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Biosynthesis and PBAN-Regulated Transport of Pheromone Polyenes in the Winter Moth, *Operophtera brumata*

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Abstract The trienoic and tetraenoic polyenes, (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene, (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene, and (3*Z*,6*Z*,9*Z*)-1,3,6,9-henicosatetraene were found in the abdominal cuticle and pheromone gland of the winter moth *Operophtera brumata* L. (Lepidoptera: Geometridae), in addition to the previously identified single component sex pheromone (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene. The pheromone biosynthesis activating neuropeptide (PBAN) is involved in the regulation of polyene transport from abdominal cuticle to the pheromone gland. *In vivo* deuterium labeling experiments showed that (11*Z*,14*Z*,17*Z*)-11,14,17-icosatrienoic acid, the malonate elongation product of linolenic acid, (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoic acid, is used to produce (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene and (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene.

Deceased May 1st 2011 in the course of this study. The other authors dedicate the publication to the memory of Prof. Cheng-Hua Zhao and his contributions to chemical ecology.

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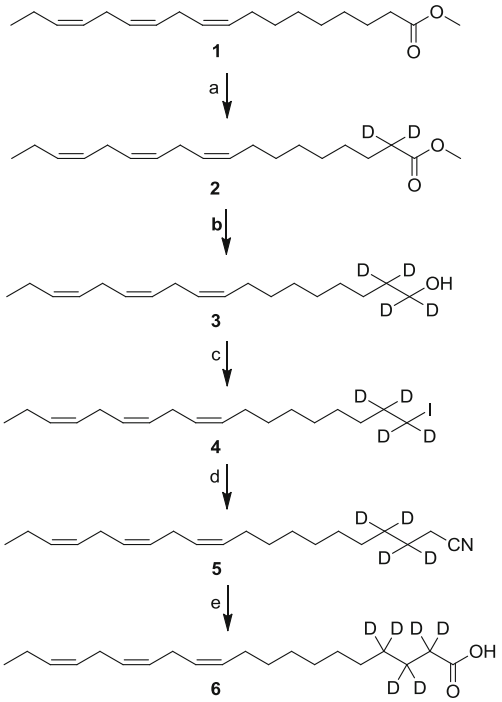
Keywords (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene · (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene · (3*Z*,6*Z*,9*Z*)-1,3,6,9-henicosatetraene · (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene. PBAN · Decarboxylation · *In vivo* labeling · Lepidoptera · Geometridae

Introduction

Trienoic and tetraenoic hydrocarbons with a chain length of 19 and/or 21 carbon atoms are used as sex pheromones by many arctiid, geometrid and noctuid moths. Examples include (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (Z3,Z6,Z9-19:H) in *Ascotis selenaria cretacea* (Butler) (Witjaksono et al. 1999), (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene (Z3,Z6,Z9-21:H) in *Caenurgina erechtea* (Cramer) (Underhill et al. 1983) and *Anticarsia gemmatalis* (Hübner) (Heath et al. 1983), (3*Z*,6*Z*,9*Z*)-1,3,6,9-nonadecatetraene (1,Z3,Z6,Z9-19:H) in *Operophtera fagata* (Scharfenberg) (Szöcs et al. 2004), as well as Z3,Z6,Z9-21:H and (3*Z*,6*Z*,9*Z*)-1,3,6,9-henicosatetraene (1,Z3,Z6,Z9-21:H) in *Utetheisa ornatrix* (Linnaeus) (Conner et al. 1980; Jain et al. 1983). These polyenes are produced in the oenocytes associated with the abdominal epidermis, starting with one- or two-cycles of 2C unit chain elongation of diet-derived linolenic acid, (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoic acid. The elongated acids, possibly conjugated to CoA, are either reduced to corresponding aldehydes followed by decarbonylation to form trienes with one less carbon atom, or undergone a further desaturation to form the fourth double bond in the terminal of the molecule, and then reduced and decarbonylated to the corresponding tetraenes. (Millar 2000; Howard and Blomquist 2005; Choi et al. 2007; Goller et al. 2007; Ding et al. 2011). The polyenes subsequently are taken up by the hemolymph lipophorin and transported to the pheromone gland for release

or further epoxidation (Schal et al. 1998a, b; Jurenka et al. 2003; Wei et al. 2003, 2004; Matsuoka et al. 2006). In the geometrid moths *Ascotis selenaria cretacea* (Bulter) (Ando et al. 1997; Wei et al. 2004) and the lymantrid moth *Lymantria dispar* (L.) (Jurenka et al. 2003), polyene production or transportation is regulated by the pheromone biosynthesis activating neuropeptide (PBAN), whereas in the arctiid moth *U. ornatrix* and the pyralid moth *Amyelois transitella* (Walker), PBAN does not appear to have any influence (Choi et al. 2007; Wang et al. 2010).

The winter moth, *Operophtera brumata* (L.) (Lepidoptera, Geometridae), uses a single tetraene, 1,Z3,Z6,Z9-19:H, as its sex pheromone (Bestmann et al. 1982; Roelofs et al. 1982). In the present study, we compared the polyene composition in pheromone gland and abdominal cuticle of the species and investigated the effect of PBAN on the production and transportation of polyenes in adult females by monitoring the titer of pheromone and other polyenes after decapitation. We also studied polyene production in this species by *in vivo* labeling experiments coupled with gas chromatography/mass spectrometry (GC/MS) analysis.



Scheme 1 **a** Na, MeOD, 4h reflux, 71 %, **b** $LiAlD_4$, abs. diethyl ether, reflux, 95 %, **c** imidazole, PPh_3, I_2, 0 °C, 84 %, **d** THF, n-BuLi, MeCN, −78 °C, 30 %, **e** NaOD, 60 % EtOD, 24 h

Methods and Materials

Chemicals The polyenes, 3Z,6Z,9Z-19:H, 1,3Z,6Z,9Z-19:H, 3Z,6Z,9Z-21:H and 1,3Z,6Z,9Z-21:H were a gift from Dr. Wittko Francke (Institute of Organic Chemistry, University of Hamburg). (11*Z*,14*Z*,17*Z*)-[2,2,3,3,4,4-$^2H_6$]-11,14,17-icosatrienoic acid (D6-Z11,Z14,Z17-20:Acid) was synthesized according to Scheme 1. Thus, methyl linolenate (**1**) was deuterated under basic conditions at C-2. Reaction with lithium alumininium deuteride furnished the labeled alcohol **3** that was transformed into iodide **4**. The chain was elongated by substitution with the acetonitrile anion. Finally, saponification under basic conditions in deuterated ethanol furnished the target (11*Z*,14*Z*,17*Z*)-[2,2,3,3,4,4-$^2H_6$]-11,14,17-icosatrienoic acid (**6**). Details are given in the Electronic Supplementary Material. The ethyl ester of (9*Z*,12*Z*,15*Z*)-[17,17,18,18,18-$^2H_5$]-9,12,15-octadecatrienoic acid was purchased from Cambridge Isotope Lab, Inc. This labeled ester was hydrolyzed to the corresponding acid (D_5-Z9,Z12,Z15-18:Acid) by methanolic potassium hydroxide. The synthetic PBAN used in the present study was purchased from Peninsula Laboratories Inc. (Belmont, CA, USA).

Insects The larvae of *O. brumata* complete feeding in summer, and drop down to the ground to pupate in the soil. The adults start emerging after sunset in late fall, when the temperatures drop to around 7 °C (MacPhee 1967). The wingless female adults climb up the trunk immediately after emerging, and start releasing sex pheromone at a height of 1–1.5 m above the ground by stretching out their abdominal tips. The insects used in this study were collected mainly in early November of 2009–2011 in Lund from a small wood (55°42′ N, 13°12′ E) where a mix of oak and chestnut trees grew. In addition, some insects were collected in Hungary in 2008. The collected female adults were kept under outdoor conditions, and those displaying calling behavior were regarded as sexually active and producing-releasing pheromone and were used in the experiments.

Polyene Extracts The pheromone glands of 1- to 3-d-old calling female *O. brumata* were dissected 0.5–1 hr into the scotophase. Each gland was extracted for 30 min in 10 μl of hexane containing 1 ng of (6*Z*,9*Z*)-6,9-nonadecadiene (Z6, Z9-19:H) as internal standard. After removal of the pheromone gland, the abdomen was separated from the thorax at the junction and cut laterally. The ovaries were removed along with the majority of the remaining fat body and internal organs. The abdominal cuticle along with the epidermal tissue then was extracted in 100 μl of hexane containing 10 ng of Z6,Z9-19:H as internal standard.

PBAN Functional Assay Females of 1- to 2-d-old were decapitated at 2 hr before the onset of darkness and kept in plastic containers (200 ml) for 24 hr. To prevent desiccation of the decapitated insect, a piece of wet filter paper was placed inside each container to maintain high humidity. The heads of *O. brumata* saved from the decapitation were homogenized on ice with a modified Weever's saline (21 mM KCl, 12 mM NaCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 170 mM glucose, 5 mM

Table 1 Polyunsaturated hydrocarbons in the pheromone gland and abdominal cuticle of female *Operophtera brumata*

Polyene	Absolute amount (ng/fe)		Blend ratio (%)		Percentage in gland (%)[a]
	Gland	Abdomen	Gland	Abdomen	
Z3,Z6,Z9-19:H	0.8±0.7	19.1±11.3	19.2±10.3	17.8±9.6	4.4±2.4
1,Z3,Z6,Z9-19:H	1.9±1.4	55.7±29.0	45.9±8.6	54.8±10.0	3.6±2.6
Z3,Z6,Z9-21:H	1.1±0.7	17.7±5.1	26.2±10.3	20.3±7.9	5.8±4.3
1,Z3,Z6,Z9-21:H	0.4±0.3	6.1±2.0	8.8±5.0	7.2±3.8	5.4±4.0

Means ± SD, N=10; [a] Hydrocarbons present in the hemolymph were not included in the calculation of percentages

PIPES, 9 mM KOH, adjusted to pH 6.6 according to Carrow et al. 1981). The homogenate was centrifuged at 11,000g for 10 min at 4 °C, and the supernatant was recovered and stored at −80 °C. The head extract (0.2 or 2 female equivalents in 2 µl saline) or the synthetic PBAN (0.1, 1 or 10 pM in 2 µl saline) was injected into the decapitated female 1 h before dark. The pheromone glands of the decapitated females, decapitated females with PBAN injected, and intact females were separately extracted as described above. In another experiment, females that had been decapitated for 4 d were injected with synthetic PBAN (5 pM in 2 µl saline). Polyenes from both pheromone glands and abdominal cuticle were extracted 2 hr after injection.

In vivo Labeling In order to investigate the decarboxylation process, two deuterium-labeled fatty acids, the D_5-Z9,Z12, Z15-18:Acid and the D_6-Z11,Z14,Z17-20:Acid were diluted in vegetable cooking oil (75 % rapeseed and 25 % sunflower oil, ICA, Sweden) in a concentration of 10 µg/µl. One µl was injected into the abdomen of a female. After 24-h incubation, both the pheromone gland and the abdominal cuticle were extracted in hexane to measure the biosynthetic incorporation of deuterium label into polyenes. Females without any labeled compound applied were used as controls.

GC/MS Analysis The composition of polyenes in pheromone glands and abdominal cuticle was determined by analysis on a Hewlett-Packard 5972 mass selective detector coupled to a Hewlett-Packard 5890 series II GC. An HP-INNOWax capillary column (30 m×0.25 mm i.d.) was used for the analyses. The oven program was set to 80 °C for 2 min after injection, then increased to 230 °C at 10 °C/min, and held at the final temperature for 15 min.

Both full scan and selected ion monitoring (SIM) modes were used to monitor the polyenes. The absolute amounts of the polyenes were quantified using the internal standard Z6,Z9-19:H. The native trienes, (3*Z*,6*Z*,9*Z*)-3,6,9-heptadecatriene (Z3,Z6, Z9-17:H), Z3,Z6,Z9-19:H, and Z3,Z6,Z9-21:H were monitored by the base peak *m/z* 79, the ion *m/z* 108 that indicates the ω-3 double bond ($C_2H_5[(CH{=}CH)_3H]^+$), the ion *m/z* 178 (for Z3,Z6, Z9-17:H), 206 (for Z3,Z6,Z9-19:H), 234 (for Z3,Z6,Z9-21:H) that show the fragment (M-$[C_2H_5(CH{=}CH)H]^+$), and the molecular ions *m/z* 234, 262, and 290, respectively (Witte et al. 2009).

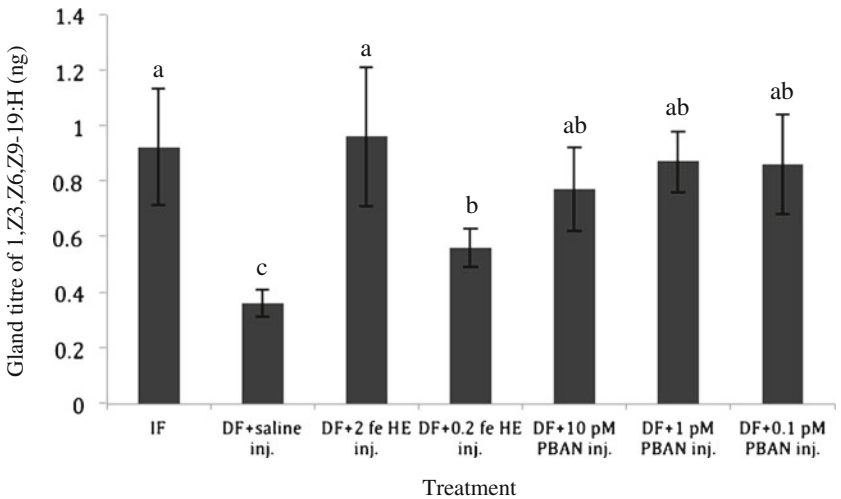


Fig. 1 PBAN effect on the glandular sex pheromone titer. IF intact female (N=14), DF+ saline inj. = decapitated female with saline injection (N=16), DF+2fe HE inj. = decapitated female with 2-female equivalent head extract (N=14), DF+0.2fe HE inj. = decapitated female with 0.2-female equivalent head extract (N=11), DF+10 pM PBAN inj. = decapitated female with 10 pM PBAN injection (N=13), DF+1 pM PBAN inj. = decapitated female with 1 pM PBAN injection (N=12), DF+0.1 pM PBAN inj. = decapitated female with 0.1 pM PBAN injection (N=12). Means with different letters are significantly different (P<0.05, one-way-ANOVA followed by LSD test)

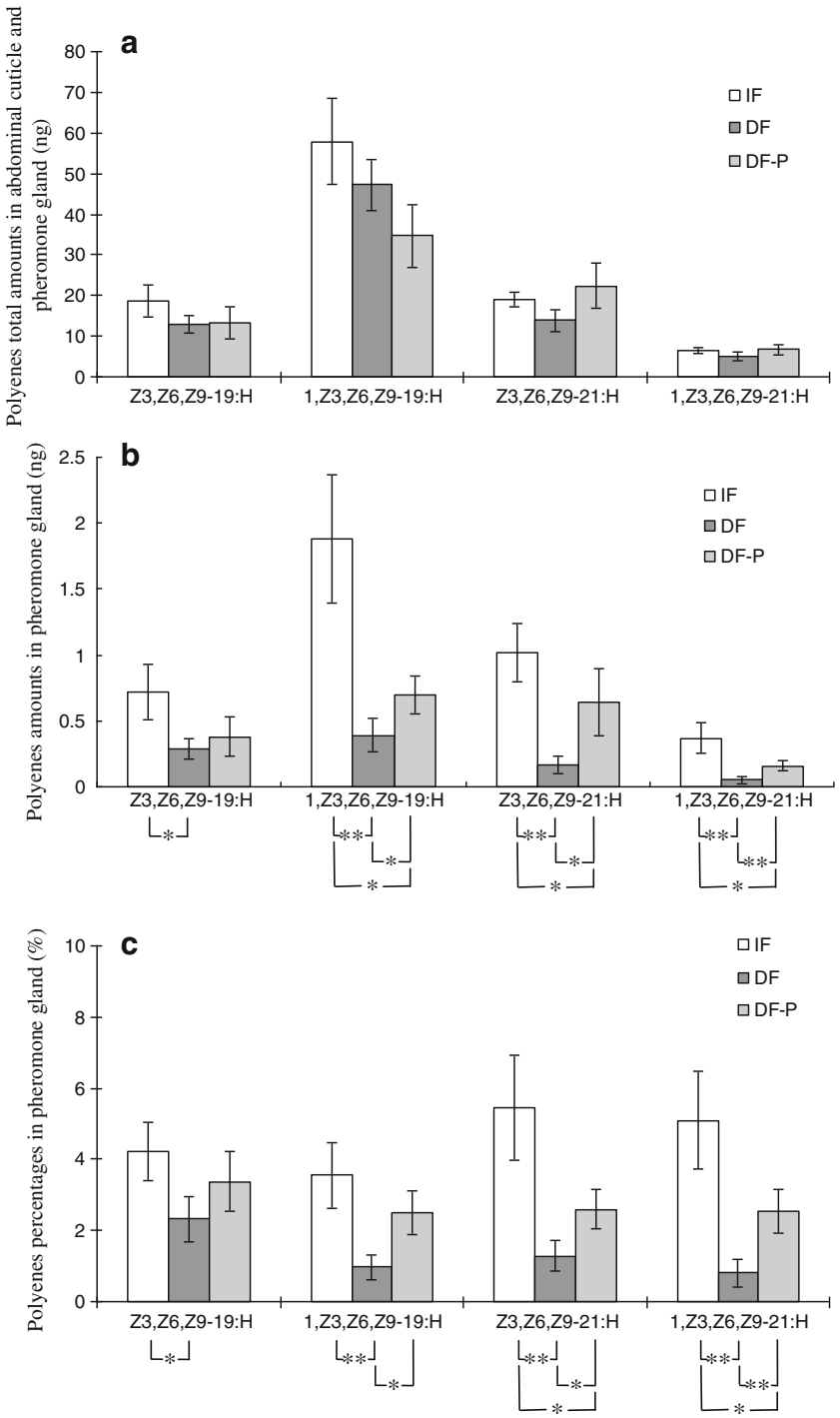


Fig. 2 Polyene amount after decapitation and PBAN injection. **a** total amount (ng) of polyenes in abdomen and gland; **b** gland polyenes titer variation (ng); and (**c**) percentage of polyenes in the pheromone gland relative to the total amount. IF intact female (n=10), DF decapitated female with saline injection (n=10), DF-P = decapitated female with 5 pM PBAN injection (n=10). The *asterisks* show significance level at $P<0.05$ (*) and $P<0.01$ (**), one-way-ANOVA followed by a LSD test

The two tetraenes, 1,Z3,Z6,Z9-19:H and 1,Z3,Z6,Z9-21:H were monitored by ions m/z 79 (base peak), 206/234 (M-[CH_2(CH=CH=CH)H]$^+$), 260/288 (molecular ion), as well as the tropylium rearrangement ion 91 ([C_7H_7]$^+$) and methyltropylium rearrangement ion 105 ([C_8H_9]$^+$) produced from the tetraenoic structure. When D_5-Z9,Z12,Z15-18:Acid and D_6-Z11,Z14,Z17-20:Acid were applied, the deuterated hydrocarbon products D_5-Z3,Z6,Z9-17:H, D_5-/D_6-Z3,Z6,Z9-19:H and D_5-/D_6-1,Z3,Z6,Z9-19:H were monitored by ions showing 5 or 6 amu more respectively than the characteristic ions mentioned.

Results and Discussion

Polyene Composition In addition to the sex pheromone 1,3Z,6Z,9Z-19:H, three other polyenes, 3Z,6Z,9Z-19:H, 3Z,6Z,9Z-21:H, and 1,3Z,6Z,9Z-21:H, were found in both the

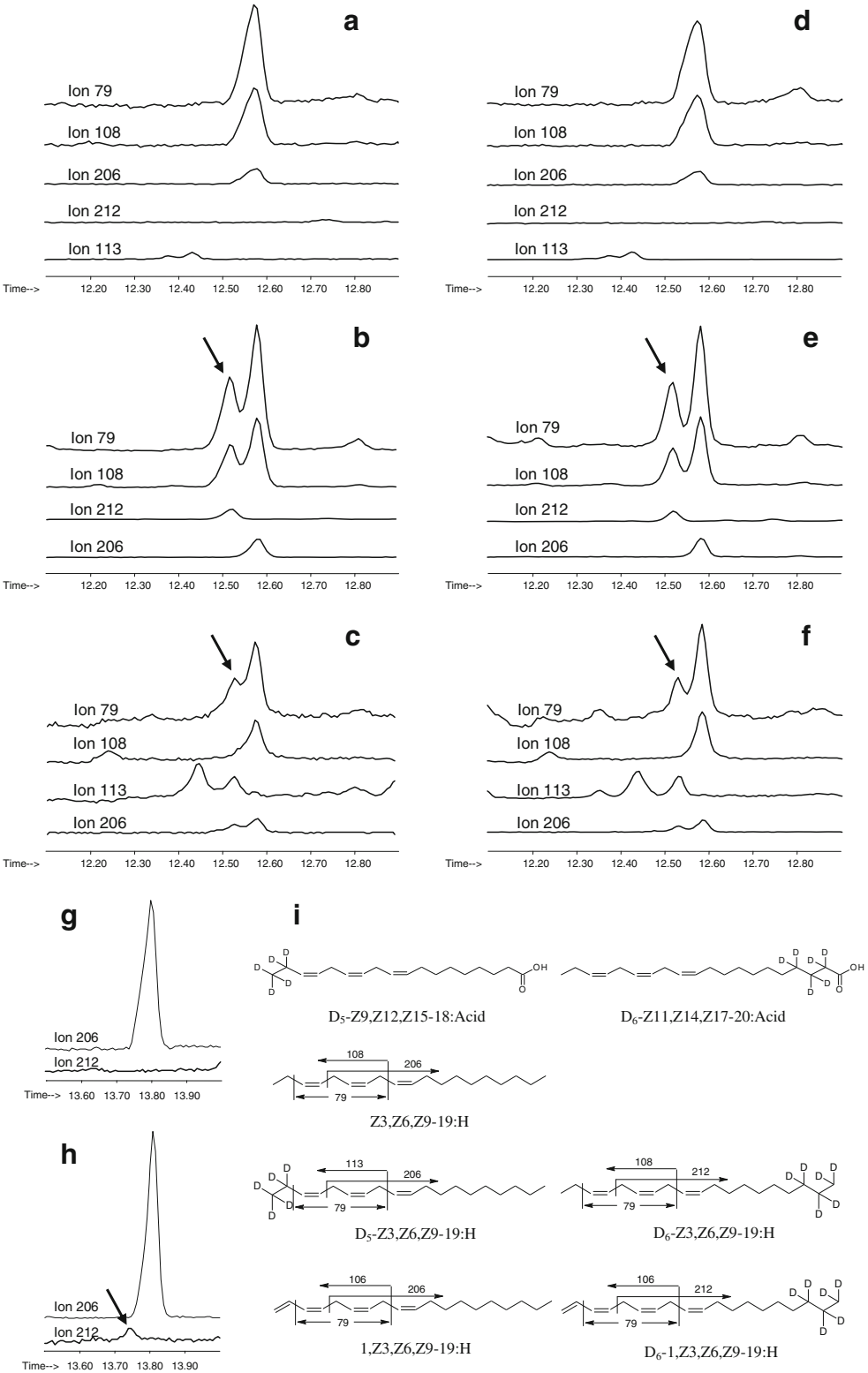


Fig. 3 Incorporation of D_5-Z9,Z12,Z15-18:Acid and D_6-Z11,Z14,Z17-20:Acid into Z3,Z6,Z9-19:H and 1,Z3,Z6,Z9-19:H in pheromone gland and abdominal cuticle. Native Z3,Z6,Z9-19:H in the pheromone glands (**a**) and abdominal cuticles (**d**) of control females injected with vegetable oil without labeled compounds (2fe); D_6-labeled Z3,Z6,Z9-19:H in the pheromone glands (**b**) and abdominal cuticles (**e**) of females injected with D_6-Z11,Z14,Z17-20:Acid (3fe); D_5-labeled Z3,Z6,Z9-19:H in the pheromone glands (**c**) and abdominal cuticles (**f**) of females injected with D_5-Z9,Z12,Z15-18:Acid (3fe); Native 1,Z3,Z6,Z9-19:H (**g**) and D_6-labeled 1,Z3,Z6,Z9-19:H (**h**) in the pheromone glands of females injected with D_6-Z11,Z14,Z17-20:Acid (3fe); structures of deuterium labeled acids and corresponding decarboxylated products with proposed fragmentation (**i**). *Arrows* show the deuterium labeled polyenes

pheromone glands and abdominal cuticle of female *O. brumata*. The component ratios of these polyenes in pheromone gland compared with abdominal cuticle were very similar. However, only about 3–6 % of each polyene was found in the gland, and the majority was in the abdominal cuticle (Table 1).

PBAN Effect on Polyene Transportation In the first experiment, the pheromone titer in the gland was significantly decreased 24 hr after decapitation and restored to a normal level after injection of 0.2 or 2 female equivalents of *O. brumata* head extract. This PBAN-like activity of the head

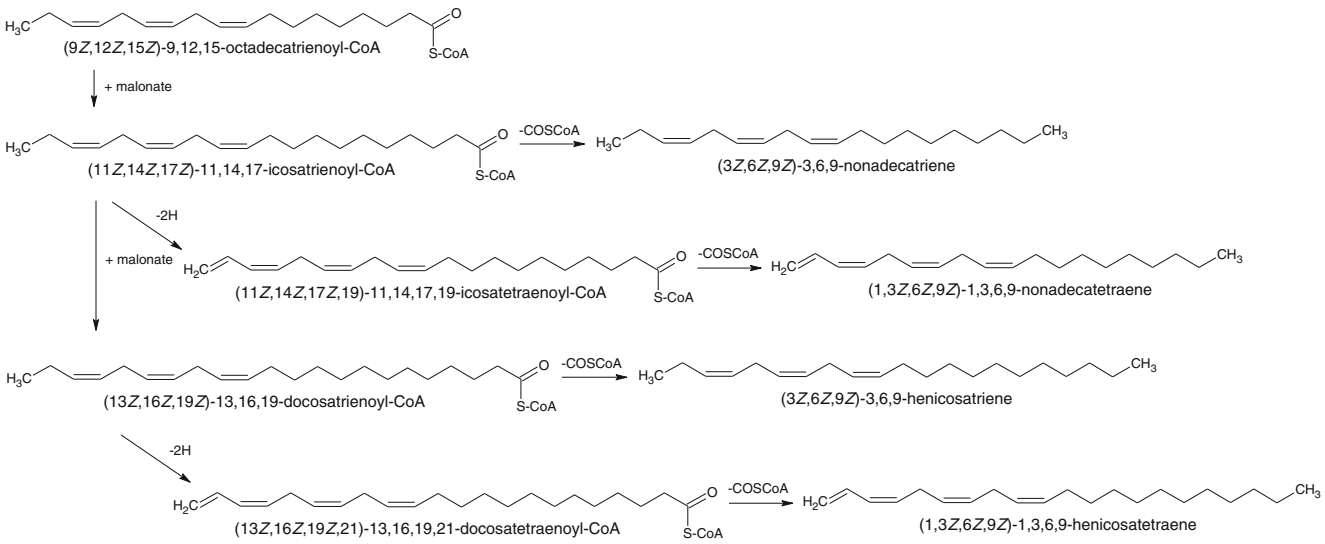


Fig. 4 Proposed biosynthetic pathway to polyenes in *Operophtera brumata*

extract was similar to that observed in decapitated females after injection of synthetic PBAN (Fig. 1). In the second experiment, no significant differences in total amounts of the polyenes were found among the groups of intact females, decapitated females, and decapitated-plus-supplementary-PBAN females (Fig. 2a). However, both the titer and the proportion of polyenes in the pheromone gland was significantly reduced 4 days after decapitation, but partly restored when supplementary synthetic PBAN was injected (Fig. 2b and c). Waiting 4 days after decapitation was done to minimize the remaining endogenous PBAN and glandular pheromone, but this probably also led to unhealthy and weakened insects, which could explain why the titers of glandular polyenes in the second experiment were not fully restored as much as observed in the first experiment. These results indicate that PBAN does not influence polyene production in the abdominal cuticle, but more likely affects the transport of polyene from the abdomen to the pheromone gland in this species.

Polyene Biosynthesis The injection of labeled acids into the female abdomen led to apparent deuterium incorporation in Z3,Z6,Z9-19:H extracted from both the abdominal cuticle and the pheromone gland (Fig. 3), whereas no labelled Z3,Z6,Z9-17:H, the direct decarboxylation product of linolenic acid, was produced. D_5-linolenic acid was incorporated into the elongation-decarboxylation product, Z3,Z6,Z9-19:H by an approximate rate of 50 % in gland and 35.4 % in the abdominal cuticle (Fig. 3c, f). Trace label from the acid also was found in Z3,Z6,Z9-21:H in both pheromone gland and abdominal cuticle extracts, by an approximate incorporation rate of 8.2 % and 15.7 %, respectively (data not shown). However, no label incorporation was detected in the tetraenes 1,Z3,Z6,Z9-19:H and 1,Z3,Z6,Z9-21:H in this experiment. When D_6-Z11,Z14,Z17-20:Acid was applied, the deuterium was incorporated into Z3,Z6,Z9-19:H by an approximate rate of 21.2 % in gland and 54.7 % in abdominal cuticle (Fig. 3b, e). A trace of D_6-Z11,Z14,Z17-20:Acid also was incorporated into the tetraene 1,Z3,Z6,Z9-19:H in the pheromone gland by approximately 2.1 % (Fig. 3h), and in abdominal cuticle (2.4 %), as well as into the longer triene Z3,Z6,Z9-21:H in gland (1.4 %) and abdominal cuticle (3.3 %) (data not shown), which were much lower relative to Z3,Z6,Z9-19:H (Fig. 3b, e). In addition, no label incorporation from this acid was detected in 1,Z3,Z6,Z9-21:H. The different label incorporation efficiencies among C19 and C21 trienes and tetraenes reflect the complexities of elongation-(desaturation)-decarboxylation reactions. These results are in agreement with a proposed pathway starting from acetate/malonate elongation of linolenic acid to Z11,Z14,Z17-20:Acid, which is subsequently used to produce Z3,Z6,Z9-19:H possibly via an aldehyde intermediate followed by decarbonylation (Fig. 4). Our previous *in vitro* characterization of a terminal desaturase activity before the removal of the carboxy group lends further support to the proposed pathway leading to the production of tetraenes (Ding et al. 2011).

The elongation-decarboxylation pathway involved in insect hydrocarbon biosynthesis was first found in the American cockroach (Major and Blomquist 1978), and thereafter the reductive decarboxylation of long-chain fatty acid was suggested as the principal mechanism to produce insect cuticle hydrocarbons (Howard and Blomquist 1982). The mechanism leading to the production of long chain unsaturated hydrocarbons has been studied previously in the termite *Zootermopsis angusticollis* where α-hydroxylation might produce α-hydroxylated acids as the first key intermediate (Chu and Blomquist 1979). Whereas in the house fly, *Musca domestica*, the decarbonylation reaction was demonstrated in hydrocarbons production (Reed et al. 1994, 1995). In this reaction, an acyl-CoA conjugate was first reduced to an aldehyde intermediate, which was then converted to a hydrocarbon by a Cytochrome P450 enzyme, with release of

carbon dioxide. More recently, an insect-specific CYP gene encoding the aldehyde decarbonylase was functionally characterized in the fruit fly, *Drosophila melanogaster*, further confirming the oxidative decarbonylation process (Qiu et al. 2012). Cloning and characterization of the homologous decarbonylase gene would clarify the detailed mechanism of hydrocarbon production in *O. brumata* and other lepidopteran insects.

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