



## PBAN gene architecture and expression in the fire ant, *Solenopsis invicta*

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### ABSTRACT

The PBAN/pyrokinin peptides are a major neuropeptide family characterized by a common FXPRLamide at the C-termini. These peptides are distributed ubiquitously in the Insecta and are involved in many essential endocrine functions, e.g. pheromone production. We report the gene architecture of the fire ant *Solenopsis invicta* PBAN (Soi-PBAN) gene, including the exon and intron boundaries. Furthermore, we quantified expression of the Soi-PBAN mRNA in the head, thorax and abdomen of the fire ant. The Soi-PBAN gene is comprised three exons and two introns, all composed of 13,358 nucleotides, which is 2–4 times larger than lepidopteran PBAN genes. The overall pattern of the PBAN immunoreactive neuron number and localization was similar for female and male alates, inseminated female delates, workers and queens. The Soi-PBAN mRNA expression level was highest in the head, followed by the thorax, and abdomen of adult ants. Expression in the abdominal tissues was expected to be similar to the head, or at least higher than thorax because strong PBAN immunoreactive neurons were detected previously in brain–subesophageal and abdominal ganglia. This result suggests that another FXPRL gene could be dominant in the abdomen rather than Soi-PBAN gene.

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### 1. Introduction

Insect neurohormones function as intercellular communicators that regulate a variety of physiological and behavioral events during development and reproduction. One major class of neuropeptide hormones is the pheromone biosynthesis activating neuropeptide (PBAN)/pyrokinin peptide family, which is defined by a common FXPRLamide or similar amino acid fragment at the C-terminal end. The PBAN/pyrokinin family of peptides is produced in the central nervous system (CNS) of insects, with homologous peptides and receptors in vertebrates. These peptides have been conserved throughout evolution, because they are expected to be found in all insects (reviewed by [Rafaeli, 2009](#)), in Crustacea ([Torfs et al., 2001](#)), as well as in *Caenorhabditis elegans* ([Lindemans et al., 2009](#)). PBAN has been reported to regulate pheromone biosynthesis in a variety of moths. Pyrokinins are peptides that activate visceral muscle contraction of mid- and/or hind-guts in a variety of insects. In addition, the family of peptides containing the FXPRL sequence in the C-terminus have other physiological roles in insects, including egg and pupal diapause, cuticle melanization, and puparium formation (reviewed by [Rafaeli, 2009](#)).

Two decades ago, the first PBAN molecule identified was a 33-amino acid peptide from *Helicoverpa zea*, Hez-PBAN (*Helicoverpa zea* PBAN) ([Raina et al., 1989](#)). Subsequently PBAN amino acid sequences were determined through direct isolation and purification of peptides, or DNA cloning methods ([Choi et al., 2010](#); [Rafaeli, 2009](#)). The results yielded additional PBAN encoding genes from 18 lepidopteran moths and several non-lepidopteran insects (summarized by [Choi et al., 2010](#)). In addition to PBAN, a diapause hormone and three additional FXPR/KL neuropeptides (NPs:  $\alpha$ ,  $\beta$ ,  $\gamma$ ) were deduced from the same gene and are well conserved in moths. The neurohormonal action of PBAN for pheromone biosynthesis in lepidopteran moths has been well studied ([Rafaeli, 2009](#); [Rafaeli and Jurenka, 2003](#)), but the physiological functions of PBAN peptides in the other species are unknown. PBAN is synthesized in the subesophageal ganglion (SG) and is released into the hemolymph via the corpora cardiaca, a neurohemal organ, in moth species.

The red imported fire ant, *S. invicta*, is among world's 100 worst invasive alien species ([Lowe et al., 2000](#)). The affected economic sectors are broad ranging and include households, electric service and communications, agriculture, schools and recreation areas ([Lard et al., 2006](#)). Workers (sterile females) may number 250,000 in mature colonies, female alate sexuals (winged female potential queens) and male alate sexuals (winged males) represent the reproductive offspring of the colony queen(s). The fire ant is probably the most studied invasive ant species in the world and a

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great deal is known about the pheromone systems used to reduce reproductive competition, recruit resources, and maintain colony social structure and territoriality (Vander Meer and 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 endocrinal processes during development. Recently, we demonstrated the presence of PBAN/pyrokinin immunoreactive neurons in the central nervous system of the fire ant, *S. invicta*, and identified and characterized PBAN and three additional F/PXPRL peptides from the *Soi*-PBAN cDNA (Choi et al., 2009; Choi and Vander Meer, 2009). In the present study we have mapped the *Soi*-PBAN gene structure and demonstrated similar PBAN immunoreactive neuron patterns from the different sexual forms, including female and male alates, workers and queens. We determined the tissue expression level of the *Soi*-PBAN mRNA using RT- and Q-PCRs from the head, thorax, and abdomen. We also discovered the PBAN gene structure and immunoreactivity in the different sexual forms, and discuss the possibility of different genes encoding PBAN/pyrokinin family like peptides in the central nervous system of the fire ant.

## 2. Materials and methods

### 2.1. Fire ants

All *S. invicta* samples used in this study were from monogyne (single egg laying queen) colonies collected from the Gainesville area in Florida by excavation of nests or by establishing colonies directly from newly mated queens. Colonies collected by excavation were removed from the soil by floating out the workers and brood as previously described (Banks et al., 1981). All colonies were fed crickets and 10% sugar solution absorbed onto wads of tissue and maintained under standard laboratory conditions.

### 2.2. *Soi*-PBAN genomic DNA

DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Sequencing libraries were constructed according to protocols recommended by the respective manufacturers. A 454 shotgun sequencing library and an Illumina 330bp-insert paired-end library were built from the DNA. Details regarding the sequencing of libraries and subsequent assembly of genomic data are summarized in Wurm et al. (2010). The sequences of *Soi*-PBAN cDNA and translated amino acid (Choi and Vander Meer, 2009) were used for blast searching the fire ant, *S. invicta*, genome database (Wurm et al., 2010).

### 2.3. Immunocytochemistry

The distribution of PBAN-like immunoreactivity in the CNS of different fire ant sexual form was observed using whole-mount immunocytochemistry as described previously (Choi et al., 2009). No staining was observed when PBAN and leucopyrokinin were used to preabsorb the antisera.

### 2.4. Reverse transcriptase (RT)-PCR

Total RNA was isolated from head, thorax, and abdomen of female alates using the PureLink<sup>TM</sup> RNA Kit (Invitrogen, Carlsbad, CA, USA). The total RNA (50 ng) was used to amplify a 501-bp DNA fragment of *Soi*-PBAN with a *Soi*-PBAN specific primer set (5'-AGGAATTCGGTAAATCGTGC-3' and 5'-GTTGCGTAATTGACGTCGG-3'). A 100-bp fragment of the fire ant 18S rRNA gene was also amplified for a positive control using primers (5'-CCCGTAATCG-

GAATGAGTACACTTT-3' and 5'-ACGCTATTGGAGCTGGAATTACC-3'). The one-step RT-PCR was performed as follows: 1 cycle at 50 °C for 30 min, 40 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min using RT/Taq mix polymerase (Invitrogen, Carlsbad, CA, USA). Then PCR products were checked for the amplification by 1.5% agarose gel electrophoresis following ethidium bromide visualization under a UV light. Expected PCR products were purified and cloned, then confirmed by DNA sequence at ICBR of University of Florida.

### 2.5. Quantitative (Q)-PCR

Total RNA samples (50 ng RNA) from different tissues were treated with 1 unit of DNase (Invitrogen, Carlsbad, CA, USA) for 15 min at 25 °C. The reaction was terminated by the addition of 1 μl of 25 mM EDTA and the sample heated to 65 °C for 10 min. Expression of the PBAN gene was normalized to the 18S gene. The DNase-treated RNA was reverse transcribed (55 °C for 30 min) in separate tubes using the PBAN-specific (5'-AATTCCTGTTTCTCATCTGACTTGATCGCTTTCCTA) and 18S-specific (5'-GCTAGCTGGCATCGTTTATGGTTAG) oligonucleotide primers using superscript III according to manufacturer's directions (Invitrogen, Carlsbad, CA, USA). One quarter of the cDNA reaction was used subsequently as template in QPCR using oligonucleotide primers for *S. invicta* PBAN (5'-AATTCCTGTTTCTCATCTGACTTGATCGCTTTCCTA and 5'-ATCGTGCAACTATCGTCTGCATTATGGCC) and 18S rRNA (5'-CCCCTAATCGGAATGAGTACACTTT and 5'-ACGCTATTGGAGCTGGAATTACC) with SYBR green dye in separate reactions. The PBAN QPCR was conducted as follows: 1 cycle of 50 °C for 2 min, 1 cycle at 95 °C for 2 min, 35 cycles of 95 °C for 15 s, 65 °C for 15 s, 72 °C for 1 min. The 18S rRNA QPCR was conducted as follows: 1 cycle of 50 °C for 2 min, 1 cycle at 95 °C for 2 min, 35 cycles of 95 °C for 15 s, 54 °C for 15 s, 72 °C for 1 min. Plasmid constructs of the PBAN and 18S gene were serially diluted and run alongside the unknowns. These standards were used to construct a standard curve for each gene Critical threshold (Ct) versus log PBAN or 18S gene. The number of copies of each respective gene was calculated from the standard curve for each treatment (tissue). The PBAN gene copy was normalized to the 18S gene by division (PBAN copies/18S copies). The PBAN gene copy number was calibrated to the lowest target (tissue). All QPCR experiments were verified by conducting dissociation analysis.

## 3. Results and discussion

### 3.1. Architecture of the fire ant PBAN gene and comparison

The entire *S. invicta* PBAN gene is composed of 13,358 nucleotides (Fig. 1 and see Supplementary data). The gene is comprised three exons (94, 242, and 195 nucleotides translated, respectively) interrupted by two large intron sequences; intron 1 contains 8727 nucleotides and intron 2 contains 3906 nucleotides (Figs. 1 and 2). Untranslated regions (UTR) were observed at the 5' end of exon 1 and 3' end of exon 3. Nucleotide sequences of the three concatenated exons (727-nt) are identical with the *Soi*-PBAN cDNA identified previously (Choi and Vander Meer, 2009). *Soi*-PBAN cDNA encodes a DH-homologue peptide, which is translated from the second exon (242-nt), and the other peptides, β-NP, γ-NP and *Soi*-PBAN, are translated (195-nt) from the third exon. The *Soi*-PBAN gene has three exons and relatively large intron gaps compared to lepidopteran PBAN genes that consist of six exons (Jing et al., 2007; Xu et al., 1995; Zhang et al., 2005). So, the fire ant PBAN gene is 2–4 times larger than moth PBAN genes. A search of two hymenopteran species, *Apis mellifera* (accession No: NM\_001110712) and *Nasonia vitripennis* (accession No.: NM\_001167725)

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-323 gcgcacaagaatcgcatacgcagctttcattcactgaaaccgaaagaattaatgaatagctcaagttttttccgagaaacgaagatagaggctggtcctgtgc
gtgggagcgacattcccaagactctcgggtgtgaaacctggatccaggaaacctcattgctgatgtaggacagtcagccgagatttcaataaagacgaatcgtttctcgc
gacgatcgtgattggtctcagaggggatcgcaaaaacaacgtcgacgttcttgcaacgacgttaactccgacgcacctctataaaaccccaaggatcacctgctgtc

+1 GAACATTACTGCTGCGGAGTGTGAGGAGCGACGTGCCGAG 44
ATGATCGTCACCAGGAATCCGTTAATCGTGCAACTATCGCTGCATTATGCCCATGCTGCTCTGCTGGGGTCTCGCGCTTCTGGTGAATACG 138
M I V T R N S V N R A T I V C I M A M L L C L G S R A S G E Y E 32

ggtaataccgattttaatacagcacgtaattattaaaaatgtacatctattttttaattgttaatttcatagaaaaaagggtgatattacagggactcattta 245
catttcatttaacaactttgttaataatcaaaattacatattgaaacttaacaattatactgatcaaaacggtgtttaaataagatttcaaaactccaagttatgtaata 352
tttattac ..... ttattatttttaaaaaataaatgtaatacccaagaatcctaatttttatttataaacccgctgtttaca 8863

AATCAAGGGAATGGCTCTAACGGCGGATCGAGTGAGAGTAGATCTCCGAGCAACGATTTGGTTCCTGTATCGACGGCAATGTATCAAGCGCACCTCGCGATATCG
S R E I G S N G G S S E S R S P S N D F G S C I D G K C I K R T S Q D I A
CCAGCGCATGTGGTTCGGCCCGCTTTAGGAAAGCGATACAAGTCAGATGAGAAACAGGAATTGAGTTCGAGATCGAGATCCTTGCAGACCGCTTAGATGGCGTGCCTT
S G M W F G P R L G K R Y K S D E K Q E L S S E I E I L A N A L D G V R W
GGCGGTTCATCACAATCCG 9105
A V I T I P 112

ggtgagaatcaaaatTTTTTaaagcattcatcactaggcagattaaagcgatactggaccgagatttctgtacgcaattattttgaacagcctaactcttttaa 9211

ttaaatttacagatttcaaaatccttttttacagtttaaaaaagtgaacaaaatataattgCGgaagcaataatttaataacgaaaaacaaaatagattaaaaaa 9317
gttagttta ..... taacaaacaaaataatttca 13011

GCTAGTGACAAGAGACAGCCTCAATTTACTCCGCTCTGGGACGAGGATCAGGTGAGGACCTCTCTTACGGAGACGCATACGAAGTCGACGAAGACGACCATCCATTATTC
A S D K R Q P Q F T P R L G R G S G E D L S Y G D A Y E V D E D D H P L F
GTGCCCCGTTTAGGACGACGGCTTCCTGGATACCATCACCAGACTCGGACGTCAATTACGCAACGTATTACGAAAACCTA TAG 13206
V P R L G R R L P W I P S P R L G R Q L R N V L R K L * 176

GCAACTATCCGAGTTAGAAGATCATTGAAAATTTGGAGGAAAAAGTATGATTTTCGTAGATTTGCGCGATTGTGACGATCTTGATGAAACGATAACCCAGTTATGGCATGAT
TATGTGAAGATATAGAGA AATAAA GTATATTGTATTGCG 13358

aaactaactgtcatcttttcttagtatcttaaaaatagaaaatataactaaaaatgttttacattgtttaacccttaacacacaggtgccatagagatacagaaat
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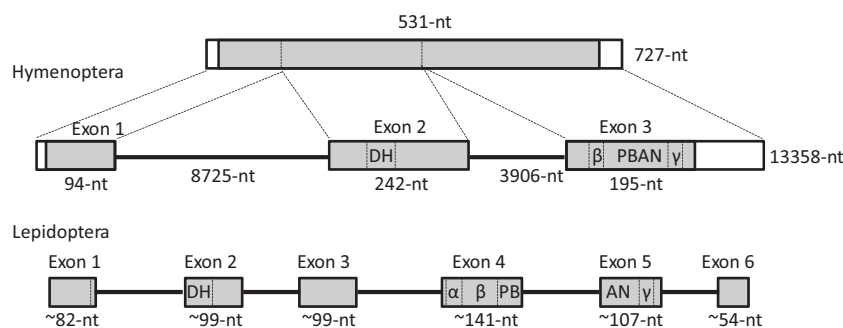
**Fig. 1.** Nucleotides and amino acid sequences of the fire ant PBAN gene. The putative *Soi*-DH, PBAN,  $\beta$  and  $\gamma$  neuropeptides (underlined) are positioned between six predicted endoproteolytic cleavage sites indicated by bold italic. The gaps of intron nucleotides indicated with dot-shade lines can be found in [Supplementary data](#). Expected TATA box as promoter binding site, the first ATG as the initiation codon, TAG as the termination codon, and AATAAA as the polyadenylation signal are indicated in boxes.

genomic DNAs from the NCBI database revealed that these PBAN genes are also composed of three exons as in the fire ant PBAN gene, but contrary to the fire ant PBAN gene they contain very short intron sequence gaps. A comparison of Hymenoptera and Lepidoptera PBAN genes suggests that exon 1 for both translates the signal peptide. The hymenopteran exon 2 and 3 corresponds to moth PBAN gene exons 2–3 and exons 4–6, respectively (Fig. 2). The reduced or fused exons, and intron loss in the hymenopteran PBAN genes may be attributable to the evolutionary process.

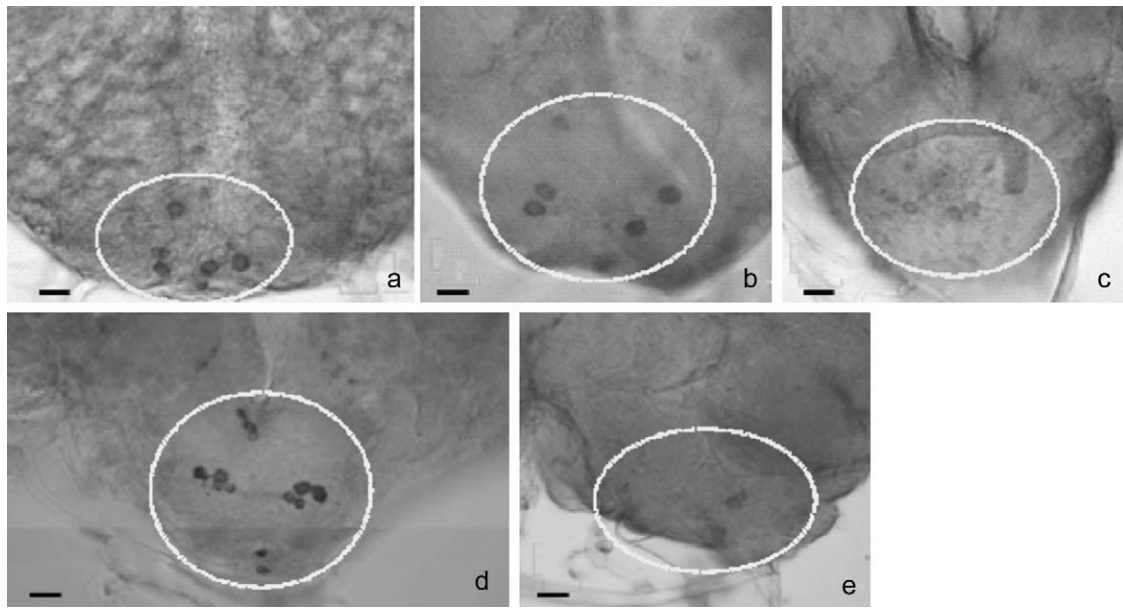
### 3.2. PBAN-like immunoreactivity of different sexual forms and worker ants

The localization of *S. invicta* PBAN (*Soi*-PBAN) immunoreactive neurons was initiated to compare results for female and male alates, unseminated female delates, workers and queens. Three clusters of neurons from queen Br-SG were previously reported

(Choi et al., 2009). In this study, the overall pattern of the PBAN immunoreactive neuron number and localization was similar in each of the female and male categories evaluated (Fig. 3). This result was expected because the pheromonotropic activity of Br-SG extracts from the fire ant female and male categories were not significantly different (Choi et al., 2009). There are three cell clusters in the subesophageal ganglion (SG), two pairs in the thoracic ganglia, and three pairs are found in the abdominal ganglia (AG) (Fig. 4). Strong PBAN immunoreactivity from the post-translated PBAN-like peptide was always detected in Br-SG, as well as the abdominal ganglia from all the adult forms (Fig. 4). We observed well developed perisymphatic organs (PSO), which are neurohemal organs associated with the PBAN-like immunoreactive neurons in each AG that function to release neuropeptide into the hemocoel (Fig. 4). Therefore, PBAN-like peptides detected in AG neurons are expected to be synthesized in the AG neurons and released into the hemocoel via the PSO, as opposed to PBAN-like



**Fig. 2.** Schematic diagram of the fire ant PBAN (*Soi*-PBAN) cDNA (top) and gene architecture (middle), and lepidopteran PBAN gene (bottom). The nucleotide numbers of each exons in lepidopteran PBAN represent means of three moth species. DH: diapause hormone homologue;  $\alpha$ ,  $\beta$  and  $\gamma$ : neuropeptides encoded from the cDNA; PBANs are translated from exon 3 in Hymenoptera, and exons 4 and 5 in Lepidoptera, respectively. The shaded area represents *Soi*-PBAN coding regions. Open areas represent untranslated regions.



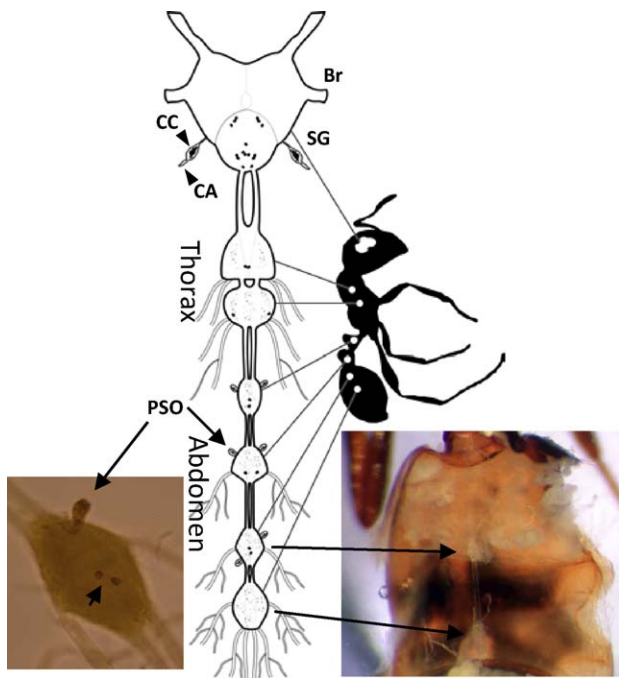
**Fig. 3.** Photomicrographs of PBAN-like immunoreactivity found in the Br-SG of the fire ant sexual adults, female alate (a), female dealate unseminated (b), male alate (c), queen (d) and worker (e). Immunoreactive neurons are indicated with circles in each adult SG area.

peptides synthesized in the Br-SG and transported to the AG. However, a number of reports regarding PBAN gene mRNA expression measured by RT-PCR in the abdominal nerve tissues showed that gene expression was absent or barely detectable in several moth species (Lee and Boo, 2005; Sun et al., 2003; Wei et al., 2004, 2008; Xu and Denlinger, 2003, 2004). In this study, we determined the PBAN mRNA transcription level and quantified the

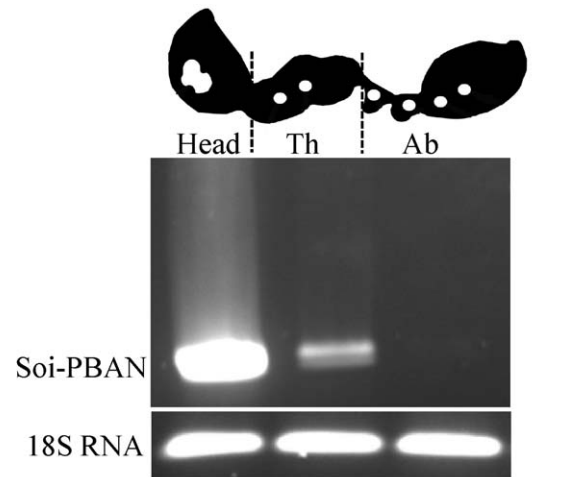
gene copies in abdominal tissue, as well as other tissues, using two different PCR amplification methods.

3.3. *Soi-PBAN* expression from different tissues

Quantification of *S. invicta* PBAN transcripts from head, thorax and abdominal tissues of female adults revealed a specific expression by RT- and Q-PCRs (Fig. 5). As anticipated, the strongest transcriptional signal and maximum number of *Soi-PBAN* gene copies ( $1990 \pm 280$  copies/ng RNA) were detected in the head (Fig. 5) indicating that the gene products including *Soi-PBAN* are actively



**Fig. 4.** Schematic drawing of fire ant central nervous system (CNS) and PBAN-like immunoreactivity neurosecretory cells, and photomicrographs of abdominal ganglion (left) and anatomical dissection (right). The ganglia in the ventral nerve cord contained five pairs of PBAN-like immunoreactive neurons (arrowhead), and three pairs are found in the abdominal tissue. Br: brain; SG: subesophageal ganglion; CC: corpora cardiaca; CA: corpora allata PSO: perisymphatic organ. The schematic drawing was adapted from Choi et al. (2009).



tissue	Head	Thorax	Abdomen
copy	1990±280	149±47	6±2

**Fig. 5.** Schematic drawing of the fire ant tissues for *Soi-PBAN* gene mRNA expression measured by RT-PCR (upper) and Q-PCR (lower). Same amount of total RNAs from head, thorax (Th) and abdomen (Ab) were amplified by RT-PCR and gene copies were quantified by Q-PCR ( $N = 3$ ). Tissue expression of the fire ant PBAN gene was investigated by RT-PCR and was repeated two times with similar results. The fire ant 18S rRNA gene was amplified for a positive control.



synthesized in this area. This result supports the strong PBAN immunoreactive response detected from the fire ant Br-SG in the present and previous studies (Choi et al., 2009). SoI-PBAN gene mRNA transcription and gene copies in the thoracic tissue were lower ( $149 \pm 47$  copies/ng RNA). However, abdominal tissue gave minimal amplification ( $6 \pm 2$  copies/ng RNA) of the SoI-PBAN gene (Fig. 5). This result contradicts the strong PBAN-like immuno-response observed from abdominal neurons and the associated PSO in the AG (Fig. 4). If these peptides were from only SoI-PBAN gene products, the gene expression and copy number in abdominal tissue would be expected to be at least similar to or higher than the thorax tissue. Therefore, these results suggest that a FXPRL peptide is dominantly expressed in the abdomen that is derived from a gene other than the SoI-PBAN gene. The FXPRL (= PBAN/pyrokinin) family of peptides is translated from specific genes in insects. Thus far, only two gene families are known to produce FXPRL peptides: the PBAN and the capability (CAPA) genes. The PBAN gene transcripts include four or five species of FXPRL peptides, including DH,  $\alpha$  (in lepidopteran moths only),  $\beta$ , PBAN, and  $\gamma$ -neuropeptides (NP) (Choi and Vander Meer, 2009). The CAPA gene encodes one FXPRL neuropeptide with a very conserved motif, WFGPRL at the C-termini (Predel and Wegener, 2006).

PBAN anti-sera reacts with any C-terminus FXPRL peptide sequence regardless of what gene is producing the peptide, thus the cross-reactivity does not allow the determination of the number genes responsible for the observed reactivity. Interestingly, the immunocytochemistry and the transcription studies strongly suggest that PBAN/pyrokinin-like peptides are encoded from multiple genes. Many CAPA gene encoding FXPRL peptides have been isolated from the abdominal neurohemal organs of several insect groups (Predel and Wegener, 2006). Two different genes express FXPRL peptides in the *Drosophila* CNS. They are known as Capa and Hugin genes (Choi et al., 2001; Kean et al., 2002; Meng et al., 2002). The Capa gene encodes one FXPRLamide pyrokinin (PK-1 = Cap2b-3). The equivalent gene in Lepidoptera is the Hugin gene that encodes one FXPRLamide pyrokinin (PK-2). Though PBAN/pyrokinin family peptides, characterized by the conserved penta-peptide (FXPRLamide) at the C-terminus, have been found independently in various insect groups, it is clear that the PBAN gene is well conserved in Insecta, and probably extends to the entire Arthropod phylum. In spite of several different functions reported for these peptides during insect development, the mode of action and functions for the specific peptides produced by *S. invicta* await further research.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2010.10.008.

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