Identification of a new member of the PBAN family of neuropeptides from the fire ant, *Solenopsis invicta*

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**Abstract**

Neuropeptide hormones produced by neurosecretory cells in the central or peripheral nervous systems regulate various physiological and behavioral events during insect development and reproduction. PBAN/Pyrokinin is a major neuropeptide family, characterized by a 5-amino-acid C-terminal sequence, FXPRLamide. This family of peptides has been implicated in regulating various physiological functions including, pheromone biosynthesis, muscle contraction, diapause induction or termination, melanization, and puparium formation in different insect species. In the present study, we report a new member of the PBAN family from the red imported fire ant, *Solenopsis invicta*, Soi-PBAN, composed of 26-AA (GSGEDLSYGDAYEVDEDDHPLFVPRL). Three additional peptides were deduced from Soi-PBAN cDNA: 15-AA (TSQDIASGMWFGPRL), 8-AA (QPQFTPRL) and 9-AA (LPWIPSPRL), that correspond to diapause hormone (DH), β-neuropeptide (NP), and γ-NP, which are found in many lepidopteran moths. Five peptides, DH, α, β, γ NPs, and PBAN are encoded from PBAN genes of lepidopteran moths, but in the fire ant the α-NP is missing. Each of the four synthetic peptides from the fire ant Soi-PBAN cDNA showed significant pheromonotropic activity in a moth model, indicating that these peptides are cross-reactive. Soi-β-NP induced the highest amount of pheromone production of the four peptides evaluated. The Soi-DH homologue had the lowest pheromonotropic activity, but was still significantly greater than control values. When the deduced amino acid sequences (entire ORF domains) from Soi-PBAN cDNA were compared with other known sequences, the fire ant was most similar to the honey bee, but phylogenetically distant from moth and beetle species. Soi-PBAN (26-AA) unlike the other three peptides shows a low degree of sequence identity with honeybee PBAN (33-AA). Based on the amino acid sequences encoded from insect PBAN genes identified to date, neuropeptide diversity is correlated with the taxonomic or phylogenetic classification of Insecta. From the present study we report the first neuropeptide identified and characterized from the central nervous system of Formicidae.

**Keywords:** Fire ant, PBAN, Neuropeptide, Pheromone, *Solenopsis invicta*.

**Introduction**

Neuropeptides are the largest group of insect hormones and are produced in the central and peripheral nervous systems, where they are released into the hemolymph, affecting development and reproduction. A variety of peptide families have been identified from insects (Gade et al., 1997). One of these families is the Pheromone Biosynthesis Activating Neuropeptide (PBAN)/Pyrokinin family defined by a conserved C-terminal pentapeptide (FXPRLamide) that is the active core fragment for peptide function (Raina & Kempe, 1992). A pyrokinin (leucopyrokinin) from the cockroach, *Leucophaea maderae*, was first isolated and characterized (Holman et al., 1986) as a myotropin with subsequent myotropic peptides being identified from various insect orders (Nachman et al., 1986). PBAN has been the subject of a great deal of interest especially for lepidopteran moths, since the first PBAN was identified from *Helicoverpa zea* adults two decades ago (Raina et al., 1989). Now, the PBAN/pyrokinin peptide family with a FXPRLamide functional epitope is expected to be widely distributed in Insecta with various physiological functions already documented: (1) stimulation of pheromone biosynthesis in female moths (Raina et al., 1989); (2) induction of melanization in moth larvae (Matsumoto et al., 1990;
Raina et al., 2003); (3) induction of embryonic diapause in Bombyx mori (Suwan et al., 1994); (4) stimulation of visceral muscle contraction in cockroaches (Predel & Nachman, 2001); (5) acceleration of puparium formation in the flesh fly (Zdarek et al., 1997); and (6) termination of development of pupal diapause in heliothine moths (Xu & Denlinger, 2003). The PBAN/pyrokinin family peptides are cross-reactive in that each peptide can activate all physiological functions noted above in experimental models.

The neurohormonal action of PBAN for pheromone biosynthesis in lepidopteran moths is well studied (Rafaeli & Jurenka, 2003). PBAN is synthesized in the subesophageal ganglion (SG) and is released into the hemolymph via the corpora cardiaca (CC), a neurohemal organ in H. zea (Raina and Klun, 1984). The first PBAN identified was the 33-amino acid peptide from H. zea, Hez-PBAN (Helicoverpa zea PBAN) (Raina et al., 1989). Subsequently PBAN amino acid sequences were determined from B. mori (Kitamura et al., 1989; Kitamura et al., 1990) and Lymantria dispar (Masler et al., 1994), through direct isolation and purification of peptides. Using DNA cloning tools more PBAN encoding genes have been identified from the moths, B. mori (Kawano et al., 1992; Sato et al., 1993), H. zea (Davis et al., 1992; Ma et al., 1994), Mamestra brassicaceae (Jacquin-Joly et al., 1998), Helicoverpa assulta (Choi et al., 1998), Helicoverpa armigera (Choi, 1999; Zhang et al., 2004), Agrotis ipsilon (Duportets et al., 1999), Bombbyx mandarina (Xu et al., 1999), Spodoptera littoralis (Iglesias et al., 2002), Heliothis virescens (Xu & Denlinger, 2003), Manduca sexta (Xu & Denlinger, 2004), Adoxophyes sp. (Choi et al., 2004), Samia cynthia ricini (Wei et al., 2004), Plutella xylostella (Lee & Boo, 2005), Ascosia selenaria cretacea (Kawai et al., 2007), Clostera anastomosis (Jing et al., 2007), Spodoptera exigua (Xu et al., 2007), Orgyia thyellina (Uehara et al., 2007) and Antheraea pernyi (Wei et al., 2008). From the identification of PBAN cDNAs, PBAN, diapause hormone (DH) and three additional F/PXPRL type peptides deduced from Soi-PBAN cDNA stimulate significant pheromone production in a moth model. This is the first time that a PBAN/pyrokinin family peptide from an ant species has been shown to have pheromontropic activity.

Results

Structure of PBAN cDNA

A PCR amplified 360-bp long product was obtained that included a possible PBAN and three F/PXPRL peptide domains. Based on this sequence additional gene specific primers were designed to extend the 5’ and 3’ ends of the PBAN cDNA. Using 5’- & 3’-RACE, a 754bp-long full cDNA was obtained that contained an entire open reading frame (ORF) of 531 nucleotides encoding 176 amino acids from the first initiation codon (ATG) to the termination codon (TAG) indicated in the boxes (Fig. 1). The TATA box was located 34-bp upstream of the transcription start site in the cDNA. The cleavage site for the signal peptide is predicted between the first 29 and 30 amino acids. The cDNA has four putative peptides based on six possible endoproteolytic cleavage sites (Veenastra, 2000; Southey et al., 2008) K_{62}R_{63}, G_{79}K_{80}R_{81}, K_{116}R_{117}, G_{126}R_{127}, G_{154}R_{155}R_{156}, and G_{166}R_{167}, indicated in italics in Fig. 1. The peptides cleaved are predicted to have a C-terminal amide group provided by glycine (G).

The third domain with a 26-amino acid (AA), GSGEDLYEVEDDEDDHPLFVRamide, is considered a putative PBAN homologue, So. invicta PBAN (Soi-PBAN). There are three additional putative peptides deduced from the Soi-PBAN cDNA. One of these peptides, 15-AA, TSO-DIASGMWFGPRLamide, is homologous to the diapause economic sectors include: residential households, electric and communication systems, agriculture, golf courses, and recreational areas. The fire ant is probably the most studied ant species in the world and a great deal is known about the pheromone systems used to reduce reproductive competition, recruit to resources, and other pheromones necessary to maintain colony social structure and territoriality (Vander Meer & Alonso, 1998, 2002; Vargo, 1998). In spite of decades of study on fire ant pheromones, virtually nothing is known about how pheromone production and release are regulated, nor whether protein hormones, especially neuropeptides, are involved in key physiological and endocrinial processes during development.

We previously demonstrated the presence of PBAN/pyrokinin family peptides and localization of immunoreactive neurons by applying an immunocytochemical technique to the central nervous system of the fire ant (Choi et al., 2009). In the present study, we report a new member of the PBAN/pyrokinin family from the red imported fire ant, So. invicta, PBAN and three additional F/PXPRL type peptides from the Soi-PBAN gene. We demonstrate that synthetic peptides deduced from Soi-PBAN cDNA stimulate significant pheromone production in a moth model. This is the first time that a PBAN/pyrokinin family peptide from an ant species has been shown to have pheromontropic activity.
hormone (DH) or PBAN-encoding gene neuropeptide-24 (PGN-24) domain in lepidopteran moths (Fig. 2). So far, this type of peptide possesses a conserved WFGPRL sequence in the C-terminus, except in Adoxophyes moth species (Fig. 2). The 8-AA, QPQFTPRL positioned in the second domain, has a relatively short sequence and is likely a β-type neuropeptide (β-NP) with an FTPRLamide at the C-terminus. The fourth domain, 9-AA, LPWIPSPLamide, has a Proline (P) instead of Phenylalanine (F) at the C-terminus of the pentapeptide, and likely corresponds to the γ-NP homologue in moths (Figs 1 and 2). Unlike moth PBAN genes the fire ant PBAN cDNA does not contain a α-NP homologue, VIFTPKLamide, which is highly conserved in moths (Figs 1 and 2). When the fire ant is compared to the honeybee (both species belong to the Order Hymenoptera) the length of the Soi-invicta PBAN gene (176-AA) is shorter than A. mellifera (195-AA). The latter is similar in size to ORFs found in moth PBAN genes (Fig. 3). The entire ORF sequence identity from the two species showed 56% similarity.

Pheromonotropic activity by Soi-invicta neuropeptides in moths

Generally, the H. zea female does not produce pheromones during the photophase or when decapitated. However, the amount of pheromone produced after injection with 3 pmol of Soi-PBAN or the three other peptides encoded from Soi-PBAN cDNA were all significantly higher than the saline injections ($P < 0.0154$), but lower than for 3 pmol synthetic Hez-PBAN (H. zea PBAN) injections (Fig. 4). The pheromonotropic activities of Soi-PBAN, β- and γ-neuropeptides (NP) were not significantly different. Soi-β-NP, which is the shortest peptide (8-AA), elicited the greatest pheromonotropic activity in the moths (Fig. 4). Soi-DH corresponded to a homologue of the diapause hormone (DH) or PGN-24 in moths and had the lowest pheromonotropic activity, but still significantly greater than saline injections. These results indicated that all putative fire ant PBAN/pyrokinin neuropeptides can stimulate pheromone biosynthesis in moths.

Discussion

Although PBAN and pyrokinin family peptides have been found independently from many insects based on their different functions, they are both characterized by a conserved pentapeptide (FXPRLamide) in their C-termini. These peptides are expected to be ubiquitous in insects and affect various physiological events, such as pheromone production and diapause during development and reproduction. However, regulation of pheromone biosynthesis by PBAN has only been determined in lepidopteran moths (Rafaeli & Jurenka, 2003). Although two new PBAN/pyrokinin-like peptides have been recently found from Hymenoptera...
Insect PBANs and Neuropeptides

<table>
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<tr>
<th>DH</th>
<th>α-NP</th>
<th>β-NP</th>
<th>PBAN</th>
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Figure 2. Comparison of PBAN and other neuropeptides in moths (top) and schematic diagram of PBAN-DH gene structures from Lepidoptera and Hymenoptera (bottom). The full PBAN genes of Lymantria dispar (Masler et al., 1994) and Pse-PT of Plutella separata (Matsumoto et al., 1992) have not been identified. Compared full or partial amino acid sequences are from lepidopteran species, Helicoverpa zea (Ma et al., 1994), Helicoverpa assulta (Choi et al., 1998), Helicoverpa armigera (Zhang et al., 2004), Heliothis virescens (Xu & Denlinger, 2003), Agrotis ipsilon (Duponnois et al., 1999), Mamestra brassicae (Jacquin-Joly et al., 1998), Spodoptera littoralis (Iglesias et al., 2002), Spodoptera exigua (Xu et al., 2007), Plutella xylostella (Lee & Boo, 2005), Ascots sellenaria cretacea (Kawai et al., 2007), hymenopteran species, Solenopsis invicta (this study), Apis mellifera (Hummon et al., 2006), and pyrokinin from coleopteran species, Tribolium castaneum (Li et al., 2008); DH: Diapause Hormone, NP: Neuropeptide, PBAN: Pheromone Biosynthesis Activating Neuropeptide.

and Coleoptera (Hummon et al., 2006; Li et al., 2008), their physiological role(s) have not been characterized. During the last four decades the fire ant has been intensively studied, due to the broad range of economic damage caused by this destructive, omnivorous species. As in other social insects the fire ant has evolved a sophisticated pheromonal communication system to maintain colony cohesiveness and sociality. Several pheromone components from fire ants have been identified (Vander Meer & Alonso, 1998). However, knowledge of pheromone production and release is still unknown, as is the role of neuropeptides in the physiology of PBAN related to pheromone production or other endoclinical processes in the fire ant. Here we report the first identification and structural characterization of a PBAN/pyrokinin family peptide from Hymenoptera, specifically, So. invicta PBAN (Soi-PBAN).

To date, PBANs from 19 species of Lepidopteran moths have been identified: super families Noctuoidea (Noctuidae: eight species; Lymantriidae: two species; Notodontidae: one species); Bombycoidea (Saturniidae: two species; Lymantriidae: one species; Bombycidae: two species); Geometridae (one species); Tortricidae (one species), and Plutellidae (one species) (Figs 2 and 5). These PBANs are expected to functionally stimulate pheromone production in different moths, as well as their own species. Although the other PBAN family peptides found in honeybees and beetles are not characterized functionally, the phylogenetic relationships of all known PBAN genes were analyzed in the present study. The phylogenetic analysis of PBAN genes matches the phylogeny based on the family level of classification and evolutionary diversity in Insecta, indicating that the neuropeptide sequences could be applied to study insect phylogenetic relationships (Fig. 5). From the PBAN phylogenetic relationship, the fire ant is closest to the honeybee within Hymenoptera followed by the beetle (Coleoptera), and then moths (Lepidoptera). The amino acid sequences from PBAN genes for So. invicta and A. mellifera showed 56% identical, which decreases to about 30% when compared to lepidopteran moths. Fire ant PBAN, Soi-PBAN (26-AA), is significantly shorter than the PBAN one species).
Fire ant PBAN

S. invicta: MI---VTRNSVNRATIV---CIMAMLLCLGRASGEYESREIGSNSSSGSSESRSSPSNDGFSC 55
A. melifera: MIGFAVIS---SFNRFTTIFVCVULLCVVYLSSYASGEYDGRDSSGSSNSN---D---RAPSNEFGSC 57

S. invicta: TDGKCICRTSQIDSASMGFPRGLGKRYKSDQELSSFESILANALGVRWAVITIPASD 115
A. melifera: TDGKCICRTSQIDTSMGFPGLRGRARRDRAKPEINSDEAFANAFEPEHWAIVITIPETE 117

S. invicta: KRQ---FQFTPLRLGSGSD---LGYG---DAYEV---DED---H---PLFVPRLGRWIPSPRGLR 167
A. melifera: KRQ---FQFTPLRLGSGEDYFYSFPGKQDEELTEQYIYPLFLASRLGRVFWTPSPRGLR 177

** Similarity of PBAN genes from two hymenopteran species, Solenopsis invicta (present study) and Apis mellifera (GenBank Accession number: NM_001110 712). The fire ant PBAN gene is about 56% similarity with honeybee. Identical amino acids in two sequences are indicated with asterisks. Dashed lines indicate gaps.

Figure 3.

Figure 4. Pheromonotropic activity of synthetic peptides deduced from Soi-PBAN cDNA and Hez-PBAN in Helicoverpa zea female moths (top) and four synthetic peptide sequences (bottom). Bars represent the means SEM of at least 5 replications. Bars with the same letters are not statistically different by analysis of Fisher PLSD (ANOVA) (P < 0.05).

Similar to leucopyrokinin (PETSFTPRL) because Glutamine (Q), Proline (P), Serine (S) and Threonine (T) belong to the same polar and uncharged amino acid residues. To date, all β-NPs discovered from insects contain the FTPRL epitope at their C-termini (Fig. 2). The pheromonotropic activity was highest for Soi-β-NP and was not significantly different from H. zea's own PBAN (Hez-PBAN, Fig. 4). Half-maximum effective concentration (EC_{50}) values of the H. zea moth α- and β-NPs binding the PBAN receptor were similar, and active at lower concentrations than any other FXPRL peptides (Choi et al., 2003). The previous and current results indicate that the C-terminal ends of α-NP (FTPKL), β-NP (FTPRL) and PBAN (FSPRL) could have similar conformational structures when bound to the Hez-PBAN receptor. This can be explained in that different amino acids are substituted, but they belong to the same functional family at the C-termini (e.g., Serine (S) and Threonine (T) belong to the polar and uncharged R group, and Lysine (K) and Arginine (R) to the non-polar and aromatic R group). Also, the lack of an α-NP in fire ants could be explained if the domains for α-NP and β-NP in the fire ant were fused together to form a single peptide (Soi-β-NP). The Soi-β-NP (PQOFTPRL) of the fire ant is similar in structure to Neb-pyrokinin-2, SVQFKPRL, identified as a flesh fly pupariation factor, which is known to accelerate pupariation (Verleyen et al., 2004). But, the physiological role of Soi-β-NP in fire ant remains to be determined. The low pheromone production induced by Soi-PBAN and Soi-γ-NP in the moth compared to Soi-β-NP could be due to a non-polar aliphatic R group, Valine (V), positioned in FVPRL of the C-terminus instead of Serine (S), and Proline (P) positioned in PSPRL instead of Phenylalanine (F).

The putative Soi-DH homologue (TSQDIASMWFGRPL) positioned in the first cleaved domain of Soi-PBAN cDNA, 15-AA, corresponds to the PBAN-encoding gene neuropeptide-24 (PGN-24), a diapause hormone (DH) found in lepidopteran moths. This peptide, with a conserved domain (33-AA) of A. mellifera and they share only 35% identity, mostly in their C- and N-terminal regions. However, the other three neuropeptide homologues (Soi-DH, β and γ) have similar sequences to the analogous peptides found in the honeybee.

The shortest and well conserved neuropeptide, α-NP (VI FTPKL), sometimes referred to as PBAN-encoding gene Neuropeptide-7 (PGN-7) is found in all moth PBAN genes and is similar to leucopyrokinin peptides, however, this peptide is not encoded from Soi-PBAN cDNA (Figs 1 and 2). Similarly, this peptide domain is also absent from honeybee and beetle PBAN genes (Hummon et al., 2006; Li et al., 2008). Leucopyrokinin is known to stimulate the contraction of cockroach hindgut muscles (Holman et al., 1986), but it is not known if the peptide plays the same role in lepidopteran moths. Soi-β-NP (8-AA), PQOFTPRL, is relatively shorter than β-NPs found in moths, and is more
WFGPRLamide sequence at the C-terminal end, was initially identified from the silkworm, where it induced embryonic diapause in *B. mori* (Imai *et al.*, 1991), but the function of this peptide is unknown in other moths. This type of peptide, however, has been found in the PBAN/Pyrokinin family of peptides identified from many insect groups indicating that the peptide could be involved in a common function related to insect development including metamorphosis. Recently, the peptide motif has been demonstrated to terminate pupal diapause in heliothine moths (Sun *et al.*, 2003; Xu & Denlinger, 2003). The DH type peptide, with a conserved WFGPRLamide sequence at the C-terminus, showed the lowest pheromontropic activity in the fire ant (this study), and the lowest activity (highest EC₅₀) of the expressed PBAN receptor when compared to the four other *H. zea* FXPRL peptides (Choi *et al.*, 2003). These results imply that the WFGPRLamide structure does not bind effectively with the PBAN receptor. The function(s) of the PBAN/pyrokinin family of peptides identified from fire ants has yet to be determined. However, from the previous fire ant PBAN immunocytochemical study (Choi *et al.*, 2009), PBAN/pyrokinin family peptides are produced from neurosecretory cells in the SG and abdominal ganglia of all fire ant adult forms and released into the hemolymph. This indicates that these peptides are likely involved in currently unknown physiological event(s) in fire ant adults.

In conclusion, we identified *So. invicta* PBAN/pyrokinin cDNA that encodes four putative peptides, Soi-DH, Soi-β-NP, Soi-PBAN and Soi-γ-NP. The cDNA sequence is phylogenetically distant from lepidopteran PBAN/pyrokinin sequences. Moth PBAN/pyrokinin genes also code for an α-NP homologue, VIFTPKLamide, which is highly conserved, but is absent from the fire ant PBAN/pyrokinin cDNA. Fire ant PBAN (Soi-PBAN) is a relatively short peptide with a low degree of similarity to PBANs identified from other insects. All peptides from Soi-PBAN cDNA have pheromontropic activity in a moth model, but their own function remains to be elucidated. Based on amino acid sequences encoded from insect PBAN genes identified, neuropeptide hormone diversity is correlated with basic taxonomical or phylogenetic classification of Insecta. This is the first neuropeptide identified and characterized from the central nervous system of Formicidae.

**Experimental procedures**

**Insects**

**Fire ants.** Field collected monogyne fire ant colonies, *Solenopsis invicta*, were collected from Gainesville area, Florida, USA, and maintained at room temperature in the laboratory using standard procedures described previously (Banks *et al.*, 1981). The fire ant brain-subesophageal ganglion (Br-SG) was dissected from female alates, and used to isolate mRNA, as described below.

**Moths.** Pupae of Corn Earworm (*Helicoverpa zea*) moths were shipped from North Carolina State University (Department of Entomology) and maintained at room temperature under L/D regimen of 15:9 until they emerged as adults. Virgin female adults
were decapitated at ca. 24-h early in their second or third photophase and were used throughout this study.

**Peptides**

Synthetic Soi-PBAN and three fire ant neuropeptides (Sigma Genosys, The Woodlands, TX, USA) and Hez-PBAN (Peninsula Laboratories, San Carlos, CA, USA) were each dissolved in lepidopteran saline (21 mM KCl, 12 mM NaCl, 3 mM CaCl$_2$, 18 mM MgCl$_2$, 85 mM trehalose and 5 mM pipes adjusted to pH 6.6 with KOH), and evaluated for pheromonotropic activity.

**Poly (A) RNA isolation and cDNA synthesis**

One hundred brain-subesophageal ganglia (Br-SGs) were dissected from fire ant female alates in an autoclaved cold hymenopteran saline (130 mM NaCl, 6 mM KCl, 4 mM MgCl$_2$, 5 mM CaCl$_2$, 160 mM sucrose, 25 mM glucose, and 10 mM HEPES, adjusted to pH 7.2 with NaOH) and stored at −80°C until use. Poly (A)* RNA was isolated from the dissected Br-SGs by Micro Fast mRNA purification kit (Invitrogen, Carlsbad, CA, USA), and used to synthesize cDNA with the GeneRacer cDNA synthesis kit (Invitrogen).

**Molecular cloning and characterization**

The synthesized cDNA above was amplified with a degenerate primer set: a sense primer, 5′-GGNATGGGTGGNCCNGNMTGNNTGNGNMG-3′ and antisense primer, 5′-CKNCNA RNCKNGNNCCRAACACATNCC-3′ based on PBAN cDNA conserved sequences deduced from other PBAN genes, using a PCR-based method. PCR was performed with the following temperature program: 5 cycles at 95°C for 30 s, 67°C for 30 s, and 72°C for 1 min, 5 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min, and 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR product was gel purified and cloned using TOPO TA cloning kit (Invitrogen) and sequenced. Based on this partial sequence, the further identification of the full cDNA of Soi-PBAN was performed using gene specific primers indicated with arrows (Fig. 1); sense primer, 5′-ATCGCCAGCGGCATG-GGTGTC CC-3′ for 3′-RACE and antisense primer, 5′-GCCAG- GAAATGGATGTCGC TC TCC-3′ for 5′-RACE with GeneRacer kits (Invitrogen). The RACE PCR products were ligated into the PCR2.1 vector from the TOPO TA cloning kit (Invitrogen) for sequencing. The obtained full-length sequence information was aligned and sequences compared with our partial sequence using Genetyx DNA software (Genetyx Co., Tokyo, Japan).

**Pheromonotropic activity**

Synthetic fire ant PBAN (Soi-PBAN) or other peptides deduced from the Soi-PBAN cDNA, or Hez-PBAN were dissolved in lepidopteran saline (1 pmol/μl), and injected (3 μl) between the fourth and the fifth abdominal segments of decapitated *H. zeas* females during mid-photophase. The *H. zeas* pheromone glands were dissected after 0.5–1 h incubation at room temperature and extracted with hexane containing (Z)-9-tetradecenal (100 ng) as an internal standard. A GC 6890N (Agilent Technologies) equipped with a capillary column (30 m × 0.25 mm, DB-23, J&W) was used to measure the amount of pheromone. The oven temperature was programmed at 80°C for 1 min, then 10°C/min to 230°C and held for 8 min. The results were analyzed by non-parametric analysis as ranks (Fisher PLSD, ANOVA) using STATVIEW 5.0 software.

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**References**


