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RAPID COMMUNICATION

Similar Differentiation Patterns Between *PBP* Expression Levels and Pheromone Component Ratios in Two Populations of *Sesamia nonagrioides*

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Abstract Pheromone-binding proteins (PBPs) are thought to contribute to the specificity of the pheromone detection system through an initial selective binding with pheromone molecules. Here, we report different expression levels of *PBP* transcripts in the antennae of two populations of the stemborer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), one collected in Europe and one in sub-Saharan Africa. The three *PBP* transcripts previously identified in this species were found to be expressed in both male and female antennae. Whereas *PBP3* did not show any differential expression, *PBP1* and *PBP2* appeared to be expressed differently according to the population origin and sex. Simultaneously, we measured and compared the ratio of the three components of the *S. nonagrioides* pheromone blend (Z11-16:Ac; Z11-16:OH; Z11–16:Ald) in females of the two populations. The ratio of Z11-16:OH and Z11–16:Ald varied significantly according to the population origin of this species. Cluster analyses revealed similar differentiation patterns between *PBP1* and *PBP2* expression levels and the ratios of Z11-16:OH and Z11–16:Ald. Different female sexual signals may thus correspond to different male reception systems, which are adjusted by the *PBP* expression levels, thereby ensuring optimal communication within populations.

Keywords Stemborer · Lepidoptera · Noctuidae · Pheromone-binding proteins · Quantitative PCR · Sex pheromones

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Introduction

Chemosensory functions are essential for insects to interact with their environment and with their congeners. Among the semiochemicals involved in those interactions, sex pheromones are intraspecific chemical signals that are particularly important for mating processes. In many insects, detection of sex pheromones takes place in chemosensory sensilla on the antennae. Within these sensilla, sex pheromone molecules are detected by highly-specific pheromone receptors in the dendritic membrane of olfactory sensory neurons. In most Lepidoptera, sex pheromones consist of a blend of molecules of fatty acid origin. They are highly hydrophobic and poorly soluble in the sensillum lymph. A class of small soluble proteins (14–15 kDa) mediates solubilization of pheromone molecules at the sensilla surface and transport to the receptor neurons. These proteins are named pheromone-binding proteins (PBPs) and multiple PBPs are expressed in a single species (Leal 2013). Therefore, the interaction of PBPs with pheromone components is an important aspect of the intraspecific communication systems of insects.

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The stem borer *Sesamia nonagrioides* Lef (Lepidoptera: Noctuidae) is a major pest of maize in Mediterranean Europe. The species has a fragmented distribution from Mediterranean Europe to West and East Africa. Moyal et al. (2011), using mitochondrial and nuclear markers, demonstrated that the European and African populations belong to the same species and that the European population originated in both West and East Africa.

The pheromone blend from European *S. nonagrioides* populations is a mixture of two main components, (*Z*)-11-hexadecenyl acetate (Z11-16:Ac) and (*Z*)-11-hexadecen-1-ol (Z11-16:OH), and one minor component, (Z)-11-hexadecenal (Z11–16:Ald) (Krokos et al. 2002). It is hypothesized that differences in pheromone composition probably occur between European and African populations since they are well separated as populations, and they have been reproductively isolated *in natura* for about 100,000 years (since the last interglacial period); in fact, the genitalia vary slightly between the two populations (Moyal et al. 2011). We tested this hypothesis by investigating two populations, one collected in Europe and one in sub-Saharan Africa. We studied both the expression levels of the three genes coding for the three PBPs reported by Glaser et al. (2013) and the composition of the pheromones. Any similar differentiation patterns between *PBP* expression levels and pheromone component ratios in these two *S. nonagrioides* populations could account for differences in their intraspecific communication.

Methods and Materials

Insect Collection and Rearing For the European population, *S. nonagrioides* larvae were collected on *Zea mays* (Monocot, Poaceae) in Rieumes (Southwest of France, 43°22′N, 1°11′E, 300 m a.s.l.) during the winters of 2011–2012, and are referred to as the French population. For the African population, larvae were collected on *Typha domingensis* (Monocot, Typhaceae) in Makindu (Eastern Kenya, 1°29′S, 37°16′E, 990 m a.s.l.) several times in 2011 and 2012 and are referred to as the Kenyan population.

To eliminate any possible effects of differences in environmental factors such as temperature and humidity, and in stress effects due to shipping, the two populations of *S. nonagrioides* were reared in the same laboratory and conditions at 24.4± 0.7 °C, 54.4±5.8 % relative humidity (means±SD), and an L16:D8 h reversed photoperiod for three generations. Larvae were reared on a modified artificial diet from Poitout and Bues (1970). For each population, 2-d-old virgin males and females were used for molecular analyses, and 2-d-old virgin females for pheromone analyses.

Molecular Analyses (qPCR) Total RNAs were extracted from antennae dissected from male and female adults coming for three different pools of pupae from each population, since all individuals did not pupate at the same time (biological replicates $N=3$ for each population). RNAs were extracted with the RNeasy MicroKit (Qiagen, Hilden, Germany) which included a DNase treatment. cDNAs were synthetized using the advantage RT-for-PCR Kit (Clontech, Mountain View, CA, USA).

Gene-specific primers for *S. nonagrioides PBP* genes were designed from the sequences identified previously by Glaser et al. (2013) These were SnonPBP1-F (5′-GGGCGAACAC ATAATGCAAG-3′), SnonPBP1-R (5′-CCTTTCCATGGT GCATCTTC-3′), SnonPBP2-F (5′-TATGGAGACAATCC GCGAAC-3′), SnonPBP2-R (5′-CAAGACTTCTCCCACG ATGAG-3′), SnonPBP3-F (5′-TCAAGGTTTTGGAGGA GTGC-3′), and SnonPBP3-R (5′-CAAAAGGTCCAGCTTC TTGC-3′). Gene-specific primers for the reference genes *rpL13*, *rpL17*, and *rpS3* were designed from conserved regions after alignment of several Lepidoptera homologous genes, i.e., SnonrpL13-F (5′-TACCGCAGAAAACAAAAC AGGAT-3′), SnonrpL13-R (5′-GCGGGGTTAAGACCAG ATGC-3′), SnonrpL17-F (5′-TTTGGCACCACACAAG GTC-3′), SnonrpL17-R (5′-GGCACGGTATGTGCGTCTG-3′), SnonrpS3-F (5′-GCTGAATCGCTCAGATACAAACT-3′), and SnonrpS3-R (5′-GCTTGCCAGAGACTACCACCT-3′), yielding PCR products ranging from 100 to 200 bp.

qPCR mix was prepared in a total volume of 12 µl with 6 µl of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 3 µl of diluted cDNA (or water for the negative control or RNA for controlling the absence of genomic DNA) and 200 nM of each primer. qPCRs were run using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) as follows: 95 °C for 3 min, 40 cycles of 20 sec at 95 °C, 15 sec at 58–62 °C (depending on the primer pair), and 20 sec at 72 °C. To assess the specificity of the PCR reactions, a dissociation curve of the amplified products was obtained by gradual heating from 50 °C to 95 °C at 0.2 °C/s. Standard curves were generated by a five-fold dilution series of a cDNA pool evaluating primer efficiency E ($E=10(-1/\text{slope})$). For each case, the presence of only one amplified product was verified. All reactions were performed in duplicate on the three biological replicates. *PBP* expression levels between male and female antennae and between the French and the Kenyan populations were calculated relatively to the expression of the reference genes using the ΔΔCq method (ABI User Bulletin [11–15]).

Pheromone Analyses Female *S. nonagrioides* moths from both populations were observed during the scotophase. When females remained in the calling posture for 10 min, the glands were dissected and used for solid-phase microextraction (SPME) collections using the method developed by Frérot et al. (1997), according to the Ameline (1999) protocol. For each population, pheromone was collected from three batches of five glands using a Supelco™ SPME (Bellefonte, PA,

USA) holder equipped with a fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DBV/CAR/PDMS; StableFlex, 50/30 μm). Collections were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) using a Bruker Scion 436-GC linked to a Bruker Scion SQ detector. The fused silica capillary column (30 m× 0.32 mm i.d.) was coated with Rxi®-5Sil MS (0.25 μm film thickness, Restek, France), the column temperature was programmed from 50 to 300 °C at 8 °C/min and carrier gas was helium N60 at constant flow of 2 ml/min. The fiber was subjected to thermal desorption in the split/splitless injector at 250 °C for 30 sec. Mass spectra were recorded in the electron impact mode (EI) at 70 eV, and the identity of each component was validated by retention time and mass spectra comparison with synthetic reference compounds. The relative abundances of each component were determined.

Statistical Analyses All statistical analyses were performed in R (R Core Team 2013). The relative abundances of pheromone components were transformed to √x. Data normality and variance homogeneity of the *PBP* and pheromone data sets were first verified by Shapiro and Bartlett tests, respectively. The means of *PBP* transcript expression levels were compared by Student's *t*-test following two-way analysis of variance (ANOVA2) considering sex and population as factors. The means of relative abundances of each pheromone component were compared between populations by Student's *t*-test. The inter-population differentiation patterns of male *PBPs* and female pheromones were compared with two cluster analyses performed with the UPGMA (unweighted pair group method with arithmetic means) clustering method on a Euclidean distances matrix (R-package APE) (Paradis et al. 2004) on two data sets: (i) expression levels of male *PBPs*; (ii) relative abundance of female pheromone compounds. For each cluster analysis, *PBPs* or compounds specific and frequent in each population were determined by the indicator value (IndVal) method (Dufrêne and Legendre 1997). The value given is the product of relative abundance and relative frequency of occurrence of a *PBP*/compound within a group; a high value is obtained when the *PBP*/compound is specific and reproducible in a particular group compared to the whole set of observations. The statistical significance of a *PBP*/compound as an indicator was evaluated with a randomization procedure.

Results and Discussion

In addition to two *PBPs* (named *PBP1* and *PBP2*) described by de Santis et al. (2006) in *S. nonagrioides,* we recently identified a third one using transcriptome sequencing, named *PBP3* (Glaser et al. 2013). The transcriptome was obtained from a mix of the two populations investigated here, and no difference in the three *PBP* sequences could be detected. Interestingly, although some polymorphisms could be found in the genomic sequences of PBPs between the pheromone strains of the European corn borer, most cause no change in amino acids (Willett and Harrison 1999), as observed here. An increased rate of non-synonymous substitutions was observed in the Asian corn borer, *Ostrinia furnicalis,* which uses a different pheromone blend (Allen and Wanner 2011). Our results thus show that the two *S. nonagrioides* populations, although geographically separated, belong to the same species. Using qPCR, we confirmed the expression of the *S. nonagrioides PBP* genes in both male and female antennae (Table 1), as previously suggested by RNAseq analyses (Glaser et al. 2013) and RT-PCR (de Santis et al. 2006). Within each population, the expression levels of *PBP1* and *PBP2* were higher in males than in females whereas *PBP3* was equally expressed in both sexes, whatever the population (Table 1). The expression of PBPs in antennae of both sexes has already been observed, particularly in noctuids (Acin et al. 2009; Callahan et al. 2000; Liu et al. 2013; Konstantopoulou et al. 2006; Maïbèche-Coisné et al. 1997; Zhang et al. 2011), and this suggested that both males and females detect the sex pheromone emitted by the female; females may use this signal to detect competing conspecific females and space themselves in the environment relative to one another, or to detect their own production for quantity adjustment.

We also demonstrated that, among the *PBP* genes expressed in *S. nonagrioides*, *PBP1* and *PBP2* were expressed differently according to the population origin whatever the sex, whereas both populations expressed similar level of *PBP3* transcripts (Table 1). For both sexes, *PBP1* transcripts appeared to be more expressed in the French population, whereas *PBP2* was more expressed in the Kenyan population.

GC/MS analyses for the three components of the *S. nonagrioides* pheromone blend, Z11-16:Ac, Z11-16:OH, and Z11-16:Ald, showed that Z11-16:Ac was present in similar amounts in the pheromone blends of both populations. Z11-16:OH was absent in the Kenyan population, and Z11-16:Ald was more abundant in the Kenyan than the French population (Table 1).

We, thus, demonstrated differences in both the expression levels of *PBPs* and pheromone blend composition between the two *S. nonagrioides* populations. If PBPs do participate in pheromone component discrimination, one would expect a relationship between *PBP* expression levels and pheromone composition. This was tested by cluster analyses (Fig. 1). For each group, the IndVal methods revealed one significant and one indicator *PBP*/compound association (Fig. 1); while the French groups were characterized by *PBP1* (IndVal value= 0.74, *P*-value<0.05)/Z11-16:OH (IndVal value=1, *P*-value< 0.05), the Kenyan groups were distinguished by *PBP2* (IndVal

Table 1 Expression levels of *PBP1*, *PBP2,* and *PBP3* in *Sesamia nonagrioides* male and female antennae from Kenyan and French populations after qRT-PCR analyses and abundance percentages of the three pheromone components emitted by *Sesamia nonagrioides* females from the Kenyan and the French populations after GC/MS analyses (means[a]± SD, *N*=3)

	Population	Sex	Expression level		Population	%
PBP1	Kenyan	Male	0.36±0.05 a	Z11-16:Ac	Kenyan	63.2±12.0
	French	Male	1.02±0.25 b		French	70.0±7.2
	Kenyan	Female	0.05±0.01 a			
	French	Female	0.11±0.04 a			
PBP2	Kenyan	Male	1.45±0.21 c	Z11-16:OH	Kenyan	0 a
	French	Male	1.02±0.25 b		French	13.8±9.8 b
	Kenyan	Female	0.23±0.03 a			
	French	Female	0.09±0.02 a			
PBP3	Kenyan	Male	1.11±0.19	Z11-16:Ald	Kenyan	36.7±11.9 b
	French	Male	1.01±0.18		French	16.2±6.0 a
	Kenyan	Female	1.17±0.63			
	French	Female	1.02±0.16			

[a] Means with different letters indicate statistical differences at 5 % level according to the Student's *t*-test following ANOVAs

value=0.59, *P*-value<0.05)/Z11-16:Ald (IndVal value=0.61, *P*-value<0.05).

These cluster analyses revealed similar differentiation patterns between *PBP1* and *PBP2* expression levels and the ratio of Z11-16:OH and Z11–16:Ald. There was a clear link between the expression level of *PBP2* in Kenyan males with an increase in the % of Z11-16:Ald produced by females, and another link between *PBP1* over-expressed in the French population and the presence of Z11-16:OH only in this population. Although functional assays to determine the binding properties of *S. nonagrioides* PBPs would be required, we can speculate that PBP2 is involved in Z11-16:Ald binding, and that PBP1 is involved in Z11-16:OH binding. Thus, different female sexual signals may be associated with different male reception systems, adjusted by the *PBP* expression levels, ensuring optimal communication within populations.

In summary, our results indicate a difference in the intraspecific communication system between the *S. nonagrioides* populations from Europe and Sub-Saharan Africa. This is the first report, to our knowledge, on differential expression levels of *PBPs* in populations of Lepidoptera among the same

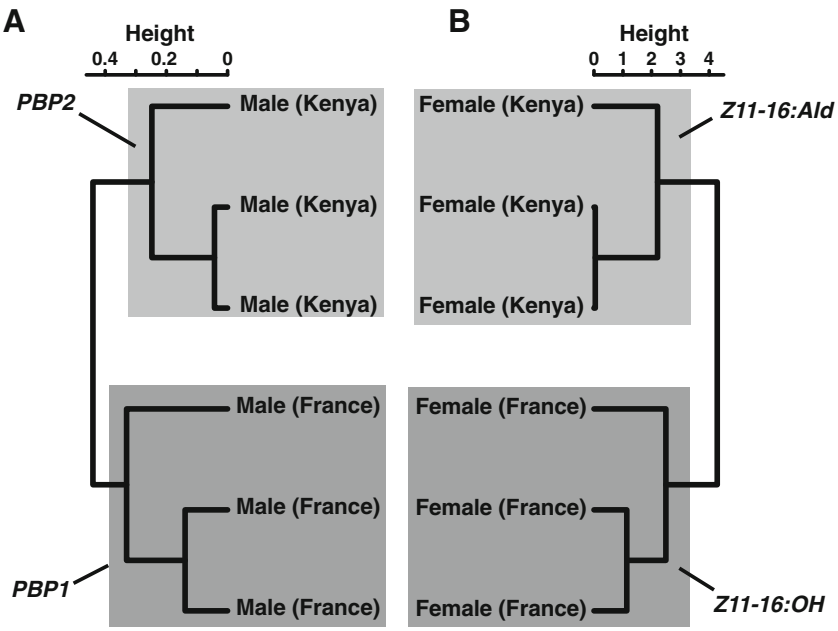

Fig. 1 Unweighted pair group method with arithmetic mean clusters based on a Euclidean matrix calculated from the *PBP* expression level and the abundances of pheromone components of *Sesamia nonagrioides*: **a** Male *PBP* data matrix; **b** Female pheromone data matrix. The *grey squares* are the differentiated groups. The names in *italic* near the squares are indicator *PBP*/pheromone compound of each group detected by IndVal analyses

species and on differences in pheromone blends between populations of *S. nonagrioides*.

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