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# Molecular cloning, mRNA expression and biological activity of the pheromone biosynthesis activating neuropeptide (PBAN) from the European corn borer, Ostrinia nubilalis

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## Abstract

Pheromone biosynthesis activating neuropeptide AQ3 (PBAN) is a member of the pyrokinin (FXPRLamide) insect neuropeptides. Here, we report the cloning of AQ4 the gene Ostnu-PBAN from the E and Z pheromone strains of the European corn borer (ECB), Ostrinia nubilalis (Lepidoptera: Crambidae), a major pest of maize. The Ostnu-PBAN genomic sequence is > 5 kb in length and consists of six exons. The deduced amino acid sequence revealed a 200-residue precursor protein including a signal peptide, a 24-amino AQ5 acid (aa) diapause hormone, a 37-aa PBAN and three other FXPRLamide neuropeptides. Our in vivo assays suggest that the 37-aa synthetic Ostnu-PBAN is hormonally active in the pheromone gland. It restores sex pheromone production to normal levels in mated females and decapitated virgins of both E and Z cultures. The results of a real-time PCR analysis AQ6 indicated that Ostnu-PBAN mRNA levels reached a plateau in the brain-suboesophageal ganglion complexes 1 day after eclosion, and mating did not affect the mRNA expression. Three size classes of Ostnu-PBAN mRNA (1.9, 2.0 and 2.1 kb) were obtained, differing only in the length of the 3′ untranslated region. However, there was no correlation between sequence divergence and the pheromone composition, voltinism or geographical origin (Hungary, Slovenia, Sweden, Turkey) of ECB moths.

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## Introduction

In many moth species, the female releases a volatile sex pheromone that attracts conspecific males for mating (Tamaki, 1985). Sex pheromones are typically synthesized de novo from acetyl-CoA in the pheromone gland (PG), which is located between the eighth and ninth abdominal segments of females (Percy-Cunningham & McDonald, 1987). Most moths utilize Type I sex pheromones that are composed of fatty acid derivatives (C10–C18 straight chain aldehydes, alcohols or their acetate esters) usually containing double bonds in defined positions and geometric configurations (Bjostad et al., 1987; Ando et al., 2004). The blend ratio of sex pheromone components is species specific, and the structural and compositional variations of the components serve as the basis for mate recognition (Roelofs & Rooney, 2003). Female moths often exhibit a diel periodicity in pheromone production that is regulated by the pheromone biosynthesis activating neuropeptide (PBAN). The first PBAN was identified from brain-suboesophageal ganglion (SG) complexes of Helicoverpa zea as a 33-amino acid (aa) C-terminal amidated peptide that is released into the haemolymph only during the scotophase (Raina et al., 1989). Subsequently, neuropeptides with similar functionalities and sequence homologies to Helze-PBAN were identified from Bombyx mori (Kitamura et al., 1989) and Lymantria dispar (Masler et al., 1994). Almost simultaneously, a 24-residue peptide with egg diapause-inducing activity was isolated from SG extracts of B. mori (Imai et al., 1991). This work revealed that the AQ7





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identified diapause hormone (Bommo-DH) carries a FGPRLamide C-terminus that is almost identical to those of PBANs and myotropic pyrokinin (PK) insect peptides (Gäde, 1997; Predel & Nachman, 2006). Structure-activity relationship analyses showed that the highly conserved C-terminal pentapeptide characterized by FXPRLamide or a similar sequence (where X is a variable amino acid) is the active core region, representing the minimum number of residues required for physiological activity (Nachman et al., 1986; Raina & Kempe, 1990; Kuniyoshi et al., 1992). The FXPRLamide neuropeptides are widely distributed in insects and control diverse physiological processes (reviewed in Predel & Nachman, 2006; Jurenka & Nusawardani, 2011; Altstein et al., 2013; Jurenka, 2015). Following purification of the Bommo-DH, Bommo-PBAN and Helze-PBAN neuropeptides, molecular cloning techniques allowed identification of the DH-PBAN gene from B. mori and H. zea (Davis et al., 1992; Kawano et al., 1992; Ma et al., 1994). DNA sequencing revealed that the DH-PBAN gene encodes a larger precursor whose post-translational proteolytic processing yields DH, PBAN and three additional short peptides
(a-, b- and g-SG neuropeptides, ie SGNPs), all sharing the common C-terminal FXP(R/K)Lamide motif. Since then, cDNAs encoding PK/PBAN neuropeptides sharing an overall conserved structure have been published for more than 20 lepidopteran species (Jurenka, 2015). To date, however, no sequence information has been provided for the PBAN gene in the European corn borer (ECB), Ostrinia nubilalis Hbn. (Lepidoptera: Crambidae, Pyraustinae), which is an important economic pest of corn, hop, millet and hemp in the Northern Hemisphere (Caffrey & Worthley, 1927; Dillehay et al., 2005) and a model species for the study of evolutionary changes in sex pheromone communication (Lassance, 2010).

ECB populations are characterized by a variation in life history traits, such as voltinism. In Hungary, survey evidence indicates the presence of both univoltine and bivoltine life cycles. Typical bivoltine ECB populations occur in southern regions of the country, whereas univoltine populations are found in the north (Mészáros, 1969; Keszthelyi, 2010). Female ECB moths exhibit a diel periodicity in the emission of sex pheromone with peaks in the late scotophase and troughs in the photophase (Foster, 2004; Kárpáti et al., 2007). In the early 1970s two pheromonally distinct but otherwise indistinguishable races of Os. nubilalis were identified both in its native Palearctic range and its introduced range in North America (Klun & Cooperators, 1975). The females produce mixtures of (Z)-11- and (E)-11-tetradecenyl acetate (Z11–14:Ac and E11-14:Ac) as their sex pheromone (Klun et al., 1973). The so-called Z-strain is characterized by the production and perception of a 97:3 molar ratio of Z11–14:Ac and E11–14:Ac, whereas the E-strain ECB utilizes a 1:99 blend of Z11-/E11–14:Ac (Kochansky et al., 1975). Substantial research has been conducted to define the genetic basis of pheromone production in Os. nubilalis, and accumulating evidence indicates that the ratio of Z11-/E11–14:Ac in the final pheromone blend is determined by the fatty-acyl reduction step (Roelofs et al., 1987; Zhu et al., 1996), which is the proposed site of PBAN action as shown in B. mori (Ozawa & Matsumoto, 1996). Then, a locus encoding a stereo-selective PG fatty-acyl reductase (pgFAR) was identified as the gene responsible for the dimorphism in female pheromone production (Lassance et al., 2010). The Z-strain ECB is more widespread in both Europe and North America, whereas the E-strain occurs in certain areas within the geographical range of the Z-strain, often in sympatry (Klun & Cooperators, 1975; Anglade et al., 1984; Peña et al., 1988; O'Rourke et al., 2010). In Hungary, the Z-strain is likely to be the only one present based on male trapping surveys and chemical analyses of field-collected females (Anglade et al., 1984; Peña et al., 1988; Kárpáti et al., 2016).

In ECB moths, previous research has detected PBAN-like biological activity and immunoreactivity in three sets of neurosecretory cells of the SG and in the corpora cardiaca (Ma & Roelofs, 1995a,b). These observations, along with in vitro experiments employing synthetic Bommo-PBAN and isolated PGs, have indicated that PBAN-related neuropeptides in Os. nubilalis act directly on the PG cells to stimulate sex pheromone production (Ma & Roelofs, 1995c). The involvement of PBAN-related peptides in the sex pheromone biosynthesis of ECB has been further supported by identification of the receptors of the Ostnu-PBAN and Ostnu-DH neuropeptides (Nusawardani et al., 2013). Here, we describe the exon–intron structure of the Ostnu-PBAN gene and the amino acid sequence of the Ostnu-PBAN precursor deduced from the cDNA sequence. Developmental- and tissue-specific mRNA expression of the Ostnu-PBAN gene was examined using quantitative real-time PCR. Functional analyses on the hormonal activity of the newly identified Ostnu-PBAN were also performed.

## Results

### Pheromone strain typing

Consistent with previous observations, we found only Z-strain ECB moths in Hungary. Furthermore, Z-strain ECBs were also collected from Sweden and Turkey and those belonging to the E type were sampled from Slovenia. Details of collection procedures and pheromone identification are provided in the Experimental procedures section.

### Cloning of Ostnu-PBAN cDNA

In an attempt to isolate cDNA(s) encoding the DH-PBAN precursor in ECB, we used degenerate primers designed from two conserved regions of known lepidopteran DH-PBAN precursors. Single reverse transcription PCR (RT-PCR) products of the expected size of 288 bp were generated from brain-SG complexes of both E- and Z-strain females separately and cloned into the pJET1.2 vector. Conceptual translation of these sequences revealed 84 and 64% amino acid identity with DH-PBAN precursors of Omphisa fuscidentalis and B. mori, respectively. This homology indicates that the predicted peptide belongs to the family of DH-PBAN precursors.

The full-length DH-PBAN cDNA was obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) using eight total RNA samples extracted independently from ECBs collected at different geographical sites representing bivoltine E, univoltine Z and bivoltine Z populations. Subsequent RT-PCR amplifications were conducted using primers designed based on the terminal segments of the 5′- and 3′-RACE products. A total of 39 full-length transcripts were assembled, which were assigned to the ECB collections designated as SIE (Slovenia, E-strain, laboratory culture), KHuZ (Kéty, Hungary, Z-strain, laboratory culture,) BHuZ (Bicske, Hungary, Z-strain), RSwZ (Ravlunda, Sweden, Z-strain) and LSwZ (Landskrona, Sweden, Z-strain), and numbered sequentially. The transcripts fell into three classes based on their 3′ untranslated region (UTR) length: short (~1.9 kb), intermediate (~2.0 kb) and long (~2.1 kb), occurring in 27, 60 and 13% of sequences, respectively. To obtain a nonredundant sequence data set for each geographical region, repeated sequences above the arbitrary 98% nucleotide identity were removed from sequences of each of the five ECB collection sites, separately. The remaining sequences were then examined using a recombination detection program RDP4 and three putative recombinants (BHuZ2, BHuZ3 and BHuZ4) were identified, all occurring in ECB females caught at Bicske, Hungary. These presumed chimeric sequences were also removed, and thus a total of 14 sequences were included in the final data set.

The full-length transcripts contained a 60-bp 5′ UTR and a 603-bp open reading frame (ORF). The deduced amino acid sequence revealed a 200-residue precursor protein (Fig. 1). A total of 40 variable sites were found within the ORF, of which 27 were parsimony informative. These base substitutions comprise 32 transitions and eight transversions (Fig. 2). Three of the mutations result in amino acid substitutions of A13S, L18F and A180S, respectively. Two mutations (A13S and A180S) are at parsimony informative positions (Fig. 2) and provide potential phosphorylation sites for serine/threonine kinases. The deduced protein contains a hydrophobic N-terminus, indicating a signal peptide with a potential cleavage site between A21 and V22 (Fig. 1). The 24 amino acid residues spanning V22 to L45 contain a conserved C-terminal pentapeptide fragment FGPRL followed by a G-K-R sequence, serving as a signal for C-terminal amidation and proteolytic processing (Bradbury & Smyth, 1991; Veenstra, 2000). This 24-residue peptide shares high sequence similarity with lepidopteran DH peptides (Fig. 3). Four additional amidated peptides sharing characteristics of the PK/PBAN family of neuropeptides are also predicted to be proteolytically processed from the 200-amino acid ECB precursor, including: a 37-aa Ostnu-PBAN (L128–L164) and three short SGNPs, a 7-aa a-SGNP (V96–L102), a 20-aa b-SGNP (S105–L124) and an 8-aa g-SGNP (T167–L174) (Fig. 1). All of these putative peptides are flanked by K-K, G-R or G-R-R sequences at their N-termini and G-R, G-K-R or G-R-R sequences at their C-termini, which are predicted recognition sites for endoproteolytic cleavage and are potentially amidated at their C-termini owing to the presence of Gly residues at the cleavage sites (Fig. 1). They share a common C-terminal pentapeptide motif F(T/S)P(R/K)L and show homologies to other members of the PK/PBAN peptide family (Fig. 3). In accordance with their close taxonomic relationship, the Ostnu-PBAN precursor showed high sequence conservation amongst the crambid species. Excluding the signal peptide, it is 87 and 76% identical to DH-PBAN precursors of the bamboo borer (Om. fuscidentalis) (GenBank AFP87384), and the legume pod borer (Maruca vitrata) (AFX71575), respectively (Fig. 3). Lower homology (66–68%) was observed with the DH-PBAN precursors of B. mori and H. zea (Fig. 3). Last but not least, Ostnu-PBAN had 98.4% nucleotide identity and complete amino acid sequence identity to a hypothetical gene (GenBank accession number LC002981) in the Asian corn borer (Ostrinia furnacalis).

The 3′ UTR sequences of Ostnu-PBAN mRNA ranged from 1250 to 1486 nucleotides, excluding the poly(A) tail. Sequence analysis of the 3′ UTR indicated that amongst the 1117 bases (excluding alignment gaps), there were 170 variable sites (15.2%) and 124 informative ones. A total of 67 indels with an average length of 11.8 bp were indicated, of which 40 were parsimony informative. Two large (> 50 bp) indels were observed, which discriminate the Ostnu-PBAN mRNA size variants from each other (Fig. 4A). A BLAST search indicated that a 165-bp portion of the 171/176 bp insertion has 94% identity to a reverse complement sequence of an intron in the cadherin-like protein gene of Os. nubilalis (GenBank DQ000165, bases 9405–9552 and 9689–9702). The other large insert of 97/98 bp contains one copy of a ~45-bp direct repeat sequence of the 5′ flanking region in which two 7–15 bp T-rich elements are

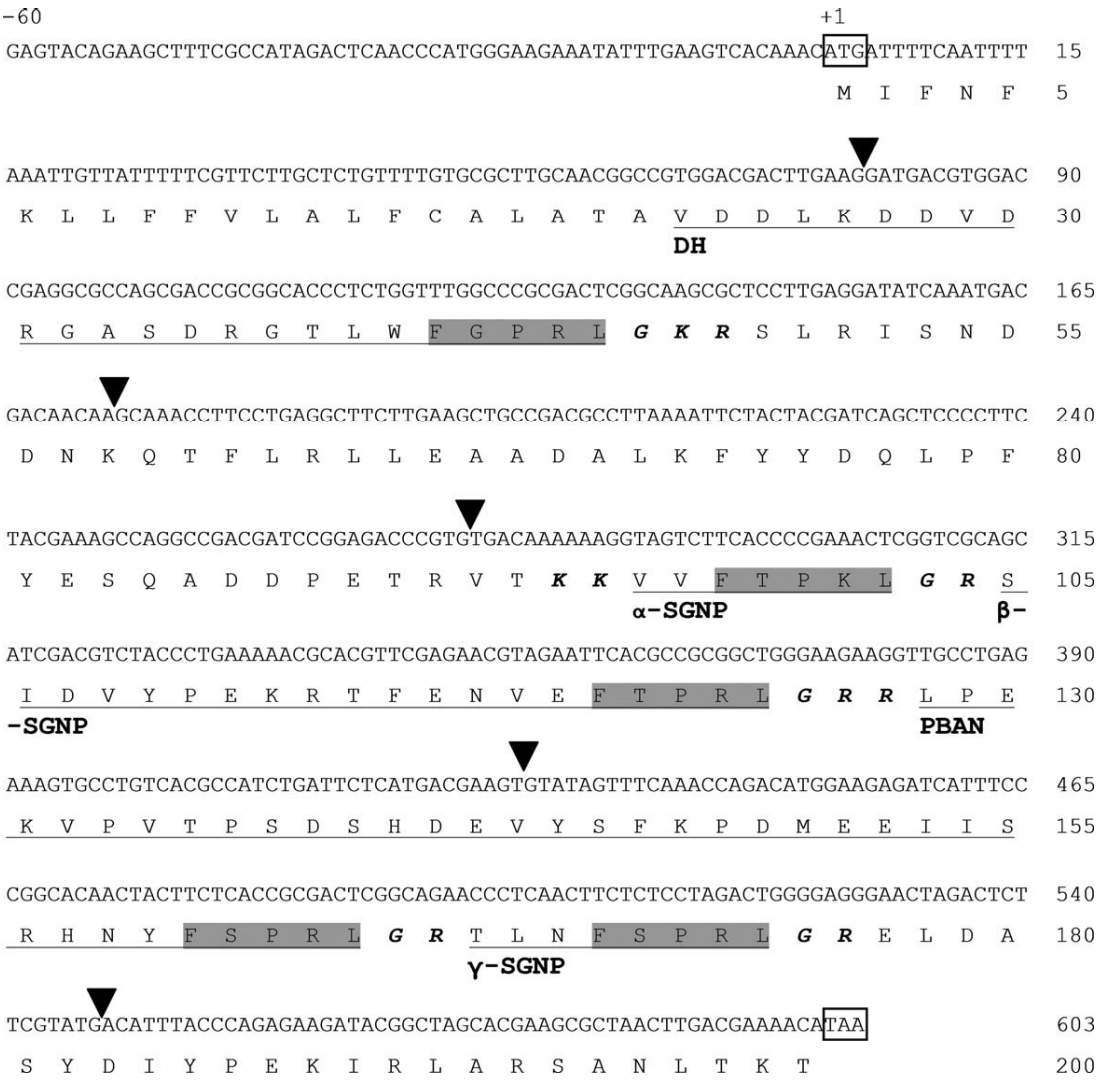



Figure 1. Nucleotide and deduced amino acid sequences of a putative Ostrinia nubilalis pheromone biosynthesis activating neuropeptide (PBAN) cDNA, designated as KHuZ1 (Kéty, Hungary, GenBank accession number: KU952100). The nucleotide 1 1 is the A of the ATG-translation initiation codon. Potential endoproteolytic cleavage sites are highlighted in bold italics. Amino acids of the five putative FXPRL-$NH_2$ peptides are underlined: diapause hormone (DH), a-, b-, g-suboesophageal ganglion neuropeptides (SGNPs) and PBAN. Residues of the conserved C-terminal pentapeptide sequence are highlighted against a grey background. The initiation and termination codons are boxed. Positions of introns are indicated by black inverted triangles.

AQ48

found (Fig. 4B). Two short (10- and 12-bp) palindromic sequences, a 10-bp direct repeat and a 16-bp inverted repeat were also found within the 3′ UTR (Fig. 4B). A search for putative regulatory sequences revealed AU-rich elements, eg ATTTA, which is present four times in the 3′ UTR. Although there is a potential polyadenylation signal sequence (AATAAA) at 288–291 bp downstream from the translational stop codon, and a second polyadenylation signal further downstream within the 171/176 bp insertion (Fig. 4B), we could not detect any mRNA species that might have resulted from the use of these upstream polyadenylation signals.

### mRNA expression during development

Expression of DH-PBAN mRNA was determined in immature and adult stages of ECBs by real-time PCR with primers designed against conserved regions. We observed very similar expression patterns between the tissues and stages of the E- and Z-strain ECB, hence, only data from E-strain borers are shown (Fig. 5). F5 Amongst the immature stages, Ostnu-PBAN mRNA was not detected in freshly laid eggs (data not shown). In whole larvae, transcript levels were relatively high in first and second instars, but declined as larvae grew older. Low expression was detected in whole pupae, about two

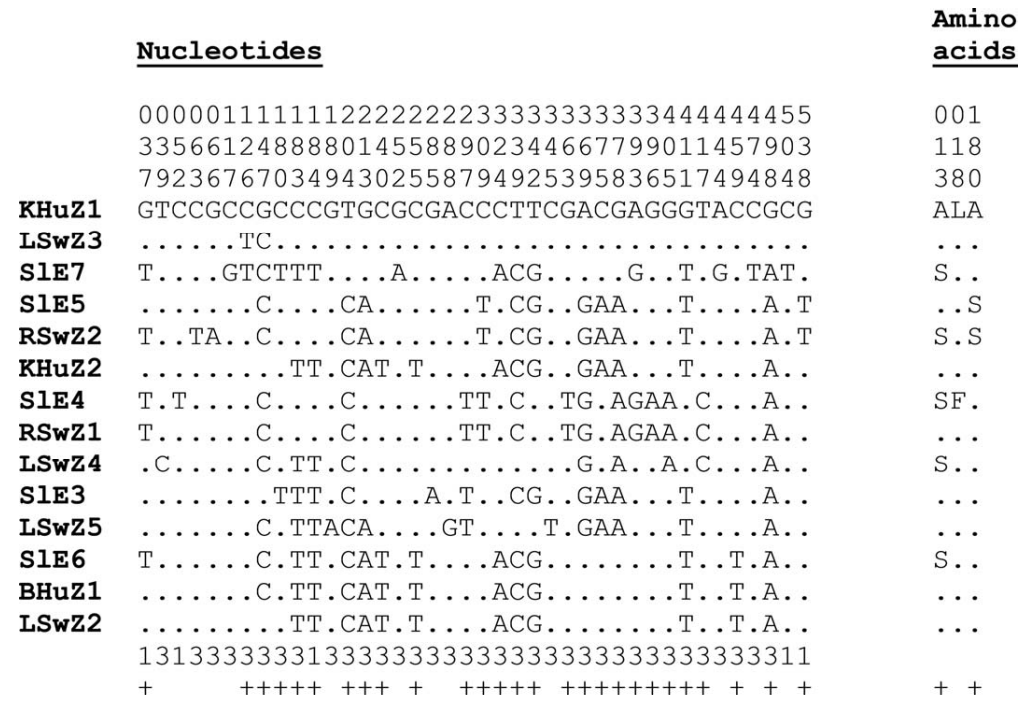
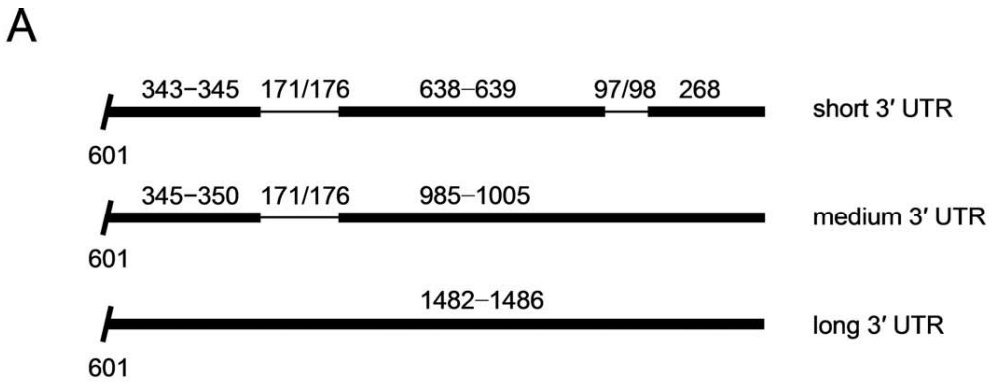



Figure 2. Distribution of polymorphic nucleotide and amino acid sites in the coding region of 14 Ostrinia nubilalis pheromone biosynthesis activating neuropeptide cDNAs from E-strain (SI, Žalec, Slovenia) and Z-strain (BHuZ, Bicske, Hungary; KHuZ, Kéty, Hungary; RSwZ, Ravlunda, Sweden; LSwZ, Landskrona, Sweden) Ostrinia nubilalis. The sequences are numbered consecutively for each geographical area. The KHuZ1 sequence (KU952100) is used as a reference. Site positions are numbered vertically from the start codon of the reference sequence. The position for each polymorphic site within a codon is shown below. Informative sites are marked by 1 signs. Dots indicate nucleotide identity.


Figure 3. Alignment of consensus sequences of the diapause hormone pheromone biosynthesis activating neuropeptide (DH-PBAN) precursor for Ostrinia nubilalis (KHuZ1, Kéty, Hungary, KU952100), Omphisa fuscidentalis (AFP87384), Maruca vitrata (AFX71575), Bombyx mori (AAB24327) and Helicoverpa zea (AAA20661). Predicted neuropeptides, such as DH, a-, b-, g-subesophageal ganglion neuropeptides (SGNPs) and PBAN, are shown in bold. Amino acid identities are indicated with asterisks and conservative substitutions are represented with colons. Grey shading within the five neuropeptides denotes residues identical to those of Ostrinia nubilalis.

AQ49

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TACATTAAACTAGATTGTAAGCGATATTTATGAGAATTTAATAAAAAATAATTGCTG(poly(A))           2082
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Figure 4. (A) Schematic representation of the distribution of long (> 50 bp) insertions/deletions in the 3′ untranslated regions (UTRs) based on a ClustalX alignment of Ostrinia nubilalis pheromone biosynthesis activating neuropeptide (Ostnu-PBAN) cDNA sequences. The lengths of the deletions and flanking sequences [without the poly(A) tail] are indicated above the lines. Nucleotide position 601 (numbered relative to the A of the ATG initiation codon) is the first base downstream from the stop codon. (B) The 3′ UTR of KHuZ1 (Kéty, Hungary, KU952100), a long variant of the putative Ostnu-PBAN cDNA (nucleotides 661 to 2142). Long (> 50 bp) insertions are shown in bold; the inverted intronic sequence present within an insert is dashed-underlined. Direct repeats are shaded in grey and marked with arrows above the sequences. Inverted repeats are indicated by arrows only. Palindromes are marked by converging arrows. Dots within the arrows indicate mismatches. Dotted lines above the sequences represent T-rich stretches adjacent to the repeated sequences. The termination codon (TAA) and the polyadenylation signals (AATAAA) are boxed.

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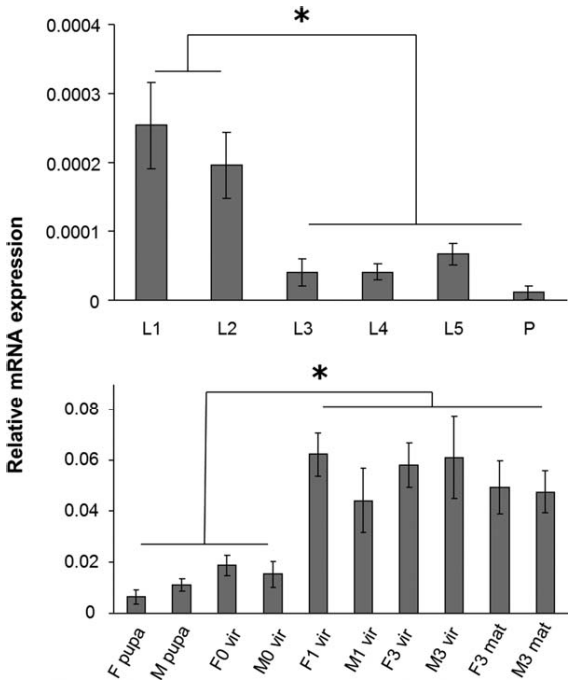


Figure 5. Relative diapause hormone pheromone biosynthesis activating neuropeptide gene expression in whole larvae and pre-emergence pupae (top) and in brain-suboesophageal ganglion complexes of adults (bottom) of Ostrinia nubilalis. The mRNA expression was normalized to expression of ribosomal protein S3. L1–L5, first to fifth instars; P, pupae; vir, virgin; mat, mated; F0, F1, F3, females on day 0, 1 and 3 after emergence, respectively; M0, M1, M3, males on day 0, 1 and 3 after emergence, respectively. Mean values 6 SEM of three biological replicates are shown. Statistical analysis was performed by analysis of variance followed by Duncan's multiple range test. Asterisks indicate significant differences at $P < 0.05$.

orders of magnitude lower than in isolated pupal brain-SG preparations (Fig. 5). Analysis of the expression pattern in brain-SG complexes revealed high amounts of Ostnu-PBAN transcript in 1–3-day-old females and males regardless of their mating status, and lower amounts in newly emerged females and pre-emergence pupae (Fig. 5). No appreciable expression of Ostnu-PBAN was detected in other tissues and organs of adults, including leg muscles, ovaries, fat bodies, PGs and hairpencil-aedeagus complexes (data not shown).

## Biological activity of Ostnu-PBAN

The predicted C-terminal amidated 37-residue Ostnu-PBAN was synthesized and injected into female ECB moths to test for pheromonotropic activity. Its dose–response relationship was examined in E-strain ECB females using a decapitated-moth bioassay. The synthetic Ostnu-PBAN significantly stimulated the production of E11–14:Ac in a dose-dependent manner (Fig. 6). Gas chromatographic and mass spectrometric (GC-MS) analysis revealed that a dose of 0.058 pmol per insect

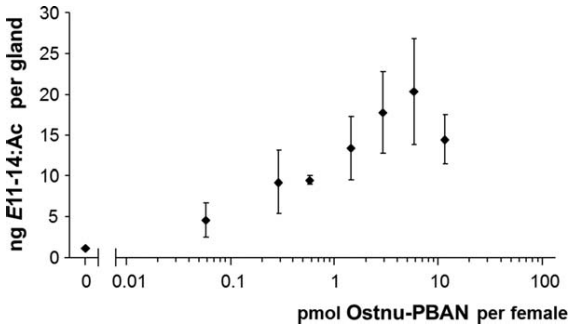


Figure 6. The dose-response effect of synthetic Ostrinia nubilalis pheromone biosynthesis activating neuropeptide (Ostnu-PBAN) on the amounts of (E)-11-tetradecenyl acetate (E11–14:Ac) in pheromone gland extracts from decapitated E-strain Os. nubilalis females. Two-day-old females were decapitated at the third hour of scotophase and 27 h later were injected with a dose series of synthetic Ostnu-PBAN (0.058, 0.29, 0.58, 1.45, 2.9, 5.8 and 11.6 pmol in 2 ml distilled water). Females injected with 2 ml water served as controls. Error bars show the SEM of six biological replicates, each containing three pheromone glands.

was sufficient for significant ($P < 0.05$) activation of pheromone biosynthesis when injected into 3-day-old decapitated females, and resulted in the production of 4.66 2.1 ng E11–14:Ac per female, as compared to the control level of 1.086 0.06 ng E11–14:Ac (Fig. 6). Maximum pheromone production (17.26 4.56 E11–14:Ac ng/PG) was achieved at a dose of 5.8 pmol Ostnu-PBAN. However, increasing the dose to 11.6 pmol per female resulted in a slight (about 28%) decrease in pheromone production (Fig. 6).

The GC-MS analysis of pheromone gland extracts revealed an average content of 14.56 5.88 ng/PG E11–14:Ac in 0- to 3-day-old virgin females and 3.26 1.16 ng/PG Z11–14:Ac in Z-strain ones (Fig. 7). Decapitation resulted in a decline in pheromone titres in both E- and Z-strain moths. As expected, mated females also produced significantly less pheromone than virgins. However, in both cases, injection of 5.8 pmol Ostnu-PBAN stimulated pheromone production close to a normal level (Fig. 7).

## Gene organization

Genomic DNA sequences were obtained from ECBs collected at four locations in Hungary (Bicske, Hódmezővásárhely, Martonvásár and Romhány), one from Adana (Turkey) and one from the Savinja valley (Slovenia). In addition, a male and a female adzuki bean borer moth (Ostrinia scapulalis Walker; caught at Matsudo, Japan) were also included in the present study. The intron–exon structure of the DH-PBAN gene was determined by alignment of the cDNA and corresponding genomic sequences. Sequencing across the intron–exon junctions of the genomic fragments revealed five introns (Fig. 1). Intron sizes and intron–exon boundaries are

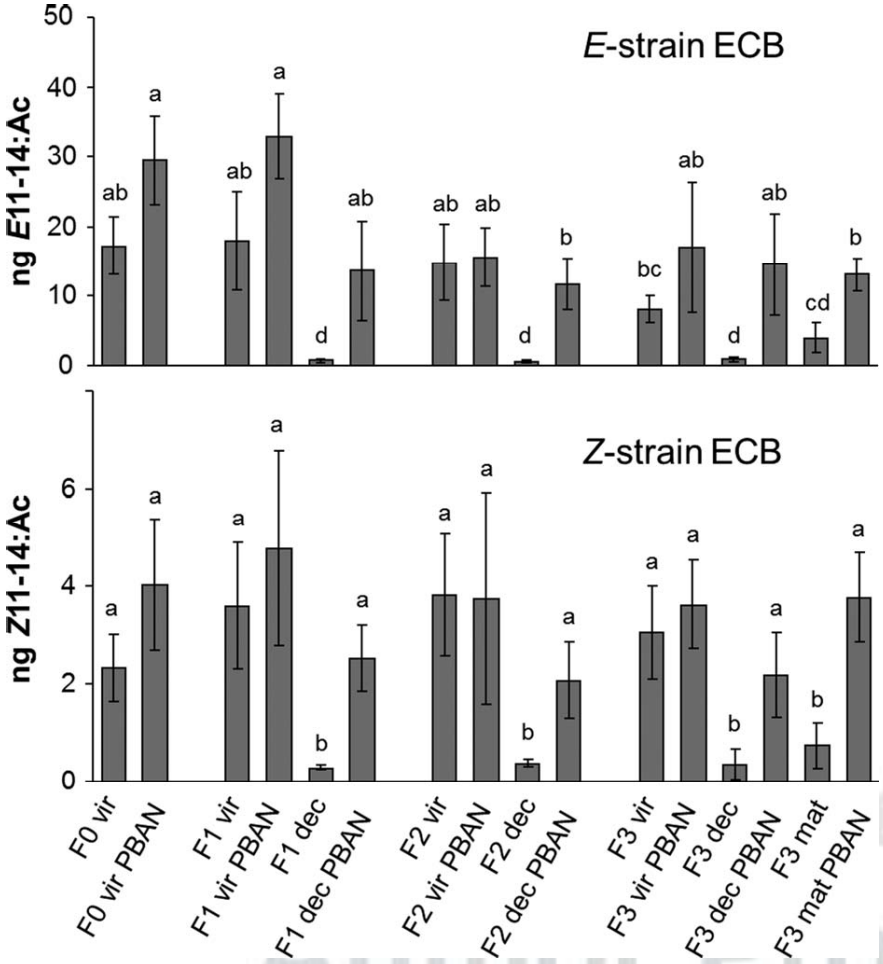


Figure 7. The amounts of the two main pheromone components, (E)-11-tetradecenyl acetate (E11–14:Ac) in the E-strain Ostrinia nubilalis females (top panel) and (Z)-11-tetradecenyl acetate (Z11-14:Ac) in the Z-strain ones (bottom panel), in pheromone gland (PG) extracts. Samples were collected at the end of the scotophase. ECB, European corn borer; F0–F3, 0–3-day-old females; vir, virgin; mat, mated; dec, decapitated; PBAN, treated with 5.8 pmol Os. nubilalis pheromone biosynthesis activating neuropeptide. Data are expressed as mean pheromone contents in nanograms per PG. Error bars show the SEM of six biological replicates, each containing three PGs. Statistical analysis was performed by analysis of variance followed by Duncan's multiple range test. Means with different letters are significantly different at $P < 0.05$.



T2 summarized in Tables 1 and 2, respectively. The putative peptides derived from these exons are: exon one–signal peptide and the N-terminal portion of DH, exon two–C-terminal portion of DH, exon four–a- and b-SGNPs along with the N-terminal portion of PBAN, exon five–the remaining portion of PBAN and the gSGNP (Fig. 1). The C-terminal codons and the complete 3′ UTR are encoded in exon six. All five intron–exon junctions in the Ostnu-PBAN gene have appropriate consensus signals for splicing: a 5′ dinucleotide GT and a 3′ AG (Table 2). Intron phases within the Ostnu-PBAN gene were 0, 2, 1, 2, 1, AQ14 respectively. Analysis of the DH-PBAN genomic sequences revealed that the deduced amino acid sequences of Os. scapulalis DNA fragments were identical to the consensus sequence of the Ostnu-PBAN precursor (Table 2).

### Neighbour-joining analysis

To examine the sequence relationships amongst Ostnu-PBAN mRNAs, a neighbour-joining tree was generated. Regardless of the pheromone composition and AQ15 geographical origin of each ECB sample, all transcripts containing a short 3′ UTR formed a separate clade with 100% bootstrap support (Fig. 8). This result can be F8 understood in the light of our observation that all sequences with a short 3′ UTR (SIE6, BHuZ1, LSwZ2) were > 99% identical to each other. Another three sequences (SIE7, KHuZ1, LSwZ3), sharing 92–97% identity, fell into the long 3′ UTR group and formed a separate clade with 85% support. However, transcripts with a medium-length 3′ UTR (SIE3–5, LSwZ5, RSwZ1–2, KHuZ2) that shared 91 to 96% identity were assigned to four distinct clades (Fig. 8).

## Discussion

By means of a molecular cloning approach, we identified PK/PBAN neuropeptides from brain-SG complexes of Os. nubilalis. Our data, presented here, indicate that the ORF of Ostnu-PBAN encodes a putative neuropeptide precursor of 200 amino acid residues. The deduced amino acid sequence revealed a 24-aa DH, a 37-aa PBAN and three additional short peptides (a-, b- and g-SGNPs) derived from post-translational proteolytic

Table 1. DNA fragments obtained from Ostrinia nubilalis and Ostrinia scapulalis diapause hormone pheromone biosynthesis activating neuropeptide (DH-PBAN)

| Origins (E/Z strain) | Male, female, larva | Exons (nucleotide positions) | Introns (intron lengths in bp) | Most similar cDNAs (% identity) |
|---|---|---|---|---|
| Os. nubilalis genomic DNA fragments. | | | | |
| Adana, Turkey (Z) | M1 | IV–V (375–529) | IV (640) | SIE6, BHuZ1, LSwZ2 (100) |
| | M2 | IV–VI (313–583) | IV (640), V (528) | SIE6, BHuZ1, LSwZ2 (100) |
| | M3 | IV–V (375–529) | IV (474) | SIE7 (100) |
| | | VI (559–1841) | – | LSwZ5 (99.5) |
| Bicske, Hungary (Z) | M4 | I–V (-49–529) | I (745), II (893), III (417), IV (534) | BHuZ1, LSwZ2 (99.2) |
| | | VI (559–1774) | – | LSwZ2 (99.7), SIE6 (99.5) |
| | | I–II (-49–128) | I (935) | SIE2, SIE5, LSwZ3 (100) |
| | M5 | IV–V (375–529) | IV (671) | LSwZ4 (99.4) |
| Martonvásár, Hungary (Z) | M6 | IV–V (375–529) | IV (640) | SIE6, BHuZ1, LSwZ2 (100) |
| Hódmező- vásárhely, Hungary (Z) | L1 | IV–V (375–529) | IV (587) | KHuZ2 (100) |
| | | IV–V (375–529) | IV (474) | SIE7 (100) |
| | | VI (559–1819) | – | KHuZ2 (99.5)* |
| | L2 | IV–V (375–529) | IV (474) | SIE7 (100) |
| | | VI (559–1820) | – | SIE4 (100), RSwZ1 (99.9) |
| Romhány, Hungary (Z) | L3 | IV–V (375–529) | IV (474) | SIE7 (100) |
| | | VI (559–1743) | – | SIE6, LSwZ1 (99.8) |
| | L4 | IV–V (375–529) | IV (474) | SIE7 (100) |
| | | IV–V (375–529) | IV (640) | SIE6, BHuZ1, LSwZ2 (100) |
| | | VI (559–1818) | – | RSwZ2 (99.5)† |
| | L5 | VI (559–2012) | – | LSwZ3 (99.2) |
| | L6 | VI (559–1974) | – | LSwZ3 (99.7) |
| Žalec, Slovenia (E) | M7 | IV–VI (346–583) | IV (664), V (534) | SIE3 (99.3) |
| | | IV–VI (346–583) | IV (436), V (511) | ND (94.6–96.7) |
| | | VI (559–1847) | – | LSwZ2 (98.7)/LSwZ5 (99.6)‡ |
| | M8 | VI (619–2066) | – | KHuZ1 (99.7) |
| | | VI (559–1821) | – | SIE3 (99.3) |
| Os. scapulalis | | | | |
| Matsudo, Japan | M9 | I–II (-41–128) | I (~ 3 kb) | BHuZ1, LSwZ2 (100) |
| | | IV–VI (285–583) | IV (639), V (528) | SIE6, BHuZ1, LSwZ2 (100) |
| | | VI (559–1743) | – | SIE6 (99.7) |
| | F1 | IV–V (313–529) | IV (650) | SIE3 (100) |
| | | VI (559–1844) | – | ND (90.8–95.0) |

Intron lengths are indicated (in bp).
Positions of the DNA fragments are given with respect to the translation initiation codon (nucleotides within introns are not numbered).
Each sequence was aligned with the cDNA-derived sequences, and the closest matches are presented in the last column.
The corresponding Ostnu-PBAN transcripts are designated as SIE3–SIE7 (Slovenia, E-strain), BHuZ1 and KHuZ1-KHuZ2 (Hungary, Z-strain), RSwZ1-RSwZ2 and LSwZ1-LSwZ5 (Sweden, Z-strain).
*, †Excluding a 29 and a 26 bp indel, respectively.
‡Mixed sequence, a 92 bp internal fragment is homologous to LSwZ2, the flanking sequences are homologous to LSwZ5.
ND, not determined: cDNA with > 98% identity was not found, the range of nucleotide identities amongst all 14 Ostnu-PBAN cDNAs are shown.
F, female; M, male; L, larva.

Table 2. Putative exon–intron structure of the Ostrinia nubilalis pheromone biosynthesis activating neuropeptide gene

| Exon number | Size (bp) | cDNA position | Splice acceptor | Splice donor | Intron phase | Amino acid |
|---|---|---|---|---|---|---|
| 1 | 5′ UTR 1 78 | –60–78 | Not determined | TTGAAG gtaatt | 0 | $Lys^{26}/Asp^{27}$ |
| 2 | 95 | 79–173 | tggcag GATGAC | CAACAA gtacgc | 2 | $Lys^{58}$ |
| 3 | 101 | 174–274 | ttgcag GCAAAC | CCCGTG gtaagt | 1 | $Val^{92}$ |
| 4 | 154 | 275–428 | caacag TGACAA | CGAAGT gtgagt | 2 | $Val^{143}$ |
| 5 | 119 | 429–547 | tttcag GTATAG | CGTATG gtatgt | 1 | $Asp^{183}$ |
| 6 | 531 3′ UTR | 548–3′ end | ttccag ACATTT | Not determined | | |

UTR, untranslated region.
Exon and intron sequences are shown in upper- and lowercase letters, respectively.
The nucleotide 1 1 is the A of the ATG-translation initiation codon.
Invariant nucleotides at the splice sites are underlined.
Amino acid(s) encoded at the splice site are indicated.
Phase 0 introns lie between two codons; phase 1 and 2 introns are located after the first and second positions of codons, respectively.
The 3′ ends are located 1850–2086 nucleotides downstream of the start codon.

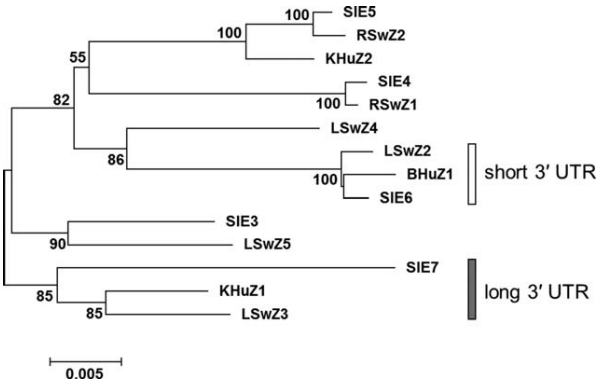


Figure 8. Neighbour-joining tree of 14 full-length cDNA sequences encoding the putative diapause hormone pheromone biosynthesis activating neuropeptide precursor from E-strain (Sl, Žalec, Slovenia) and Z-strain (BHuZ, Bicske, Hungary; KHuZ, Kéty, Hungary; RSwZ, Ravlunda, Sweden; LSwZ, Landskrona, Sweden) Ostrinia nubilalis. The sequences are numbered consecutively for each geographical area. Sequences not belonging to one of the indicated clades have medium-length 3′ untranslated regions (UTRs). All positions containing gaps and missing data were eliminated from the data set. Bootstrap values > 50% (1000 replications) are indicated at the nodes. The scale bar represents the number of base substitutions per site.

processing and subsequent carboxyl-terminal amidation. The consensus F(T/S)P(R/K)L pentapeptide motif, representing the active core sequence for diverse biological activities (Nachman et al., 1986; Raina & Kempe, 1990; Kuniyoshi et al., 1992), was found immediately upstream of the predicted endoproteolytic cleavage sites that contain a Gly residue for amidation of the C-terminus, which is critical for biological activity (Raina & Kempe, 1990). The Ostnu-PBAN precursor has closest homology (84% amino acid identity) with the DH-PBAN precursor of the bamboo borer, Om. fuscidentalis (Suang et al., 2015). Regier et al. (2012) conducted a molecular phylogenetic analysis of moth species in the Pyraloidea superfamily using sequence data from protein-coding regions of five nuclear genes. This molecular phylogeny suggested a sister relationship between the crambid subfamilies Pyraustinae (represented in the analysis by the Asian corn borer, Os. furnacalis and the fulvous-edged pyrausta moth, Pyrausta nexalis) and Spilomelinae, to which the legume pod borer, M. vitrata, and the bamboo borer, Om. fuscidentalis, belong. The close relatedness between Pyraustinae and Spilomelinae is further supported by our finding that the deduced amino acid sequence of Ostnu-PBAN consists of 37 residues, and, to our knowledge, a 37-residue PBAN has previously only been reported in Om. fuscidentalis (Suang et al., 2015).

The full-length Ostnu-PBAN cDNAs varied in size between 1910 and 2146 nucleotides due to the length variation in the 3′ UTR, which is otherwise exceptionally long (> 1250 bp) compared to the 3′ UTRs of known lepidopteran DH-PBAN genes (< 200 bp). Various structural motifs that commonly affect mRNA stability and translational efficiency are present in the 3′ UTR, suggesting the possibility that Ostnu-PBAN may be regulated by a miRNA-mediated post-transcriptional pathway (Lucas et al., 2015). AQ16

A neighbour-joining analysis of nucleotide sequences revealed that Ostnu-PBAN transcripts with relatively short and long 3′ UTR sequences fell into separate clades, whereas medium-sized transcripts comprise a heterogeneous set of sequences. However, the sequences did not show any clustering according to pheromone composition, geographical origin or life history (univoltine vs. bivoltine). In another study, ECB moths were not differentiated on the basis of D11 desaturase allelic diversity (Geiler & Harrison, 2010). The variation in intron length in the two D11 desaturase allelic classes did not separate ECB moths according to their pheromone race, geographical origin or voltine ecotypes. Currently, only a limited number of loci are known to differ between E and Z strain ECB moths. These are pgFAR (Lassance et al., 2010), and some Z chromosome-linked markers, the Tpi (Dopman et al., 2005) and three pheromone receptor genes (Lassance et al., 2011; Yasukochi et al., 2011). AQ17 AQ18

Sequencing of the entire coding sequence and exon–intron boundaries of the Ostnu-PBAN gene revealed that it is at least 5 kb long and consists of six exons and five introns with a conserved distribution of intron positions within codons (0, 2, 1, 2, 1). The Ostnu-PBAN gene has extensive structural homology to the B. mori (Xu et al., 1995a), Helicoverpa armigera (Zhang et al., 2005), Clostera anastomosis (Jing et al., 2007) and M. vitrata (Chang & Ramasamy 2014) DH-PBAN genes, which have previously been identified in the genomes of lepidopteran species belonging to various families. Short- and medium-length 3′ UTR forms were also observed in the amplification products obtained from adzuki bean borer (Os. scapulalis) DH-PBAN genomic DNA, whose deduced amino acid sequence shared 100% identity with the Ostnu-PBAN consensus sequence. An Asian corn borer, Os. furnacalis, genomic DNA sequence (LC002981) contains the entire exon 3 and exon 4 and flanking intronic sequences of a gene putatively encoding a DH-PBAN precursor. This fragment encodes for a-SGNP, b-SGNP and the N-terminal portion of Ostfu-PBAN, and exhibits 98.4% nucleotide identity and the deduced amino acid sequence of the encoded protein is 100% identical to Ostnu-PBAN SIE5 and RSwZ2. All this evidence implies that the observed nucleotide polymorphism of the Ostnu-PBAN gene pre-dates the divergence of the Asian and European corn borer moths approximately 1 000 000 years ago (Roelofs et al., 2002). AQ19

When the synthetic Ostnu-PBAN was injected into decapitated females at a dose between 1.45 and 5.8 picomoles, it restored sex pheromone production to normal levels. Furthermore, there was a significant increase in pheromone production after injection of even 0.058 pmol synthetic Ostnu-PBAN. These results indicate that it is a hormonally active peptide. In mated females, pheromone production could be restored by injecting the synthetic Ostnu-PBAN, which suggests that the pheromone biosynthetic machinery remains intact and is fully capable of de novo synthesizing the pheromone blend. This result is consistent with our previous observation on the cabbage moth (Mamestra brassicae), where the injection of an 18-aa Mambr pheromonotropin reactivated pheromone production to normal levels after 5 days of mating (Köblös et al., 2015). Confirmatory findings were reported by Tabata et al. (2003) who reactivated sex pheromone biosynthesis in Os. scapulalis by a synthetic C-terminal amidated octapeptide of Helze-PBAN. The GC-MS analysis performed here indicates that, in line with Kárpáti et al. (2007), E11–14:Ac was produced at significantly higher levels in E-strain females than Z11–14:Ac in Z-strain individuals. Roelofs et al. (1987) demonstrated that both E- and Z-strain ECB females have 14-carbon D11 unsaturated fatty acyl moieties with similar E/Z ratios of ≈ 70:30. This ratio corresponds closely to the difference in the production of the two main pheromone components between the two strains, but further studies are required to clarify any causal role.

Quantitative real-time PCR analysis showed that DH-PBAN precursor mRNA is present throughout the developmental stages from first-instar larvae to adults and in both sexes. Similar results have been reported in B. mori (Sato et al., 1994; Xu et al., 1995b), H. zea (Ma et al., 1998) and various other moths (reviewed by Rafaeli, 2009). Previous studies have demonstrated that, in addition to pheromone production, FXPRL-$NH_2$ peptides regulate other functions such as cuticular melanization in moth larvae (Matsumoto et al., 1990; Altstein et al., 1996; Raina et al., 2003), initiation of egg diapause in B. mori (Imai et al., 1991) and termination of pupal diapause in some heliothine moths (Sun et al., 2003; Xu & Denlinger, 2003) (for review, see Denlinger, 2002; Jurenka, 2015). However, the physiological functions of DH and SGNPs in the ECB remain to be established. There is considerable evidence that diapause induction in the ECB is primarily a response to seasonal changes in day length and temperature (Mutchmor & Beckel, 1958; Skopik & Bowen, 1976; Takeda & Skopik, 1985; Beck, 1989), and is mediated in part by the absence of the moulting hormone, ecdysone (Bean & Beck, 1983; Gelman et al., 1992). There was an increase in the level of Ostnu-PBAN mRNA expression following adult eclosion that plateaued after 1 day and thereafter it was not affected by mating status. This is in line with previous studies suggesting that postmating inhibition of pheromone production may be associated with cessation of PBAN release from the corpus cardiacum rather than reduced synthesis (Foster, 1993; Jurenka & Fabriás, 1993; Raina et al., 1994; Ando et al., 1996; Delisle et al., 2000). The process could be mediated by hormonal and neural mechanisms as reviewed extensively by Rafaeli (2011).

The benefits of identifying the Ostnu-PBAN precursor are manifold. In physiological studies, synthetic Ostnu-PBAN can be used to trigger in vivo and also in vitro pheromone production, which may contribute to a better understanding of the endogenous regulation of pheromone biosynthesis. It facilitates deciphering the functional role of the other PBAN family neuropeptides (DH and SGNPs) in the ECB, and can provide new insights into practical means of manipulating insect homeostasis and development. Furthermore, the ECB is an established model organism in evolutionary biology and the Ostnu-PBAN sequence information may shed more light on the divergence and evolutionary origin of the genus Ostrinia.

## Experimental procedures

### Insects

The E-strain laboratory colony of ECB was established from two dozen larvae collected from maize plants near Žalec (46814′49.3″N, 1589′50.0″E) in the Savinja valley, Slovenia, in September 2010 (Rak Cizej et al., 2010). Likewise, the Z-strain culture was established from larvae collected near Kéty (46826′41.9″N, 18831′26.8″E), southern Hungary, in April 2010. Larvae were reared on a semisynthetic diet (Nagy, 1970) under a 18:6 h light/dark cycle at 25 8C and 50% relative humidity. The resulting pupae were sexed and separated. In mating experiments, newly emerged females (~10 individuals) were placed with males (~20 individuals) in glass jars lined with plastic bags for egg laying and kept for 3 days.

Furthermore, ECB larvae were collected from corn stalks in Romhány (47855′47.6″N, 19815′45.8″E) in northern Hungary in December 2011 and 2012 (univoltine population) and in Hódmezővásárhely (46822′37.6″N, 20828′40.1″E) in southern Hungary in November 2011 (bivoltine population). In Sweden, which is the northernmost extent of the species' range in Europe, ECB larvae were captured in August 2011 from two cornfields located at opposite coasts of Skane, the southernmost Swedish province. Three and two ECB larvae were collected from univoltine populations at Landskrona (55853′0.4″N, 12851′30.6″E) and Ravlunda (55842′32.1″N, 1488′26.5″E), respectively, as described by Lehmhus et al. (2012). Adult ECB females and males were also collected from cornfields in bivoltine areas: in the Savinja valley, Slovenia, in August 2013, and at two sites in central Hungary near Martonvásár (47819′41.2″N, 18848′28.0″E) and Bicske (47828′33.9″N, 18836′29.6″E) during June and August 2015. In addition,

multivoltine ECB (Zeren et al., 1988) males were captured by delta-type sticky traps baited with the Z-type lure near Adana (37801′56.4″N, 35822′50.7″E) in southern Turkey in August 2014. Three genomic DNA samples extracted from individual males were selected from 24 samples for further analysis. Last but not least, field-collected adzuki bean borer moths (Os. scapulalis), a congener of ECB, were also included in this study. Adult Os. scapulalis moths (two males and two females) were captured in Matsudo (35847′59″N, 139854′0.8″E), Japan, in 2008–2009, stored in ethanol and later provided to us for analysis.

## Gas chromatographic analysis of pheromone components combined with electroantennographic detection

The pheromone components were monitored in ECB cultures using gas chromatography (GC) with electroantennographic detection (EAD) using antennae of adult male moths, as described by Kárpáti et al. (2007). Briefly, PGs were dissected from calling females during the second half of the scotophase and extracted in n-hexane. The extract was concentrated to 2 l l and analysed on a GC (6890 N, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a DB-Wax column (30 m 3 0.32 mm 3 0.25 l m film thickness, J&W Scientific, Folsom, CA, USA) using splitless mode injection, carrier gas helium and a temperature programme of 60 8C for 1 min, 10 8C/min to 120 8C, 5 8C/min to 220 8C, maintained for 40 min. The EAD included a micromanipulator (MP15) and an IDAC 232 amplifier (Syntech, Kirchzarten, Germany). The reference compound was n-decyl acetate. The pheromone components were identified by retention times of synthetic E11- and Z11–14:Ac and by the patterns of responses of male antennae from E and Z colonies.

## Gas chromatographic and mass spectrometric analysis of pheromone components

Hexane extracts (in 20 ml for 8 min) of three PGs were transferred to conical vials and 5 ml of 1 ng/ml E8,Z10–14:Ac (Pherobank BV) was added as an internal standard. GC-MS analysis was carried out on an Agilent 6890 GC equipped with a Rxi-5Sil (30 m 3 0.25 mm 3 0.25 l m film, Restek, Bellefonte, PA, USA) column and coupled to an Agilent 5973 mass selective detector operated in selected ion monitoring mode. Samples were injected in the splitless mode with helium used as a carrier gas at a flow rate of 2 ml/min. The oven was programmed from 100 to 300 8C at a rate of 20 8C/min after an initial delay of 1 min. All data were recorded and edited with ChemStation software D.01.02.16 (Agilent Technologies), and quantification of E11- and Z11-14:Ac was performed using the internal standard mode.

## Strain typing of wild-caught moths

For each individual used in this study, pheromone type was identified by genotyping for the pgFAR gene according to the method of Lassance et al. (2010). In a previous report, the Z allele of pgFAR was identified in ECB larvae collected in Ravlunda and Landskrona, Sweden (Lehmhus et al., 2012). Briefly, PCR amplifications were performed using the primers pgFAR-E sense 5′-GGTTTGATATTGATTGAGGAGAG-3′, pgFAR-E antisense 5′-GGTTTGTTTGGTTGTAATTTATAGG-3′, pgFAR-Z sense 5′-CGACTAGAGTAGGTATGTAATATAG-3′ and pgFAR-Z antisense 5′-TTGAGTAAGCGTTTGTATGAAG-3′ described by Lassance et al. (2010). Genomic DNA or cDNA from total RNA were used as the template as described in the next section. Cycling conditions were 95 8C for 3 min, 30 cycles at 95 8C for 30 s, 55 8C for 30 s and 72 8C for 30 s, followed by a final extension of 72 8C for 10 min with DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The 132-bp and 92-bp amplification products corresponding to pgFAR-E and pgFAR-Z, respectively, were analysed by electrophoresis in 1.5% agarose gels containing 0.5 l g/ml ethidium bromide. The specificity of the amplification was verified by sequencing of the PCR products.

In the case of ECB collections from Hungary, we also used GC-EAD or GC-MS methods to confirm the results. A number of larvae from each collection site in Hungary were reared to adulthood and the females were processed for pheromone analysis as indicated above.

## Cloning and sequence analysis

Total RNA was isolated from five individual whole larvae collected in Sweden and from pooled samples containing 15 brain-SG complexes of 1–3-day-old virgin females obtained from each of the E- and Z-strain laboratory colonies, or containing 15 heads of females collected in Bicske, central Hungary, in June 2015. Tissue samples were homogenized in 900 l l QIAzol lysis reagent (Qiagen, Valencia, CA, USA) using a microcentrifuge tube and pestle and RNA was extracted with an RNeasy Plus Universal Mini kit (Qiagen) according to the manufacturer's protocol. Isolated RNA samples were quantified by a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Total RNA preparations were treated with DNase I (Life Technologies, Rockville, MD, USA) to remove residual genomic DNA, and reverse transcribed to generate cDNA using SuperScript III (Life Technologies) and random hexamer primers. Two degenerate oligonucleotide primers (sense 5′-GATGCCYTGAARTATTAYT ACGA-3′ and antisense 5′-RAGWCGAGGBKAGAAGTA-3′), corresponding to the conserved sequence motifs DALKYYY and YFSPRL, respectively, were constructed based on the alignment of lepidopteran DH-PBAN sequences available from GenBank. PCR amplification of reverse transcribed brain-SG complex mRNA was carried out under the following conditions: 98 8C for 2 min, 35 cycles at 98 8C for 30 s, 53 8C for 30 s and 72 8C for 10 s, followed by a final extension of 72 8C for 10 min with Velocity DNA polymerase (Bioline). The amplified PCR products were gel-purified with a High Pure PCR product purification kit (Roche, Indianapolis, IN, USA), and ligated into the pJET1.2/blunt cloning vector using a CloneJET PCR cloning kit (Thermo Scientific) according to the manufacturer's protocol. The cloned PCR fragments were sequenced at Macrogen Europe (Amsterdam, The Netherlands) using pJET1.2 sequencing primers. BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) against the GenBank database revealed that the consensus sequence had 66–76% nucleotide similarity with other lepidopteran DH-PBAN sequences. Sequence information of the cloned cDNA fragments

was used to design gene-specific primers using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Full-length cDNA sequences were determined by 5′- and 3′-RACE using the SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the recommendations of the manufacturer. The two specific primers designed from the partial cDNA fragment were 5′-CGATCAGCTCCCCTTCTACGAAAGC-3′ for 3′-RACE, and 5′-CATGAGAATCAGATGGCGTGACAGG-3′ for 5′-RACE. Amplification products were cloned into the pJET1.2/blunt vector and two to 12 clones from each PCR product were sequenced as above. RT-PCR amplifications were conducted using primers designed based on the terminal segments of the 5′- and 3′-RACE products (sense 5′-GCTTTCGCCATAGACTC AACCC-3′ and antisense 5′-AATGAGGTATGAAGCGGCCAT-3′). Thermocycler conditions consisted of 98 8C for 2 min, 32–35 cycles at 98 8C for 30 s, 60 8C for 30 s and 72 8C for 40 s, followed by a final extension of 72 8C for 10 min with Velocity DNA polymerase. The obtained PCR products were cloned and sequenced as above. Nucleotide sequences were aligned using ClustalX 2.1 (Thompson et al., 1997) with default settings and refined manually. A neighbour-joining tree (Saitou & Nei, 1987) was constructed in mega 7.0.14 (Kumar et al., 2016) with 1000 bootstrap replications excluding all sites with gaps. Aligned sequences were tested for recombination using the program rdp4 (Martin et al., 2015), which is available from http://web.cbio.uct.ac.za/~darren/rdp.htm. Multiple comparison corrections were performed using the Bonferroni correction with a P-value threshold of 0.05, and polished breakpoints. Only unique events detected by at least three methods implemented in rdp4 at a significance level of $P < 0.001$ were considered.

Four collection sites were selected in Hungary and one in Slovenia as described above. Genomic DNA was isolated from two to three ECB individuals per collection site using DNAzol reagent (Molecular Research Center, Inc.) following the manufacturer's instructions. DNA was also extracted from the legs of individual males captured in Adana, Turkey, using a QIAamp DNA Micro Kit (Qiagen). Furthermore, genomic DNA was extracted from thoracic tissues of a single Os. scapulalis male and a female from Japan. Genomic DNA was quantified by a NanoDrop 1000 spectrophotometer.

Based on an alignment with B. mori DH-PBAN (GenBank D16230), the genomic organization of the DH-PBAN gene of Os. nubilalis was predicted. The exon/intron junctions were identified by PCR amplification using Os. nubilalis genomic DNA and specific primers for exon 1 (sense 5′-GCTTTCGCCAT AGACTCAACCC-3′ and sense 5′-CATAGACTCAACCCATGG AAAGGAAC-3′), exon 2 (sense 5′-CGGCACCCTCTGGTTTGG C-3′ and antisense 5′-GGGCCAAACCAGAGGGTG-3′), exon 3 (sense 5′-CGATCAGCTCCCCTTCTACGAAAGC-3′ and antisense 5′-TTCGTAGAAGGGGAGCTGATCG-3′), exon 4 (sense 5′-GGTAGTCTTCACCCCGAAACTC-3′, sense 5′-AGCATCGAC GTCTACCCTGA-3′, sense 5′-TTCGAGAACGTAGAATTCACGC -3′, sense 5′-CAGGAGGTTGCCTGAGAAG-3′, antisense 5′-C GAGTTTCGGAGTGAAGACTACT-3′ and antisense 5′-CATGAG AATCAGATGGCGTGACAGG-3′), exon 5 (antisense 5′-CGTCT AGTTCCCTCCCCAGTC-3′) and exon 6 (sense 5′-GAGAAGAT ACGGCTAGCACGAAG-3′, antisense 5′-CGCTTCGTGCTAGCC GTATC-3′, antisense 5′-AATGAGGTATGAAGCGGCCAT-3′ and antisense 5′-ATCGTCCAAAACATTAACGTGTTAC-3′). These primers were also used to amplify homologous regions from the adzuki bean borer. Thermocycler conditions consisted of 98 8C for 2 min, 32–35 cycles at 98 8C for 30 s, 60 8C for 30 s and 72 8C for 1 min, followed by a final extension of 72 8C for 10 min with Velocity DNA polymerase.

The amplification products were isolated and sequenced as described above to identify exon/intron boundaries. Nucleotide sequences assembled from overlapping clones isolated from Os. nubilalis and Os. scapulalis have been submitted to GenBank under accession numbers KT588300, KU952096–KU952114 and KX034792–KX034795. After conceptual translation, predictions of signal peptide sequences and intron splice sites were performed with the programs SignalP 4.1 and NetGene2, respectively, on CBS Prediction Servers (http://www.cbs.dtu.dk/services/; Nielsen et al., 1999; Petersen et al., 2011). Endoproteolytic cleavage sites in the peptide precursor were predicted according to the rules described by Veenstra (2000).

## Tissue mRNA expression

Developmental and tissue-specific RT-PCRs were performed using cDNA derived from total RNA extracted from laboratory cultures of the ECB (eggs, first to fifth instars, pupae and adults, as well as various organs and tissues of 3-day-old virgin adult males and females: heads, brain-SG complexes, legs, ovaries, fat bodies, pheromone glands, hairpencil-aedeagus complexes and carcasses) using a RNeasy Plus Universal Mini kit (Qiagen) and Extractme Total RNA kit (Blirt SA, DNA-Gdańsk, Poland) according to the manufacturers' protocols. Adults were considered 0 days old on the day of eclosion. Brain-SG complexes were dissected from both sexes of pre-emergence pupae, 0-, 1-, 2- and 3-day-old virgins, and 3-day old mated adults as described above. After DNAse I treatment, first-strand cDNA synthesis was performed with 1 l g total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and random hexamer primers according to the manufacturer's instructions.

Real-time PCR analysis was performed to analyse the relative mRNA expression level in various tissues and developmental stages. The oligonucleotide primer pair designed to amplify a 100-bp fragment spanning exons 3 and 4 (positions 264 to 363 relative to the translational start codon) of the Ostnu-PBAN transcript consisted of: sense 5′-GGAGACCCGTGTGACAAA-3′ and antisense 5′-CGTGAATTCTACGTTCTCGAA-3′. Quantitative PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SensiFAST SYBR No-ROX kit (Bioline) in a 20 l l reaction volume and running a standard programme (95 8C for 2 min, 40 cycles at 95 8C for 10 s, 60 8C for 10 s and 72 8C for 10 s, followed by a melt curve analysis to determine amplicon specificity using a temperature range from 65 to 95 8C with increments of 0.5 8C). The amount of DH-PBAN mRNA was normalized to ribosomal protein S3 (rpS3, GenBank AY513653 and DQ988989) mRNA levels. The oligonucleotide primer pair used to amplify a 210-bp fragment of rpS3 (from positions 178 to 378) consisted of: sense 5′-CAGAGCGTACTGGGAGAGAAG-3′ and antisense 5′-GAACCTCAGCACACCATAGCA-3′. All reactions were performed using three biological replicates in triplicate. In each

run, water-only controls and non-reverse-transcribed RNA were used as negative controls. Quantification was performed using the standard curve method (Larionov et al., 2005) by serial dilutions of plasmids containing cloned fragments of DH-PBAN or rpS3 cDNA. All PCR efficiencies ranged between 100 and 103%, with $r^2 = 0.99$ or higher. The products were purified with a High Pure PCR product purification kit (Roche) and sequenced (Macrogen Europe).

## Effect of synthetic Ostnu-PBAN

Based on the deduced amino acid sequence of residues 128–164, the 37-aa Ostnu-PBAN with an amidated C-terminus was custom synthesized by CASLO ApS (Technical University of Denmark, Lyngby, Denmark) with a purity $>$ 99% (wt/wt) determined by high pressure liquid chromatography. Dose–response relationships of the pheromonotropic effect of the synthetic peptide were tested in decapitated E-strain females according to Raina & Klun (1984). Two-day-old females were decapitated at the third hour of scotophase and 27 h later were injected with a dose series of synthetic Ostnu-PBAN (0.058, 0.29, 0.58, 1.45, 2.9, 5.8 and 11.6 pmol in 2 ml distilled water) using a 10-ll Hamilton syringe. Each dose was replicated six times. Females injected with 2-ml water served as controls. Ninety min after injection, pooled samples containing three PGs were extracted with 20 ll n-hexane for 8 min and subjected to GC-MS analysis.

The effect of the synthetic Ostnu-PBAN on pheromone production was monitored in both E- and Z-strain moths. Females were decapitated at the third hour of scotophase on days 0, 1 and 2 of adult life and 27 h later were injected with 5.8 pmol Ostnu-PBAN or water as described above. To assess the effect of mating, 3-day-old mated females were injected as above. Quantities of pheromone produced in decapitated or mated females were compared to those of 0-, 1-, 2- and 3-day-old intact virgins injected in a similar fashion. Pheromone extraction was performed 90 min after injection that occurred at the end of scotophase, when pheromone production peaked. The PGs were dissected, extracted with n-hexane, and analysed for pheromone titre as described above.

## Statistical analysis

All statistical analyses were performed using Statistica 6.1. software (StatSoft, Tulsa, OK, USA). Normality of distribution of the data was assessed by the Kolmogorov–Smirnov test. Differences between groups were evaluated by one-way analysis of variance followed by Duncan's multiple range test. Data are represented as mean ± SEM for three or more independent measurements. A P-value of $<$ 0.05 was considered statistically significant.

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