspended in saline (14) to give the dosages indicated. Brain extracts were used strictly within species.

Decapitations. At mid-photophase, 1-day-old females were segregated into two groups. One group was anesthetized (CO_2 gas and chilling) and decapitated, whereas the control group was only anesthetized.

Topical Application of Pheromone Precursors and Brain Extract Injection. The precursors used were sodium [$1\text{-}^{14}\text{C}$]acetate (56 mCi/mmol ; $1 \text{ Ci} = 37 \text{ GBq}$) in a solution of water/dimethyl sulfoxide, 1:1 (vol/vol), and [$16,16,16\text{-}^2\text{H}$]hexadecanoic acid in dimethyl sulfoxide (8–10). For the RBLR, the compound doses were, respectively, $0.2 \mu\text{Ci}$ and $0.4 \mu\text{g}$ (each used in separate experiments and applied in a $0.2\text{-}\mu\text{l}$ drop). CL females received only sodium [$1\text{-}^{14}\text{C}$]acetate (a $0.3\text{-}\mu\text{Ci}$ dose applied in a $1\text{-}\mu\text{l}$ drop). Since females of both species restrict calling periods to scotophase at the rearing temperatures used (15, 16), experiments were timed such that females could be returned to the incubators by scotophase onset. Two hours before lights-off, glands of decapitated and control 4-day-old females were forcibly extruded and the precursor was applied. After the compound was absorbed ($\approx 45 \text{ min}$ at 32°C), females were injected in the abdomen with $1 \mu\text{l}$ of saline or $1 \mu\text{l}$ of brain extract (2 head equivalents). Females were incubated for 4 hr in the dark at their respective rearing temperatures, then the pheromone glands were extracted for lipid in chloroform/methanol, 2:1 (vol/vol).

Fatty Acid Synthetase Activity. Pheromone glands from 4-day-old RBLR females were homogenized in 0.15 ml of cold 0.1 M potassium phosphate (pH 7.4), containing 0.35 M sucrose, 3 mM EDTA, and 5 mM dithiothreitol. The suspension was centrifuged at $10,000 \times g$, the supernatant was recentrifuged at $100,000 \times g$, and the resulting supernatant was used as the crude enzyme solution. Assays contained enzyme, 5 mM NADPH, 3 mM malonyl-CoA, and $0.2 \mu\text{Ci}$ of [$1\text{-}^{14}\text{C}$]acetyl-CoA (56 mCi/mmol). After incubation at 37°C for 30 min, assays were extracted with chloroform/methanol, 2:1 (vol/vol), and methyl esters formed by acid methanolysis (17). Reverse-phase TLC [Anisil RP plates, acetonitrile, 9:1 (vol/vol)] was used to separate products and radioactivity was measured on a Berthold TLC scanner.

RBLR Lipid Synthesis: Body vs. Pheromone Gland. Immediately before scotophase onset, 4-day-old females were injected in the abdomen with $0.2 \mu\text{Ci}$ of sodium [$1\text{-}^{14}\text{C}$]acetate in $1 \mu\text{l}$ of water. After a 4-hr incubation in the dark at 26°C , the pheromone gland and body were separately extracted. Tissues were homogenized in chloroform/methanol, 2:1 (vol/vol), water was added, and the chloroform layer was removed. The chloroform was evaporated under N_2 , and radioactivity was determined by liquid scintillation counting.

Organ Culture. Pheromone glands were dissected from 4-day-old RBLR females 2 hr before lights-off and washed twice in Grace's medium (18). Glands were incubated in a $30\text{-}\mu\text{l}$ drop of the basic medium [Grace's medium/bovine serum albumin ($1 \mu\text{g}/\mu\text{l}$)/sodium [$1\text{-}^{14}\text{C}$]acetate ($0.6 \mu\text{Ci}$)], or the basic medium with brain extract (1 head equivalent per μl). After a 4-hr incubation in the dark at 26°C , glands were extracted in chloroform/methanol, 2:1 (vol/vol).

Titer Determination. To quantify amounts of pheromone and intermediates, glands were extracted in chloroform/methanol, 2:1 (vol/vol), for 24 hr, with 500 ng of methyl pentadecanoate added as an internal standard. Methyl esters

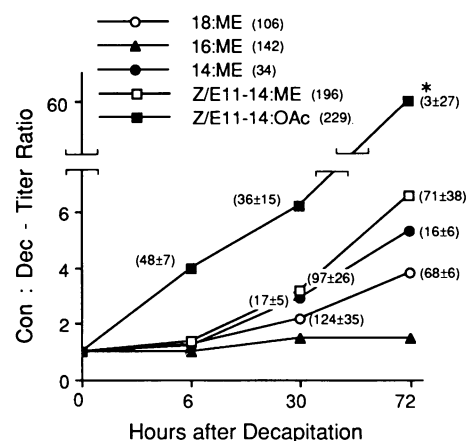


FIG. 2. Time-related changes in amounts of pheromone and biosynthetic intermediates in control (Con) versus decapitated (Dec) RBLR females (three replicates of five insects). Amounts (ng per female) obtained at time 0 hr for controls are enclosed in parentheses next to the compound key. For each interval after the time of decapitation, the titers of decapitated and control females were compared (two-tailed, unpaired t test, $P = 0.05$). Where significant differences were found, the mean from decapitated females followed by the pooled SE is indicated in parentheses beside the data point. The mean titer of control females can be calculated by multiplying the mean of decapitated females by the control/decapitated titer ratio. After extraction, esterified fatty acids were derivatized to form the corresponding methyl esters (ME). 18:ME, methyl octadecanoate. *, Logarithmic transformation was necessary to satisfy equal variances.

were formed by base methanolysis, and acetyl chloride was used to regenerate the acetates (10). Samples were analyzed by flame ionization GC on a capillary column (Supelcowax 10, $0.25 \text{ mm} \times 30 \text{ m}$). The column was programmed from 60°C (1 min) to 100°C at $10^\circ\text{C}/\text{min}$ (0 min) and then at $5^\circ\text{C}/\text{min}$ to 220°C (15 min).

Incorporation of Labeled Precursors. Separation and quantification of pheromone and intermediates in [$1\text{-}^{14}\text{C}$]acetate experiments were accomplished by thermal conductivity GC on a macrobore capillary column (SP-2380, $0.53 \text{ mm} \times 30 \text{ m}$). The column was programmed from 80°C (2 min) to 200°C at $3^\circ\text{C}/\text{min}$ and held at 200°C for 15 min. Compounds eluting from the detector were collected in cold glass micropipets, which then were washed with scintillation fluid, and radioactivity was measured.

Incorporation levels of [$16,16,16\text{-}^2\text{H}$]hexadecanoic acid into RBLR pheromone and intermediates were quantified by GC/mass spectrometry using electron-impact (70 eV) and selected-ion monitoring (10). Compounds were separated using a capillary column ($0.25 \text{ mm} \times 30 \text{ m}$) coated with crossbonded Carbowax PEG 20M (Stabilwax, Restek, Bellefonte, PA). The temperature program was the same as that used in the flame-ionization analysis. The following most abundant isotopomers (MAI) were selectively monitored: methyl tetradecanoate (14:ME), m/z 242; methyl hexadecanoate (16:ME), m/z 270; (Z)-11-tetradecenyl acetate (Z11-14:OAc), m/z 194 ($M - 60$); methyl (Z)- and (E)-11-tetradecenoate (Z/E11-14:ME), m/z 208 ($M - 32$). In addition, the (MAI + 1), the (MAI + 2), and the deuterium-enriched (MAI + 3) ions were scanned.

To quantify amounts of labeled compounds present, an aliquot of each sample was analyzed by flame-ionization GC and the total amount of each compound was determined. The total ion current for each compound [(MAI) + (MAI + 1) + (MAI + 2) + (MAI + 3)] was obtained from the GC/mass spectrometric analysis. The amount of labeled compound present (adjusted for the low, naturally occurring amounts of the MAI + 3 ion) was calculated as [(MAI + 3)/total ion

current] multiplied by the total amount of compound (from the flame-ionization GC analysis).

RESULTS

Effects of Decapitation on Titer. After 1-day-old RBLR females were decapitated, compounds in the gland that normally increased with age [i.e., the pheromone (Z/E11-14:OAc), 18:Acyl, 14:Acyl, and Z/E11-14:Acyl (17)] did not (Fig. 2). For the pheromone, significant differences between titers from decapitated and control females were found at all intervals examined. Titers from control females ranged from 4 to 61 times greater than titers from decapitated females. The significant effects of decapitation on pheromone titer also were visible in the split-plot design experiment with radiolabeled acetate (Fig. 3A). Decapitated females injected with either brain extract or saline had a significantly lower pheromone titer than control females injected with either brain extract or saline.

For all biosynthetic intermediates except 16:Acyl, significant differences were found 30 and 72 hr after the time of decapitation (Fig. 2). By 72 hr, titers from control females for these intermediates ranged from 4 to 8 times greater than

respective titers from decapitated females. Mean comparisons showed that changes in the control/decapitated titer ratio were due to an increase in titer from control females and to a decrease in titer from decapitated females. For all times examined, decapitated and control females showed no differences in titers of 16:Acyl.

Unlike the RBLR, decapitation in the CL did not result in any significant change in titer of the pheromone Z7-12:OAc (Fig. 3B). Decapitated CL females injected with either brain extract or saline had a pheromone titer similar to control females injected with either brain extract or saline.

Incorporation of Applied Precursors. The effect of brain extract injection on radiolabeled acetate incorporation into pheromone differed for the two species. In the RBLR (Fig. 3C), decapitated or control females injected with brain extract exhibited significantly higher levels of label incorporation into pheromone than decapitated or control females injected with saline. In the CL (Fig. 3D), decapitated or control females injected with brain extract had significantly lower levels of label incorporation into pheromone than decapitated or control females injected with saline.

In the same experiment using RBLR, injection of brain extract also resulted in significantly higher levels of labeled acetate incorporation into all biosynthetic intermediates except 14:Acyl (Fig. 4). Lack of significance is probably due to the low amounts of 14:Acyl found in the gland (Fig. 2). Although the split-plot design does not allow statistical comparisons among the four individual subgroups (Figs. 3C and 4), it is noteworthy that for all intermediates labeled acetate incorporation from females that were decapitated and saline-injected was always lowest (<30 dpm above background).

Effects of decapitation and brain extract injection in the RBLR on pheromone biosynthesis from deuterium-labeled hexadecanoic acid are shown in Table 1. Split-plot analysis of variance revealed no significant differences in the amounts of deuterium-labeled compound present when females were grouped according to the day-1 (decapitated or control) or the day-4 (injection with brain extract or saline) treatments.

RBLR Fatty Acid Synthetase Activity. Fatty acid synthetase activity of pheromone glands from decapitated females did

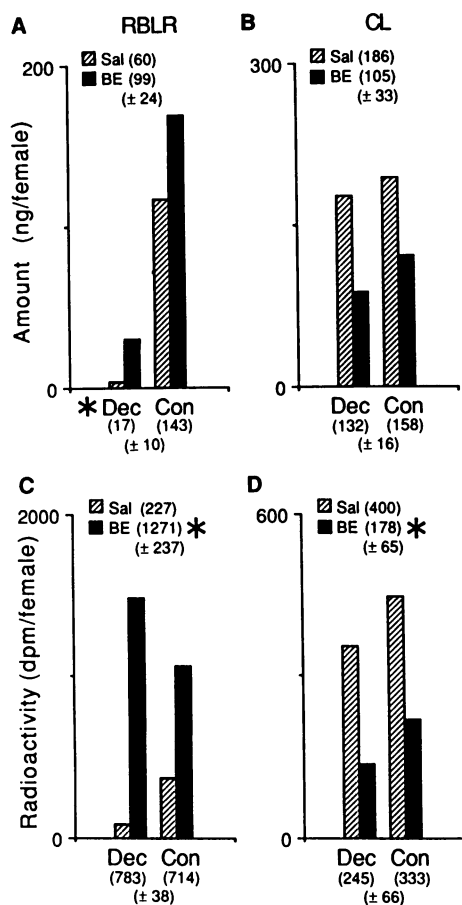


FIG. 3. Effects of decapitation and brain extract injection on pheromone titer and levels of [^{14}C]acetate incorporation in the RBLR, Z/E11-14:OAc pheromone (A and C), and the CL, Z7-12:OAc pheromone (B and D). The split-plot design was such that on day 1, females were divided into decapitated (Dec) and control (Con) groups, and then, on day 4, each group was further divided into two subgroups: one received injections of brain extract (BE) and the other injections of saline (Sal). RBLR data are based on four replicates of five insects, and CL data on three replicates of five insects. In the split-plot analysis of variance, significance (denoted by an asterisk; $P = 0.05$) was determined for the day-1 (Dec vs. Con) and for the day-4 (injected with Sal or BE) treatments. The means (\pm SE) compared are enclosed in parentheses.

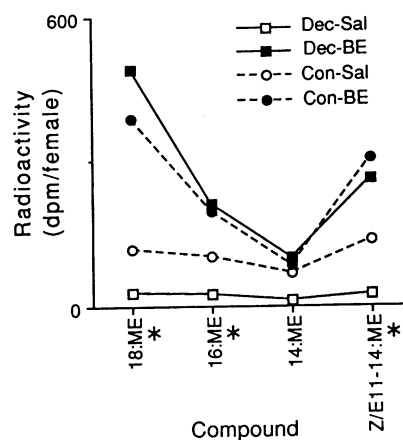


FIG. 4. Effects of decapitation (Dec) and brain extract (BE) injection in the RBLR on [^{14}C]acetate incorporation into biosynthetic intermediates (four replicates of five insects). The split-plot design and abbreviations are the same as in Fig. 3. Statistical significance ($P = 0.05$), indicated by an asterisk below the compound name, was only found when subgroups were sorted according to the day-4 treatment [injected with BE or saline (Sal)]. For these compounds, mean values are as follows: injected with BE, injected with Sal, and \pm SE. For 18:ME values are 438, 72, and \pm 100; for 16:ME values are 201, 63, and \pm 36; for Z/E11-14:ME values are 283, 79, and \pm 49. Lines connect points of each subgroup and do not imply continuity. Con, control.

Table 1. Effects of decapitation and brain extract injection on incorporation of [16,16,16-²H]hexadecanoic acid into intermediates and pheromone

Treatment		Labeled compound, ng per female			
Day 1	Day 4	16:ME	14:ME	Z/E11-14:ME	Z/E11-14:OAc
Decapitation	Saline	5.0 ± 1.2	0.3 ± 0.1	1.3 ± 0.2	1.4 ± 0.4
	Brain extract	4.5 ± 1.1	0.4 ± 0.1	1.3 ± 0.2	2.2 ± 0.7
Control	Saline	4.8 ± 1.0	0.4 ± 0.04	1.9 ± 0.4	4.2 ± 0.6
	Brain extract	4.8 ± 2.1	0.4 ± 0.1	1.7 ± 0.5	3.7 ± 1.0

Data are presented only for intermediates that occur in the pathway between hexadecanoic acid and pheromone. Values are means ± SEM based on four replicates of five females. Split-plot analysis of variance showed no significant differences in the amount of deuterium-labeled compound found when means were grouped according to the day-1 or the day-4 treatments.

not differ from that of control female glands (3 replicates of 10 females; two-tailed unpaired *t* test; *P* = 0.05). The quantities (mean ± SEM) of 18- and 16-carbon fatty acyls produced from glands of decapitated females were, respectively, 1.4 ± 0.1 and 0.3 ± 0.02 pmol per female and, for control females, 1.1 ± 0.1 and 0.2 ± 0.1 pmol per female. Interestingly, we also found that the 5:1 product ratio of the 18- to 16-carbon fatty acyls for the pheromone gland differed from the 10:1 ratio found for the rest of the body.

RBLR Lipid Synthesis: Body vs. Pheromone Gland. When radiolabeled acetate was injected into the female abdomen (three replicates of five females, two-tailed unpaired *t* test, *P* = 0.05), significantly more label (mean ± SEM) was found incorporated into lipid of control female pheromone glands (79 ± 28 dpm per female) than decapitated female pheromone glands (17 ± 4 dpm per female). The amount of label incorporated into lipid of the rest of the body, however, showed no differences between the two treatments (8687 ± 1101 dpm per female for control females and 8566 ± 378 dpm per female for decapitated females). For label incorporation into lipid of the pheromone gland, logarithmic transformation of the data was necessary to satisfy requirements of equal variances.

RBLR Pheromone Gland Organ Culture. Split-plot analysis of variance on levels of radiolabeled acetate incorporation into pheromone and all biosynthetic intermediates showed no significant differences when females were grouped according to the day-1 (decapitation or control) or the day-4 (incubation in the basic medium or the basic medium with brain extract) treatments (three replicates of five females). Although all compounds were analyzed, data are presented for the pheromone and 18:Acyl only. For label incorporation into pheromone, means (± SE for the comparison) for decapitated and control females were, respectively, 445 and 500 dpm per gland (± 110); means for glands incubated in medium alone and medium with brain extract were, respectively, 403 and 541 dpm per gland (± 98). For label incorporation into 18:Acyl, means from decapitated and control females were 145 and 131 dpm per gland (± 10); and means for glands incubated in the medium alone and medium with brain extract were 163 and 113 dpm per gland (± 33).

DISCUSSION

The RBLR and the CL moths both employ monounsaturated acetate esters as the major component of their sex pheromone blend. In addition, the reactions involved in biosynthesis are common to both species. Yet, despite these similarities, we have found that their respective control mechanisms for pheromone production appear different.

Pheromonotropic activity has been documented for CL brain extracts when injected into RBLR or European corn borer recipient females (L.S. and W.L.R., unpublished results). However, our data demonstrate that biosynthesis of the main pheromone component, Z7-12:OAc, in the CL, was not dependent on the presence of the head, and, by inference, on the brain hormone previously characterized by others (5–7). Decapitated CL females showed no differences in pheromone titer

compared to controls and exhibited no differences in levels of labeled acetate incorporation into pheromone. Both results strongly indicate that *de novo* rates of pheromone biosynthesis in decapitated and control females were similar. When brain extract was injected into decapitated or control females, we found a significant decrease in labeled acetate incorporation into pheromone as compared to females injected with saline. It is possible that by injecting brain extract, females were exposed to a factor normally not released in virgins. A factor with a negative influence on pheromone biosynthesis, however, may come into play after mating occurs.

Unlike the CL, the RBLR represents a case in which a factor, presumably similar to the brain hormone (5–7), was required for pheromone biosynthesis. Decapitation resulted in a significant decrease in pheromone titer, which can be attributed by comparison with control titers to lack of production and to a decrease in initial amounts. When decapitated or control females were injected with brain extract, significantly higher levels of labeled acetate were incorporated into pheromone and all abundant biosynthetic intermediates. Similar effects were also observed when experiments were performed at mid-photophase (J.D.T., unpublished results). These results imply that the brain hormone acted by stimulating the synthesis of the first intermediates in the pathway (i.e., the 18- and 16:Acyl products of fatty acid synthetase). Thus, in decapitated saline-injected females, one would expect no *de novo* synthesis of pathway intermediates because no brain hormone was present. Indeed, amounts of labeled acetate incorporation for intermediates were hardly above background for this treatment subgroup.

Additional evidence in support of brain hormone action in the RBLR on synthesis of the first fatty acid intermediates in the pathway, as opposed to other steps, was provided by experiments with deuterium-labeled hexadecanoic acid. The results showed that decapitated females processed similar amounts of deuterium-labeled precursor into pheromone and intermediates as controls. Furthermore, injection of brain extract had no influence on the amount of label incorporated into pheromone. Apparently, enzyme abundances and activity states for the series of reactions that convert 16:Acyl into pheromone were unaffected by decapitation or brain extract injection. Hence, the brain hormone did not influence these steps.

Previous reports have emphasized 16:Acyl as the first fatty acid intermediate formed in the pathway of pheromone biosynthesis (8–11); however, our results in the RBLR favor 18:Acyl. First, 18:Acyl was the more abundant product formed by fatty acid synthetase, and second, glandular titer of 18:Acyl decreased after decapitation, whereas the titer of 16:Acyl remained unchanged. Although we do not know the specific form in which these intermediates are used for pheromone biosynthesis, we can infer that intermediates that showed differences represented stored pools that could be added to and taken from. For 16:Acyl, status quo was maintained suggesting that the stored pool existed in a form

unavailable for pheromone biosynthesis. In addition, the form of 16:Acyl produced during pheromone biosynthesis (as a result of chain shortening of 18:Acyl) is probably short-lived, before it immediately undergoes another round of chain shortening to 14:Acyl.

Since the key to brain hormone action in the RBLR appeared to lie in the production of 18- and 16:Acyl from acetyl-CoA, we compared decapitated and control female pheromone glands for fatty acid synthetase activity. No differences were detected with regard to quantity or quality of end product formed, suggesting that fatty acid synthetase was not the target of the brain hormone. Likewise, there were no differences in labeled acetate incorporation into intermediates and pheromone when glands from decapitated and control females were incubated in gland culture with or without brain extract. Therefore, when pheromone glands were in culture, acetate freely entered the cells and all enzymes necessary for fatty acid synthesis were active.

In light of these results, it appears that in *in vivo* experiments, the topically applied acetate was not directly absorbed by the pheromone gland cells but instead was preferentially incorporated by other tissues. This is supported by the fact that analysis of female bodies after pheromone glands were removed showed that the topically applied acetate was incorporated at high levels into lipids of the rest of the body. Moreover, incorporation levels were the same regardless of treatment (decapitated or not, injected with brain extract or not—all females showed ≈ 5000 dpm per body). Injection of labeled acetate into the body also demonstrated that although decapitation hindered lipid synthesis in the pheromone gland, it did not interfere with lipid synthesis in the rest of the body. Overall, our results suggest that the brain hormone acts on some other tissue that in turn supplies substrate to the pheromone gland for fatty acid, and hence, pheromone biosynthesis.

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