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Studies of sex pheromone production under neuroendocrine control by analytical and morphological means in the oriental armyworm, *Pseudaletia separata*, Walker (Lepidoptera: Noctuidae)

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ABSTRACT

Most female moths produce species-specific sex pheromone blends in the modified epidermal pheromone gland (PG) cells generally located between the 8 and 9th abdominal segments. The biosynthesis is often regulated by pheromone biosynthesis activating neuropeptide (PBAN) either in or prior to *de novo* fatty acid synthesis or at the formation of oxygenated functional group. In *Pseudaletia separata*, information about life span, calling, PG morphology, daily fluctuation of pheromone production and its hormonal regulation is limited.

We measured pheromone titer daily (16:8; L:D) at 2 h intervals in *scotophase*. Blend ratio stabilized during the 2nd day (till 4–5th) at 6th hour of *scotophase*, with the ratio of 27.5:12.8:44.4:15.3 for Z-11-16OH:16OH:Z-11-16Ac:16Ac, respectively. Females showed calling behavior from this time. We found with light and fluorescence microscopy that PG consisted of intersegmental membrane (A part), and *dorso-lateral* region of 9th abdominal segment (B part), encountering for ~35% of total production revealed by gas chromatography. Ratios did not reveal difference. We did not find precursor (triacylglycerols) accumulation in form of lipid droplets, implying that PBAN stimulates *de novo* biosynthesis of 16:acyl precursors. *In vivo Hez*-PBAN injections (1–3 × 5 pmol, 2 h intervals) into 3 days old 16–18 h decapitated females stimulated pheromone production, both in A and B parts. Blend analyses including ratios suggest stimulation of the initial phase of synthesis, but desaturation of fatty acyl intermediates do not follow proportionally. More saturated fatty acid is converted from the available pool to the final OH and Ac, compared to females kept intact in *scotophase*. *In vitro* studies (PGs incubated 4–6 h in the presence of 0.25 or 0.5 μM *Hez*-PBAN, especially with surplus 2 mM malonyl-CoA) revealed higher saturated component ratio than the unsaturated, compared to natural blend or *in vivo* injections.

1. Introduction

Females of many moth species release species-specific sex pheromone blends, produced by the pheromone gland (PG). The PG is commonly located between the 8 and 9th abdominal segments consisting of modified epidermal cells that form a *ring gland* in most cases from the intersegmental membrane. However, there are numerous variations, to what extent these modifications occur [55].

Female sex pheromones are long-range volatile chemicals indispensable for luring potential mates by females ready to mate in most moths [72]. Synthesis of the Type I pheromone components, the most frequent type in moths, is mainly localized in the PGs [1]. This major class of sex pheromones are composed of straight-chain C10–C18 (un)saturated acyclic aliphatic compounds with limited oxygenated functional groups (aldehyde, alcohol, acetate ester). They are *de novo* synthesized from acetyl-CoA using modified fatty acid (FA) biosynthetic pathways [3,35] through 16 or 18:acyl, and follow the general scheme of desaturation and/or chain shortening followed by a final oxygenation step [77]. The hundreds of various constituents are derived from simple fatty acids by similar routes involving desaturation at one or more positions, by different rounds of chain shortening by limited beta-oxygenation, and finally modification of the functional group. The desaturases are particularly significant in generating structurally diverse lepidopteran sex pheromone components, since these enzymes have developed various substrate-, region- and stereo-specificities resulting in a range of unsaturated FA precursors. Precursors have different chain lengths, and varied positions and numbers of double bonds that

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result in *Z* (*cis*) and *E* (*trans*) double bond geometries [39]. The final reduction to alcohol or acetylation to produce acetate esters or oxidation to produce aldehydes are similarly important [77,57]. Only a small group of moths use Type II pheromone components, i.e. unsaturated hydrocarbons or hydrocarbon epoxides, as sex pheromones) [50].

In the majority of moth species, a circadian signal is indispensable for the production of pheromones. Production synchronization with environmental cues is executed through external signals via the central nervous system, and is largely regulated by neurohormones [17]. Rhythmicity in hormonal regulation of pheromone production in Lepidoptera is one of the most well-known phenomena and has been extensively covered [63,4]. Since the pioneering work of Raina and Klun [65] on the role of pheromone biosynthesis activating neuropeptide (PBAN) in the neurohormonal regulation of pheromone production in *Helicoverpa zea*, the 'brain factor' with *pheromonotropic* activity has been identified and sequenced in many moths [66,58]. The 33–34 amino acid PBAN is synthesized as a preprohormone that undergoes post-translation modification. PBAN is present in insects from a variety of orders and is characterized by a C-terminal amidated pentapeptide FXPRL motif (where *X* is: S, T, G or V) found to be the minimum essential sequence for biological activity [64]. Activity and expression of the PBAN gene has been localized in the *suboesophageal ganglion* (SOG), but connections to the *corpus cardiacum* (CC), the major neurohemal organ, imply that the product is transported to and released from here. For example, the level of PBAN in the CC is significantly higher in the *photophase*, while synthesis is continuous in the SOG of *H. armigera* and *Agrotis segetum* [61]. In *Mamestra brassicae*, the levels of PBAN-like immunoreactive material in the hemolymph have been correlated with the time of maximum calling and pheromone production [32]. PBAN depletion from the CC is rhythmic and is supported by extracellular recordings of the action potentials of innervated CC in *Bombyx mori* virgin females [74]. The hormone functions in a classical manner and its major target is the PG itself with the species-specific pheromone blends produced periodically in the gland cells following PBAN stimulation *(H. zea, H. armigera)* [59,60,62,69]. In *B. mori,* the final reduction of precursors and depletion of pheromone precursors from lipid reservoirs are under direct (circadian) control via PBAN [20]. In some *Heliothis* species and *Manduca sexta* additional innervation from the *terminal abdominal ganglion* is thought to be necessary [76], while in *Spodoptera littoralis* and *M. brassicae* pheromone biosynthesis appears to require both PBAN and the ventral nerve cord [33]. The presence of *juvenile hormone* was also found to be necessary in the armyworm *Pseudaletia unipuncta* for rhythmic PBAN-dependent pheromone production by regulating production/release from the brain–SOG [9] or by priming gland cells for PBAN action. In insect groups with relatively longer adult lives (one to several weeks), calling and emission of produced sex pheromone occur daily, which is a widespread phenomenon governed by the endogenous circadian clock. It should be noted that in some species production itself is not rhythmic, but rather the abdominal movements resulting in release of the key element during calling are clock governed (see review of Vafopoulou and Steel [79] with references therein).

For pheromonogenesis, PBAN action on the PG cells is indispensable as the process is initiated by specific binding of PBAN to its receptor. The first identification of the PBAN receptor (PBANR) as a G protein-coupled receptor was in *H. zea* [7]. PBANRs were subsequently identified in *B. mori* [29] and a number of other species [5]. There are two major types of PBANR, which are distinguished by the length of their respective C termini, a region shown to be necessary for internalization of the receptor after activation [30,38]. Regardless of species, extracellular Ca^{2+} is a prerequisite for turning the PBAN signal into the biological response of sex pheromone production (see reviews: [57,58,49]. While extracellular Ca^{2+} is an absolute requirement for pheromonotropic activity, the downstream intracellular signal transduction cascade is species-dependent. These differences are most striking when comparing *B. mori* and the heliothine species (e.g. *H. zea, H. armigera*), the two most extensively studied models of moth sex pheromone production. In the latter, the cyclic nucleotide second messenger, cAMP, is a crucial component in PBAN signaling [57,58], whereas in *B. mori* cAMP is not involved [31]. In *B. mori* and related species, PBAN stimulation results in the terminal FA reduction step, leading to the final alcohol product from the precursor FA. In brief, PBAN regulation is linked to and differentiated by adenylate cyclase activity: species in which FA synthesis is controlled by PBAN are cAMP dependent, while species, like *B. mori*, that primarily rely on PBAN activation of the terminal reductase are not [31,58,49].

In the sex pheromone biosynthetic pathway, PBAN regulates either the initial or terminal steps of the process, and as described above, strong correlations occur between PBANRs and the respective signal transduction cascades. In *Argyrotaenia velutinana* [73], *M. brassicae* [34], *H. zea* [37], and *Dendrolimus punctatus* [82] PBAN regulates step(s) in FA synthesis, most likely acting on acetyl-CoA carboxylase as presented in detail in *H. armigera* [78]. In a number of other species, including *B. mori* [2,53], *Thaumatopeia pityocampa* [14], *S. littoralis* [13] and *Ostrinia nubilalis* [43,12] PBAN was found to control FA reduction. Moreover, in *M. sexta* [15,16] and *Sesamia nonagrioides* [46] PBAN acts on mobilization and/or reduction of FA precursors in triacylglycerols (TG) and acetylation of alcohols, respectively. In *B. mori* it was demonstrated that PBAN controls lipolysis of cytoplasmic lipid droplets (LD) to liberate stored precursor FA in addition to the regulation of the terminal reduction step [20,21]. A recent study in *Heliothis virescens* also provided evidence for two-step regulation of pheromone biosynthesis by PBAN, near the beginning and end of biosynthetic process, allowing a more efficient production of pheromone in the PG than the control of only one step [11]. The molecular mechanisms underlying sex pheromone production have been extensively studied in *B. mori* as reviewed by Matsumoto [47] and in heliothine species [58].

Recently, new investigations have been launched targeting the oriental armyworm, *Pseudaletia (Mythimna/Leucania) separata*. Analytical studies by Kou and co-workers [41] revealed the presence of four blend components, two alcohols (Z-16OH (hexadecanol), and Z-11-16OH ((Z)-11-hexadecenol)) and two acetates (16Ac (hexadecanyl acetate) and Z-11-16Ac ((Z)-11-hexadecenyl acetate)). The two unsaturated compounds, however, had been identified as early as 1979, by Takahashi and co-workers [71] with an 8 (Ac):1 (OH) ratio. The ratio and presence of these components varies between mainland China, Taiwan and Japan as concluded from field trappings using the 8:1 ratio in Taiwan [42]. Moreover, Zhu and co-workers [83] stated that Z-11-16Ald + 16Ald + Z-11-16OH (in a ratio of 100:10:0.1) is the preferred blend when used in field traps in Jintan and Beijing, China, based on their extraction and blend identification studies. Studies related to temperature and photoperiod show that environmental cues affect development, reproduction and flight (migration), the latter being in strong correlation with this species' pest nature, representing good adaptation capacity [25,26,6]. Endocrinologically controlled phase polymorphism of larvae depending on population density is also known in this species [52].

Taken together, the available description about life span, calling behavior, daily fluctuation of pheromone production and exact composition of the used laboratory strain, hormonal regulation of pheromone production, PG morphology, or precise location of the pheromone producing cells is scarce or lacking in this species. Based on previous studies in *B. mori* [19,20], we initiated a series of investigations to better understand the species-specific pheromone blend production, and its hormonal regulation by PBAN in