An Antennal Circadian Clock and Circadian Rhythms in Peripheral Pheromone Reception in the Moth *Spodoptera littoralis*

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An Antennal Circadian Clock and Circadian Rhythms in Peripheral Pheromone Reception in the Moth *Spodoptera littoralis*

Christine Merlin, Philippe Lucas, Didier Rochat, Marie-Christine François, Martine Maïbèche-Coisne, and Emmanuelle Jacquin-Joly[1]

UMR 1272 INRA-UPMC-AgroParisTech "Physiologie de l'Insecte: Signalisation et Communication," INRA Centre de Versailles, Versailles cedex, France

Abstract Circadian rhythms are observed in mating behaviors in moths: females emit sex pheromones and males are attracted by these pheromones in rhythmic fashions. In the moth *Spodoptera littoralis*, we demonstrated the occurrence of a circadian oscillator in the antenna, the peripheral olfactory organ. We identified different clock genes, *period* (*per*), *cryptochrome1* (*cry1*) and *cryptochrome2* (*cry2*), in this organ. Using quantitative real-time PCR (qPCR), we found that their corresponding transcripts cycled circadianly in the antenna as well as in the brain. Electroantennogram (EAG) recordings over 24 h demonstrated for the first time a circadian rhythm in antennal responses of a moth to sex pheromone. qPCR showed that out of one pheromone-binding protein (PBP), one olfactory receptor (OR), and one odorant-degrading enzyme (ODE), all putatively involved in the pheromone reception, only the ODE transcript presented a circadian rhythm that may be related to rhythms in olfactory signal resolution. Peripheral or central circadian clock control of olfaction is then discussed in light of recent data.

Key words olfactory rhythms, circadian clock, olfaction, sex pheromone, *Spodoptera littoralis*, electroantennography, real-time PCR

Insect circadian clocks generate endogenous circadian rhythms through transcription/translation-based feedback loops of a defined set of "clock genes" (Dunlap, 1999; Shirasu et al., 2003), including *period* (*per*), *timeless*, *cycle*, *clock*, and *cryptochromes* (*cry*). The rhythmic interplay of clock genes and their products was found in a broad range of organs, including the central nervous system and numerous peripheral organs (reviewed in Giebultowicz, 2000). In particular, clocks regulating olfactory responses have been found in the antenna of *Drosophila melanogaster*, the olfactory organ. *Per*-driven luciferase or GFP (green fluorescent protein) expression in transgenic *D. melanogaster* revealed bioluminescence/fluorescent rhythms in the antennae and at the base of the chemosensory bristles, suggesting that these cells support functional clocks (Plautz et al., 1997). In addition, olfactory responses to food-related odors, measured as electroantennogram (EAG) amplitudes in both *D. melanogaster* and the cockroach *Leucophea maderae*, exhibit a circadian rhythm (Krishnan

1. To whom all correspondence should be addressed: Dr Emmanuelle Jacquin-Joly, UMR 1272 INRA-UPMC-AgroParisTech, "Physiologie de l'Insecte, Signalisation et Communication," INRA Centre de Versailles, route de Saint-Cyr F-78026 Versailles cedex, France; e-mail: jacquin@versailles.inra.fr.

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et al., 1999; Page and Koelling, 2003). Recent work in *L. maderae* revealed that EAG rhythms are extended to pheromone perception (Rymer et al., 2007). In *D. melanogaster*, EAG rhythms are abolished in *per* mutant flies that do not express *per* in peripheral oscillators (Krishnan et al., 1999). Antennal clock neurons in *cry* mutants show impaired free-running rhythms, reducing or eliminating diel rhythms in olfactory responses (Krishnan et al., 2001). Furthermore, it was demonstrated that antennal neurons are also sufficient to generate olfactory rhythms: targeted ablation of central oscillator cells had no effect on *D. melanogaster* EAG rhythms whereas clock disruption in antennal neurons abolished these rhythms, which were restored in rescued antennal functional oscillators (Tanoue et al., 2004). These findings suggest that an antennal circadian oscillator is not only necessary but also sufficient to regulate the olfactory responses. Although the mechanism by which this antennal clock could regulate EAG rhythms is unknown, microarray-based approaches revealed that genes putatively involved in odorant recognition and clearance appeared to be clock regulated (McDonald and Rosbash, 2001; Claridge-Chang et al., 2001; Ueda et al., 2002; Ceriani et al., 2002).

Moth pheromone communication displays an extensive regulation by circadian clocks: female calling behavior and pheromone release, which attract conspecific males for mating, as well as male orientation to calling females, undergo not only daily rhythms (Payne et al., 1970; Linn et al., 1996; Iglesias et al., 1999) but also circadian ones (Rosén, 2002; Rosén et al., 2003; Silvegren et al., 2005). In a previous study, we identified two clock genes, *per* and *cryptochrome1* (*cry1*), expressed in the antennae of the moth *Mamestra brassicae*, at the base of the olfactory sensilla (Merlin et al., 2006), leading to the hypothesis that a peripheral antennal clock may occur in noctuid moths, as in *D. melanogaster.* In the present study, we investigated if such clock marker genes could undergo rhythmic expression in the antennae of another noctuid, *Spodoptera littoralis.* We first isolated *S. littoralis per* cDNA and two different *cry* genes, *cry1* and *cry2*, and demonstrated that their expression did follow a circadian rhythm in the antenna, revealing the presence of an antennal clock in this organ.

The rhythmic behavioral response of male moths to sex pheromone could be due to rhythms in peripheral olfactory reception and/or in olfactory central processing and/or in general level of activity. Since antennae appear to possess circadian clocks, we investigated whether pheromone response in antennae, measured with the EAG technique, could undergo circadian rhythms in *S. littoralis.* This hypothesis is supported by the recent discovery of daily changes in the sensitivity of pheromone-sensitive antennal sensilla in *Manduca sexta* (Flecke et al., 2006), although no time-dependent difference in EAG responses to pheromones has been previously observed in *Trichoplusia ni* or *Agrotis segetum* (Payne et al., 1970; Worster and Seabrook, 1989; Rosén et al., 2003). In this study, we demonstrated for the first time in a moth that EAG responses to sex pheromone are circadianly regulated, although their regulation by the antennal clock remains to be determined.

In the first step to understanding the molecular basis of this EAG rhythm, we established temporal expression profiles of genes putatively involved in pheromone reception in this moth. Pheromone reception includes pheromone solubilization/transport by pheromone-binding proteins (PBPs) to cross the aqueous lymph of the sensilla to reach olfactory receptors (ORs) located in the dendritic membrane of the olfactory receptor neurons (ORNs), pheromone interaction with ORs, and finally pheromone degradation by efficient odorant-degrading enzymes (ODEs) (reviewed in Vogt, 2005). The ORNs express the ORs, whereas PBPs and ODEs are expressed by accessory cells in the sensillum lymph surrounding their dendrites. These olfactory proteins are then thought to participate directly in the neuron response, and therefore in the EAG response, which is a sum of receptor potentials from all ORNs responding to the stimulus applied in the recorded antenna.

We thus focused on selected genes encoding a newly identified PBP (Slit-PBP1), a newly identified OR (Slit-R2), and an ODE from the esterase family (Slit-Est) (Merlin et al., 2007) since the pheromonal blend is composed of acetate components in *S. littoralis.*

MATERIALS AND METHODS

Insects

Spodoptera littoralis were reared in the laboratory at 24 °C, 60% to 70% relative humidity, and under LD16:8 until emergence. Zeitgeber time 0 (ZT0) was designed as lights-on and ZT16 as lights-off. For studying EAG rhythms and gene expression profiles, male moths that emerged at the same time were collected and entrained for 1 day under LD16:8 followed by either LD16:8 or DD, before EAG recordings or brain and antennae collections. In DD conditions, experiments were performed on the first day of DD since it has

been demonstrated that the behavioral response of male *S. littoralis* is dampened dramatically in the second day of DD (Silvegren et al., 2005).

EAG Recording

The EAG response is the summed recording of the activity of sensory neurons of the antenna in response to an odorant stimulus applied (Schneider, 1962). Since more than 95% of neurons in moth antennae are ORNs (Lee and Strausfeld, 1991), the EAG technique has long been considered reliable for measuring the olfactory sensitivity of moths (Schneider, 1962). EAG recordings were made every 3 hours during a 24-h period on 2-day-old males either in the second day of LD or the first day of DD. Male moths were momentarily anesthetized with CO_2 and then immobilized in styrofoam holders 3 h before the beginning of the recordings. A silver wire, serving as the reference electrode, was inserted into the neck of each insect. The 2 or 3 distal segments of the left antenna were removed. To record the EAG response, the recording electrode, a glass capillary filled with Roeder's solution, was brought into contact with the cut end of the left antenna. Both electrodes were connected to a preamplifier Neurolog NL 102 (Digitimer Ltd., Hertfordshire, UK). Stimulus cartridges were Pasteur pipettes containing a filter paper loaded with a nonsaturating dose (500 ng) of *S. littoralis* main pheromonal component, (*Z*9,*E*11)-tetradecadienyl acetate (*Z*9,*E*11-14:Ac) (Tamaki and Yushima, 1974). This dose has been chosen for EAG recordings from a dose-response curve analysis: in the sigmoid curve it corresponds to the fast rising part, between the threshold, around 10 ng, and saturating doses, above 10 µg. Each insect had its own cartridge that was used for the whole experiment. Seventeen and 24 males were monitored in two independent experiments in LD conditions, and 18 males were monitored in DD conditions. Pheromone stimulations were achieved by blowing a puff of air (0.5 s; 20 $L.h^{-1}$) through the stimulating cartridge. A humidified air flow (70 $L.h^{-1}$) continuously rinsed the preparation. The EAG signal was amplified (×1000) and filtered (0 to 300 Hz). A thermistor placed upstream of the cartridge monitored the stimulation. The two signals were sampled at 1 kHz through a 12-bit acquisition card (DT3001; Data Translation, Marlboro, MA) driven by the Awave software (Marion-Poll, 1995). EAGs were analyzed with Clampfit 9.0 (Molecular Device Corp., Union City, CA). The time required to change the stimulus cartridge, to position a new insect, to connect electrodes, and to record its EAG response was approximately 2 min. Temperature was 23-24 °C at the time of recordings, and between recordings, insects were kept at 24 °C in a humidified box within a temperature-controlled incubator. The immobilization and recordings in the dark were done under dim red light (25 lux) using filters that block light of wavelengths below 600 nm (red-light).

Statistical Analysis

For each individual, raw EAGs (in mV) were converted to relative EAGs (in percentage) by dividing them by the average response of the individual over the 24-h cycle to eliminate the differences of absolute sensitivity between individuals. Circadian time effect on relative EAGs was studied by analyses of variance (ANOVAs) using a general linear model procedure (Crawley, 1994). The effect of the preparation aging was tested for a linear correlation with the duration (in h) from the beginning of the experiment. For experiments in LD conditions, we treated both factors with a common model (duration from beginning as a covariable). For DD conditions, the 2 factors were directly correlated, and both parameters were evaluated independently. First, we showed a linear decrease of the relative EAGs as a function of the time elapsed from the antenna connection. Then data were corrected for this time effect and submitted to a 1-way ANOVA with circadian time as a factor. Differences between means were assessed by Tukey multiple comparison tests at 5%. Calculations were carried out by using Minitab v12.2 software (1998; Minitab Inc., State College, PA).

Cloning of *S. littoralis period* (*per*), *cryptochrome 1* (*cry1*), and *cryptochrome 2* (*cry2*) cDNAs from Male Antennae

Per and *cry1* partial cDNA fragments were amplified via PCR from cDNA synthesized from total RNA purified from *S. littoralis* male antennae (TRIzol; Invitrogen, Carlsbad, CA). Primers were designed in conserved regions for each gene (PerF: GGAAGATCCTTCATCGATTTCGTG, PerR: GTCTTGGGGCAGATACCCCAGGTACGG and Cry1F: TTCGATGGAGAGASTGCAGGTACYAA, Cry1R: GCCGGTGCGGCCTTCGACCCATCT). The 5'-ends of the coding regions were obtained by rapid amplification of cDNA ends (SMART™ RACE kit; Clontech, Mountain View, CA) with specific primers (5'PerR: CCGCTTTTACCACCACCTCGTTCGGCG and 5'Cry1R: CGCGGAGGGTCGCCAATGGTGGC). *Cry2* was identified through the analysis of an expressed sequence tag (EST)

library made from *S. littoralis* male antennae (unpublished data). The deduced proteins of two singletons presented high sequence identities with the few insect CRY2s known to date, one corresponding to the 5′ region of the gene and the other to the 3′ region. DNA sequence between these 3′ and 5′ regions was obtained by PCR on antennal cDNA with gene specific primers (Cry2F: CCCGCGGACGCTTTACCTAAA and Cry2R: ATTGACCGACCAGTCGGCGTC). PCR products were cloned and sequenced (Genome Express, Meylan Cedex, France). Sequence analyses were performed using the BLAST program (Altschul et al., 1990).

Identification of *S. littoralis* Olfactory Genes in Male Antennae

S. littoralis OR2 (*Slit-R2*) and Esterase (*Slit-Est*) cDNA sequences were retrieved from the Genbank database (accession numbers EF395366 and DQ680828, respectively). *S. littoralis* PBP1 (*Slit-PBP1*) cDNA was identified in the antennal EST library (unpublished) by BLAST analyses compared to public databases (Genbank, trEMBL). SignalP (Nielsen et al., 1997), CLUSTALW (NPS@IBCP), and TMHMM server v. 2.0 were used for peptide signal identification, sequence alignment, and transmembrane domain identification, respectively.

Phylogenetic Analysis of Insect CRYs and Slit-R2

CRY-related amino acid sequences were retrieved from GenBank and aligned using Multalin program (Corpet, 1988). Slit-R2 was aligned with BmorOR2 (Genbank accession number AJ555487), DOR83b (Genbank accession number NM_079511) and different *Drosophila* ORs functionally characterized (Hallem et al., 2004). Neighbor joining was used to build strict consensus trees with *MEGA* v.3.1 software (Kumar et al., 2004). Branch support was assessed by bootstrap analysis based on 5000 replicates, and the bacterial DNA photolyase PHR from *Escherichia coli* was used as an outgroup for the CRY tree (*Ecol*-PRH, Genbank accession number P00914).

Real-Time PCR Assays for Gene Expression Studies

A pool of newly emerged and synchronized adult male moths were entrained for 1 day in LD at 24 °C and collected every 3 h during the next 24 h, either in LD or in DD conditions. Five brains and 10 whole antennae were collected for each time point under light in light conditions and dim red-light in dark conditions. Corresponding total RNAs were purified from each tissue and for each time point using the RNeasy® Micro kit (Qiagen, Hilden, Germany). To eliminate possible contamination by genomic DNA, a DNAse treatment was performed on the columns during the extraction procedure. cDNA synthesis was performed for each sample, using the Advantage®RT-for-PCR kit (Clontech), according to the manufacturer's instructions. cDNAs were quantified by fluorescence-based qPCR (Chromo 4 system; Bio-Rad, Hercules, CA). Primers (see Table 1) were designed using Beacon Designer 4.0 software (Bio-Rad) to amplify 100-200-bp fragments. All qPCR reactions were performed using IQ SYBR Green Supermix (Bio-Rad) in a total volume of 20 µL in the presence of 5 µL of cDNA (or water for the negative control or RNA for controlling for the absence of genomic DNA), 4 mM $MgCl_2$, and 200 nM of each primer. Real-time PCR was performed with an initial denaturation step of 3 min at 95 °C followed by 40 cycles of 94 °C for 20 s, annealing for 15 s (53 °C for *R2;* 55 °C for *cry2*, *rpL8*, *PBP1*, and *Est;* 57 °C for *per;* 58 °C for *cry1*), 72 °C for 20 s, and a melting curve ramp to confirm that each reaction did not produce nonspecific amplification. For each gene of interest a standard curve was generated from a dilution serie (0.25; 0.0625; 0.0156; 0.0039; 0.00097) of one cDNA sample, which served for relative quantification of the same gene in all other cDNA samples. Real-time PCR was performed in duplicate for each sample. After verifying that the amplification efficiencies of both the target and control genes were similar, relative quantification was performed using the comparative $2^{-\Delta\Delta CT}$

Table 1. Primers used in qPCR, designed using Beacon Designer 4.0 software.

Genes	*Primer Names*	*Primer Sequences*
Control gene	RpL8 F	ATGCCTGTGGGTGCTATGC
	RpL8 R	TGCCTCTGTTGCTTGATGGTAG
Clock genes	Period F	CAATGGTTTGTCGGATACGG
	Period R	TTTTACCACCACCTCGTTCG
	Cry1 F	CCACAGATTGAGGCTCCAC
	Cry1 R	ACACGACATTAGGCTTTCCC
	Cry2 F	TCGTGGTCATCGGTTAGGG
	Cry2 R	GGCATCACTGTCACATCTCG
Olfactory genes	PBP1 F	ATGGCGAAGAAGTTGGACCTC
	PBP1 R	CTCGTGGATCTTAGTGCGGAAG
	R2 F	TGGACAGCAGAACAACAACC
	R2 R	AGCCTGATAAGCAAGTAGAGTG
	Est F	CCTTTGCCCGTGATGGTCTTC
	Est R	TGCCGCCGAATGCCTCTATG

method (Livak and Schmittgen, 2001) to identify the relative amount of target cDNAs in time-point samples. The control used was the *S. littoralis* ribosomal protein rpL8 (*Slit*-rpL8) (Merlin et al., 2007). In the analysis of the relative fold change, the ZT3/CT3 sample, which was one of the experimental samples, was taken as the calibrator. Tissue collection, RNA extraction, cDNA synthesis, and gene expression profiling were performed separately on 2 sets of individuals that corresponded to 2 different biological repetitions.

RESULTS

Robust Circadian Rhythms of *Period*, *Cryptochrome1*, and *Cryptochrome2* Expressions in Antennae and Brain

To investigate the presence of a peripheral antennal clock in *S. littoralis* males, we first identified in male antennae three cDNAs encoding clock marker proteins, *S. littoralis* PERIOD (Slit-PER), *S. littoralis* CRYPTOCHROME 1 (Slit-CRY1), and *S. littoralis* CRYPTOCHROME 2 (Slit-CRY2) by PCR-based strategies and analysis of an antennal EST library. These cDNAs have been deposited in the Genbank database under accession numbers EF364034, EF364035, and EF396286 for *Slit-per*, *Slit-cry1*, and *Slit-cry2*, respectively. Phylogenetic analysis of the two Slit-CRYs with other insect CRYs showed that Slit-CRY1 clustered with other insects' CRY1 and the photosensitive CRY of *D. melanogaster*, whereas Slit-CRY2 clustered with insects' CRY2 and the two mouse CRYs (Fig. 1). To determine whether *per*, *cry1*, and *cry2* could function as clock genes in male antennae, we quantified by qPCR their transcripts in antennae from two sets of moths entrained under LD conditions (Fig. 2). Compared to constant levels of *rpL8* expression (10% to 20% variation), each transcript manifested ~4-fold amplitude in cycling in both repetitions. *Cry1* and *cry2* cycled in different phases, with a peak at ZT6 for *cry1* and at ZT0 (and ZT24) for *cry2*. On the contrary, *per* and *cry2* cycled in a similar phase, peaking at ZT0 (and ZT24) (Fig. 2A and B, left). To determine the endogenous character of these oscillations in the antennae, *per*, *cry1*, and *cry2* transcript levels were also measured under DD conditions. Cycling persisted with phases and amplitudes similar to those observed in LD conditions, although the waveforms were more rounded (Fig. 2A and B, right). We also quantified in LD and DD conditions temporal expression patterns of these three genes in the brain to compare their expression profiles between the central and the antennal clocks. The cerebral transcript cycling manifested phases and variations of amplitudes similar to those found in the antennae (Fig. 3).

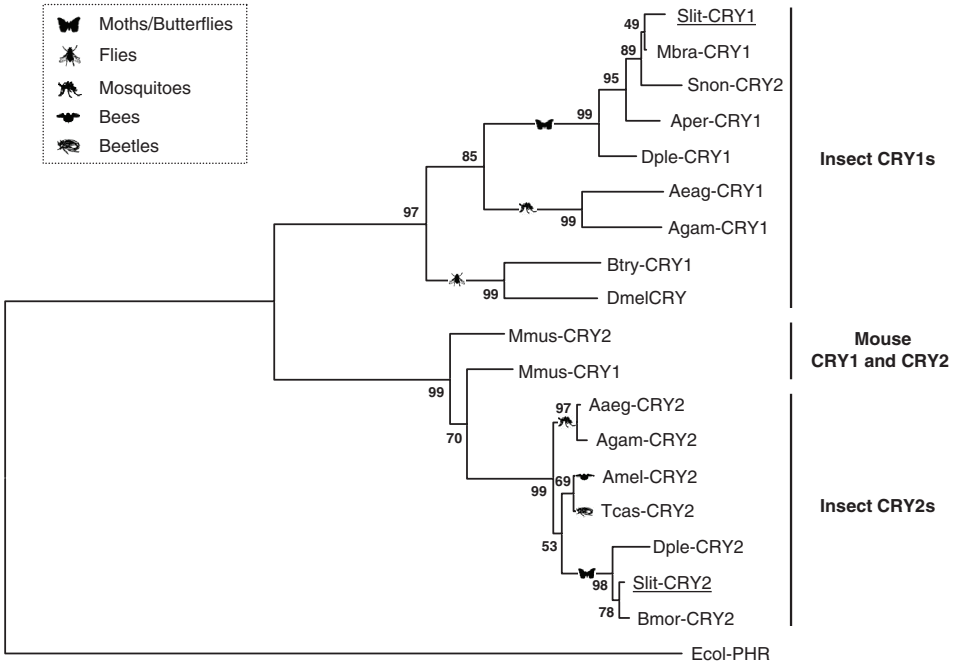

Figure 1. Neighbor-joining tree of CRYs from insects and mouse, generated with *MEGA* version 3.1 (Kumar et al., 2004). Bootstrap values (percent of branching) based on 5000 replicates are indicated on horizontal branches. Genbank accession numbers of CRY amino acid sequences are as follows: *Aedes aegypti* (*Aaeg*) CRY1 (EAT44496), CRY2 (EAT35912); *Anopheles gambiae* (*Agam*) CRY1 (XP321104), CRY2 (EAA44753); *Apis mellifera* (*Amel*) CRY2 (XP393680); *Antheraea pernyi* (*Aper*) CRY1 (AAK11644); *Bombyx mori* (*Bmor*) CRY2 (NRPG1215); *Bactrocerca tryoni* (*Btry*) CRY1 (AAU14170); *Danaus plexippus* (*Dple*) CRY1 (AY860425), CRY2 (DQ184682); *Drosophila melanogaster* (*Dmel*) CRY (AAC83828); *Escherichia coli* (*Ecol*) PHR (P00914); *Mamestra brassicae* (*Mbra*) CRY1 (AY947639); *Mus musculus* (*Mmus*) CRY1 (NM007771), CRY2 (NM009963); *Sesamia nonagrioides* (*Snon*) CRY2 (DQ243705) (in light of new available CRY sequences, this CRY may be recalled CRY1); *Spodoptera littoralis* (*Slit*) CRY1 (EF364035), CRY2 (EF396286); *Tribolium castaneum* (*Tcas*) CRY2 (XP972654). *E. coli* DNA photolyase (PHR) was used as the outgroup.

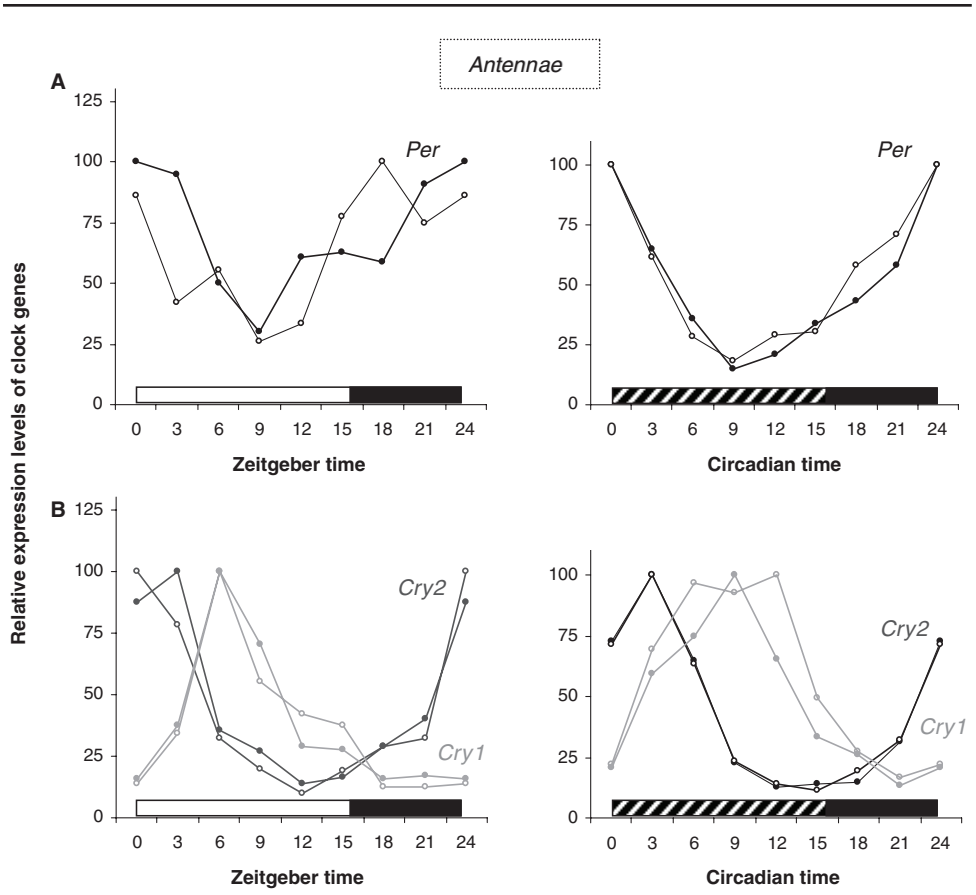

Figure 2. Temporal expression analysis of *Slit-per*, *Slit-cry1*, and *Slit-cry2* transcript levels in *S. littoralis* male antennae under LD16:8 and DD conditions by real-time PCR. Expression levels relative to the control gene *Slit-rpL8* were calculated with the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) using ZT3/CT3 as calibrators, and converted to percentage of maximal level. Points at ZT24/CT24 are duplicates of ZT0/CT0 replotted to show 24-h trends. Two replicates were performed, represented by filled and open circles. The white and black bars below the graph denote day and night, respectively. The hatched bar indicates subjective day and the black bar indicates subjective night. Daily (left) and circadian (right) rhythms of (A) *Slit-per* transcript levels, and (B) *Slit-cry1* (in gray) and *Slit-cry2* (in black) transcript levels.

Circadian Rhythms in Olfactory Responses of the Antennae to Sex Pheromone in *S. littoralis*

We observed olfactory rhythms in males of *S. littoralis* by measuring EAG responses to the main component of the sex pheromone, *Z*9,*E*11-14:Ac. To establish whether physiological olfactory responses show daily and circadian rhythms, we made EAG recordings from moths maintained in both LD and DD conditions. Preliminary analyses showed that the relative EAGs and transformed data for DD conditions followed a normal distribution and were homoscedastic for both DD and LD experiments. Under LD conditions, relative EAGs were not correlated to the time elapsed from the beginning of the experiment (no aging effect) ($F_{1,331}$ = 3.30, p = 0.07). EAG responses had comparable amplitudes throughout the day except for a statistically significant ($p < 0.05$) drop at ZT24 (8 h after lights-off; Fig. 4A). To determine whether this rhythm was endogenous, EAG responses to *Z*9,*E*11-14:Ac were recorded from moths during the first day of DD after 1 day of entrainment in LD conditions. Under DD conditions, relative EAGs (Fig. 4B: dotted curve with open circles) significantly decreased with the aging of the preparations (slope: –0.8 ± 0.2% [M + SE] per hour; $F_{1,116}$ = 9.53, $p <$ 0.001). The relative EAGs corrected for this time effect (Fig. 4B: bold curve with filled circles) clearly showed a dependence on circadian time ($F_{7,119}$ = 4.44, $p < 0.001$). We noticed a similar reduction in EAG amplitudes in DD conditions as observed in LD conditions, except that it was more gradual and the variations of the EAG amplitudes were lower (Fig. 4B). Mean EAG responses observed at circadian time CT9 to CT18 and CT6 were comparable, whereas they gradually decreased during the latter part of the subjective night (CT21) until the beginning of the subjective day (CT3; day 2 of DD) before strongly increasing at CT6. Mean amplitudes of EAG responses observed at CT24, CT21, and CT3 were significantly ($p < 0.05$) lower than those evoked at CT9, CT15, CT18, and CT6. We also compared the shape of responses at different hours to verify if the kinetics of the EAG response (phases of depolarization and repolarization) varied with time, but no differences were observed (data not shown).

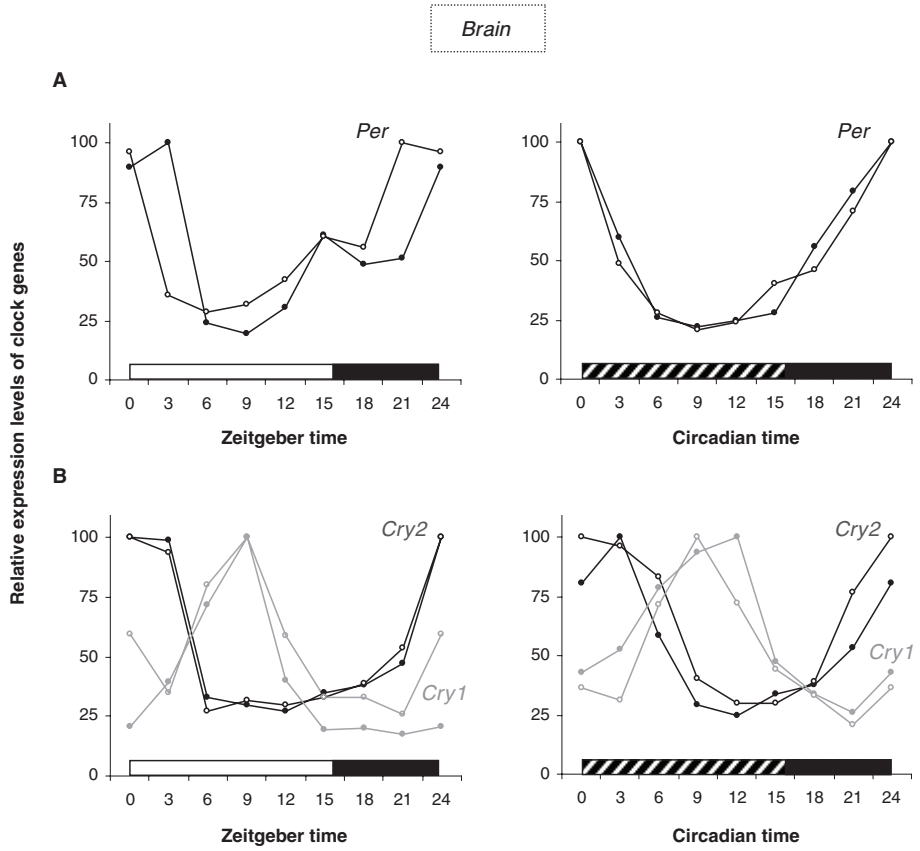

Figure 3. Temporal expression analysis of *Slit-per*, *Slit-cry1*, and *Slit-cry2* transcript levels in *S. littoralis* male brain under LD16:8 and DD conditions by real-time PCR. Quantification and normalization were performed as described in Figure 2. Two replicates were performed, represented by filled and open circles. The white and black bars below the graph denote day and night, respectively. The hatched and black bars below the graph indicate the subjective day and the subjective night, respectively. Daily (left) and circadian (right) rhythms of (A) *Slit-per* transcript levels, and (B) *Slit-cry1* (in gray) and *Slit-cry2* (in black) transcript levels.

Identification of Olfactory Genes and Temporal Expression Studies

To provide a more comprehensive understanding of the molecular basis of the EAG rhythm, we analyzed the temporal expression patterns of candidate genes belonging to different families potentially involved in pheromone reception and degradation at the peripheral level. *Slit-PBP1* encodes a PBP and was identified in a *S. littoralis* EST library established in our laboratory (unpublished data). This newly identified gene was classified in the PBP family since the corresponding protein presented all the hallmarks of insect PBPs, including a peptide signal for secretion and 6 conserved cysteine residues (Fig. 5A), as well as high similarity with previously described moth PBPs. This new cDNA sequence has been deposited in the Genbank database under the accession number EF396284. *Slit-R2* encodes a putative OR transducing the signal. The amino acid sequence contains 7 predicted transmembrane domains, common to all insect olfactory receptors known to date, and it clusters in a phylogenetic analysis with *Drosophila* DOR83b and its *B. mori* ortholog BmorOR2 (Fig. 5B). In particular, it shares 67% amino acid identities with DOR83b. *Slit-Est* encodes a putative ODE belonging to the esterase family. To examine whether these genes could be clock regulated, their temporal expression profiles were studied by qPCR in LD and in DD conditions. For *Slit-PBP1*, despite a similar temporal expression pattern in LD and DD conditions with a 1.3 factor measured between the highest and lowest expression levels (Fig. 6A), no clear rhythmicity was found. No evident rhythms of expression were discerned for *Slit-R2*, which exhibited the same range of amplitude variations as *Slit-PBP1* in LD and DD conditions, but with great variability between repetitions in LD conditions (Fig. 6B). On the contrary, *Slit-Est* transcript robustly cycled in LD as well as in DD conditions. In the LD cycle, *Slit-Est* transcript exhibited a 2-fold change in expression level with a peak at dusk (ZT12 and ZT15 for the 2 repetitions, respectively) and a trough at the end of the night (ZT24) (Fig. 6C, left). In free-running conditions, the *Slit-Est* mRNA rhythm persisted with a 3.5-fold change in expression level, with a maximum in the early part of the subjective night at CT18 and a minimum in the early part of the subjective day (CT3) (Fig. 6C, right). A 3-h phase delay was observed in DD conditions compared to LD conditions.

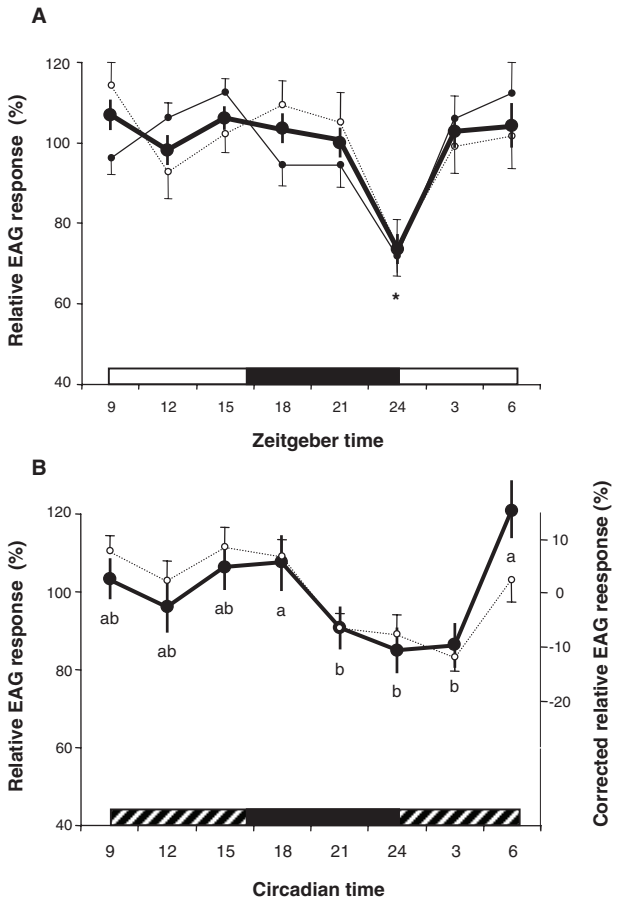

Figure 4. ***S. littoralis*** **olfactory responses at different times of day. Each point represents mean relative pheromone-evoked EAG responses to 500 ng of *Z*9,*E*11-tetradecadienyl acetate (*Z*9,*E*11-14:Ac). Error bars denote SEM. (A) Diurnal changes in mean EAG responses on day 2 of LD16:8 cycles. The white and black bars below the graph represent day and night, respectively. The asterisk denotes a significant ($p < 0.05$) difference in the EAG amplitude at ZT24 compared with those at all other times of day. Two independent experiments were performed on 17 and 24 moths, respectively, represented by small filled and open circles and thin lines. The average of the two experiments is shown as a bold curve with larger filled circles. (B) Circadian changes in EAG responses of 18 moths on day 1 of DD. Uncorrected relative EAGs are shown with small open circles joined by thin lines (left scale). EAGs corrected for the linear decrease with time are represented with large filled circles and a bold line (right scale). The hatched and black bars below the graph indicate the subjective day and the subjective night, respectively. Mean responses with same letters (a, b) do not differ significantly ($p < 0.05$).**

DISCUSSION

A Peripheral Circadian Clock in Moth Antennae

The overall organization of circadian systems in insects comprises numerous endogenous clocks organized into central and peripheral clocks. In most moth species, circadian system gates female sex pheromone emission and male pheromone responsiveness (Rosén, 2002; Rosén et al., 2003; Silvegren et al., 2005). Although an antennal peripheral clock has been shown to be necessary and sufficient to generate olfactory EAG rhythms in *D. melanogaster*, this has not been investigated in moths. Preliminary data allowed us to demonstrate that clock genes, *per* and *cry1*, were expressed in antennae of the noctuid *M. brassicae*, at the base of olfactory and pheromone-sensitive sensilla (Merlin et al., 2006), but their rhythmic expression was not studied. To date, the only study examining *per* temporal expression profile in the antennae of a Lepidoptera, *Bombyx mori*, did not revealed any fluctuation of this gene in this organ, although it oscillates in the brain (Iwai et al., 2006). On the contrary, we showed in the present study that the expression levels of the clock genes *per*, *cry1*, and *cry2* fluctuated in a circadian manner in both the antennae and brain in *S. littoralis.* These results strongly suggest the occurrence of a circadian clock in *S. littoralis* antennae.

In addition, our results support earlier evidence of different clockwork mechanisms in insect. In *D. melanogaster*, the major transcriptional repressor of the circadian clock is PER, localized in the cytoplasm during the day and transferred into the nucleus during the night (Curtin et al., 1995) to inhibit the CLOCK/CYCLE complex activity. In other insects, such as the monarch butterfly (*Danaus plexippus*), the red flour beetle (*Tribolium castaneum*), and the honeybee (*Apis mellifera*), this transcriptional activity is repressed in vitro by another clock protein, CRY2 (Zhu et al., 2005; Yuan et al., 2007). In Lepidoptera, although PER can inhibit CLOCK/CYCLE activity in vitro in some species (Chang et al., 2003), its nuclear localization in vivo has never been established (Reppert et al., 1994; Sauman et al., 2005). This lack of PER nuclear localization is then inconsistent with a potential transcriptional repressive activity in vivo. Here we showed that *cry2* cycle in a circadian manner in phase with *per* in *S. littoralis* antennae and brain, as observed in bee heads (Rubin et al., 2006), thus supporting the hypothesis of a role of transcriptional repressor for CRY2 in vivo. Furthermore, our data suggest that the transcriptional repressive activity of CRY2 could be extended at least to one peripheral clock, the antennal clock.

Evidence for a Circadian Rhythm in Moth Peripheral Olfactory Responses to the Sex Pheromone

Without EAG rhythms to pheromone in the moths *T. ni*, *C. fumiferana*, and *A. segetum*, Payne et al. (1970),

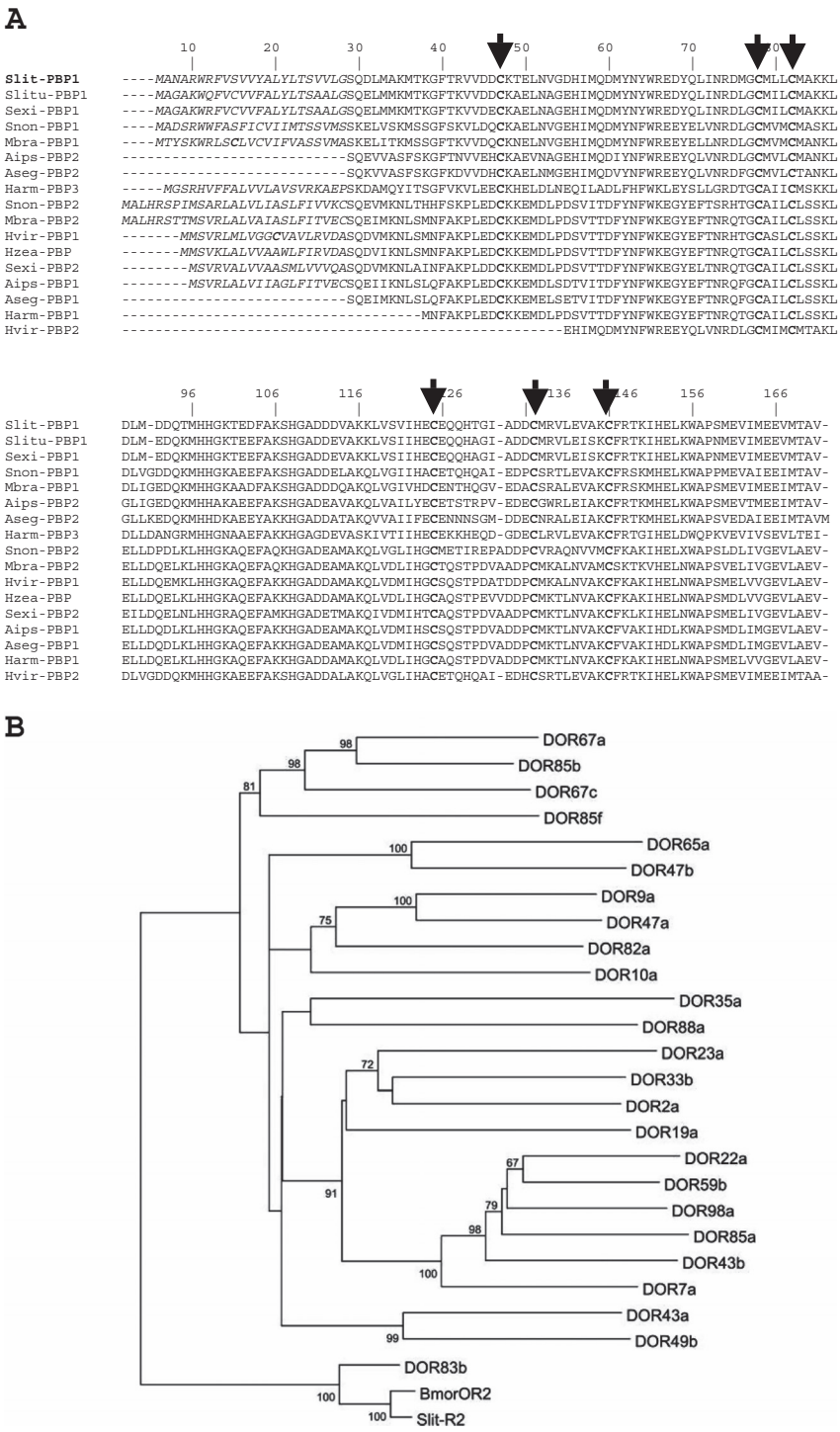

Figure 5. (A) Comparison of the predicted amino acid sequences of Slit-PBP1 with pheromone-binding proteins from other noctuids. The 6 conserved cysteines are in bold and marked by arrows. Signal peptides at the N-termini are in italics. Genbank accession numbers: Slit-PBP1: EF396284 (*Spodoptera littoralis*); Slitu-PBP1: AY743351 (*Spodoptera litura*); Sexi-PBP1: AY743351, Sexi-PBP2: AY743352 (*Spodoptera exigua*); Snon-PBP1: AY485219, Snon-PBP2: AY485220 (*Sesamia nonagrioides*); Mbra-PBP1: AF051143, Mbra-PBP2: AF051142 (*Mamestra brassicae*); Hzea-PBP1: AF090191 (*Helicoverpa zea*); Harm-PBP1: AJ278992, Harm-PBP2: AF527054 (*Heliothis armigera*); Hvir-PBP1: X96861, Hvir-PBP2: AY301988 (*Heliothis virescens*); Aips-PBP1: AY301985, Aips-PBP2: AY301986 (*Agrotis ipsilon*); and Aseg-PBP1: AF134253, Aseg-PBP2: AY301987 (*Agrotis segetum*). (B) Neighbor-joining tree of Slit-R2 with *B. mori* OR2 (BmorOR2), *Drosophila* DOR83b, and functionally characterized *Drosophila* ORs (described in Hallem et al., 2004), generated with *MEGA* version 3.1 (Kumar et al., 2004). Bootstrap values (percent of branching) based on 5000 replicates are indicated on horizontal branches.

Worster and Seabrook (1989), and Rosén et al. (2003) assumed that olfactory behavior rhythms are controlled by central mechanism. The identification of an antennal clock in a moth led us to reinvestigate a possible peripheral olfactory rhythm in *S. littoralis* antennae. The present data clearly demonstrate a circadian rhythm in EAG responses of *S. littoralis* males, consistent with previous findings of EAG rhythm in *D. melanogaster* and the cockroach *L. maderae* in response to food-related odors (Krishnan et al., 1999; Page and Koelling, 2003) and pheromones (Rymer et al., 2007). In the diurnal insect *D. melanogaster*, maximum EAG response to food odors occurs in the middle of the night (Krishnan et al., 1999), coinciding with the maximum of behavioral attractive/repulsive olfactory responses (Zhou et al., 2005). In the nocturnal moth *S. littoralis*, we observed an inverse phase relationship between EAG rhythm and olfactory behavior: the decrease in EAG amplitudes occurred in the latter part of the scotophase, the period of maximum behavioral responsiveness to pheromone (Silvegren et al., 2005). Although puzzling, this has already been observed in another nocturnal insect, *L. maderae*, in response to both food-related odors (Page and Koelling, 2003) and sex pheromone (Rymer et al., 2007). Different hypotheses

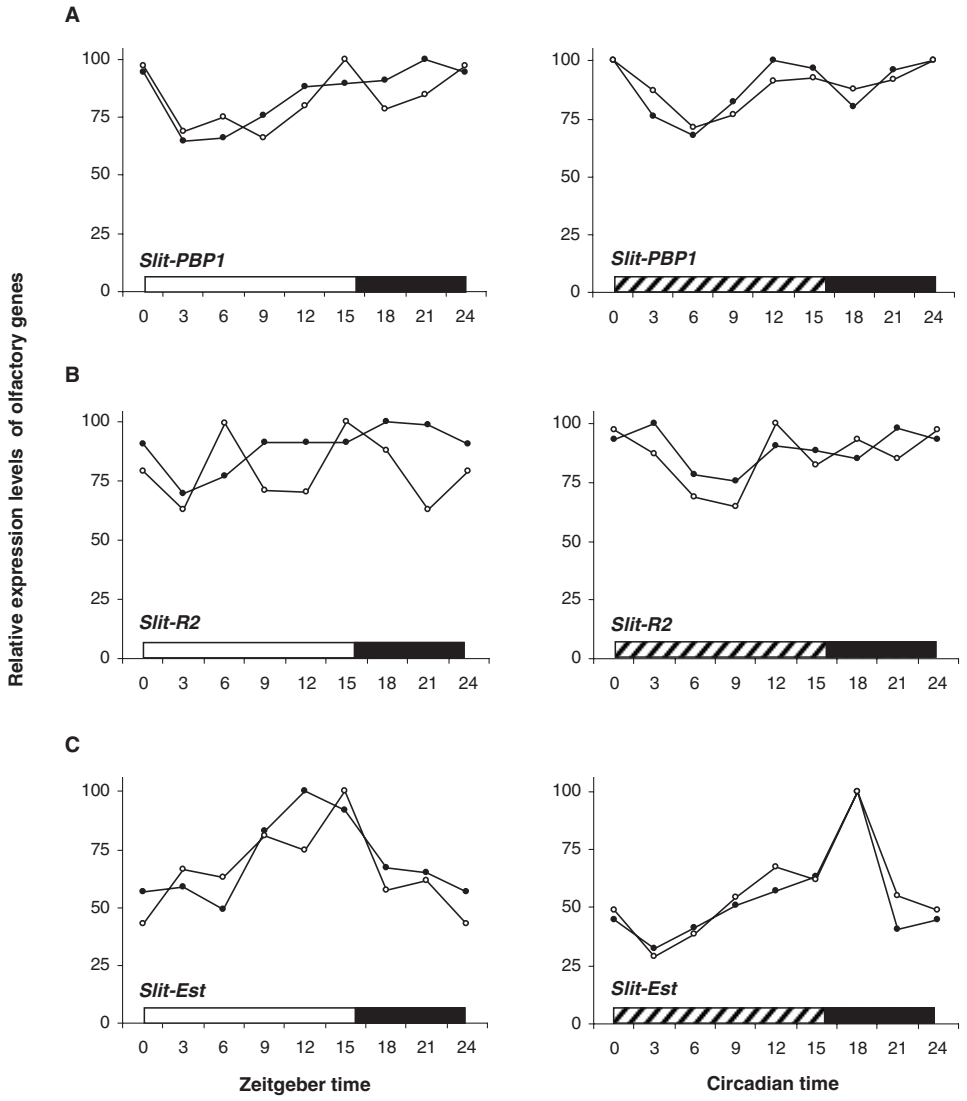

Figure 6. Temporal expression analysis of olfactory gene transcript levels in *S. littoralis* male antennae under LD16:8 (left) and DD (right) conditions by real-time PCR. Quantification and normalization were performed as described in Figure 2. Two replicates were performed, represented by filled and open circles. The horizontal white and black bars below the graph represent day and night, respectively, in LD conditions. The hatched and black bars below the graph indicate the subjective day and the subjective night, respectively, in DD conditions. Daily (left) and circadian (right) rhythms of (A) *Pheromone-Binding Protein 1* (*Slit-PBP1*) expression levels, (B) the atypical *Olfactory Receptor 2* (*Slit-R2*) expression levels, and (C) *Esterase* (*Slit-Est*) expression levels.

have been proposed to explain this apparent paradox: (1) The olfactory system may be sensitized when the insect is quiescent to function as an alarm system (Krishnan et al., 1999). (2) Despite a reduction in antennal sensitivity, animals may be more sensitive, behaviorally, to olfactory input due to circadian rhythms in central processing (Page and Koelling, 2003). In *L. maderae*, severing of the optic tracts abolished the circadian rhythm in EAG amplitudes (Page and Koelling, 2003) but did not affect the rhythm in mating behavior (Rymer et al., 2007). (3) As an alternative, EAG amplitude may not accurately reflect the ORN sensitivity (Page and Koelling, 2003). Single sensillum recording technique allows direct measurement of ORN responses through their spike frequency, and appears as a very interesting technique to confirm, or not, such an inverse phase relationship between ORN activity rhythm and olfactory behavior. Although it is known that ORNs project directly to the antennal lobes in the brain, where they activate the firing activity of their neuronal targets (the antennal lobe projecting neurons) via cholinergic synapses, the neuronal circuit between the olfactory centers in the brain and flight muscles recruited for the male flight toward the pheromonal source is not yet established. It may involve excitatory or inhibitory mechanisms, making it premature to interpret phase relationships between the EAG and behavioral rhythm and to understand the contribution of the peripheral olfactory system to olfactory rhythms in moths.

Temporal Expression of Peripheral Olfactory Genes Relevant to Pheromone Reception

In *D. melanogaster*, microarray studies highlighted expression rhythms of genes putatively involved in the peripheral reception of odors (Ceriani et al., 2002; Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ueda et al., 2002). In particular, these studies revealed that putative odorant-binding proteins

(OBPs) (McDonald and Rosbash, 2001; Ceriani et al., 2002) and enzymes, such as esterases and cytochrome P450s (McDonald and Rosbash, 2001; Ceriani et al., 2002; Ueda et al., 2002; Claridge-Chang et al., 2001), were under circadian regulation. In *S. littoralis*, an antennal EST library allowed us to identify genes putatively involved in pheromone reception. *Slit-PBP1* encodes a PBP that shares 70% to 80% identity with other noctuid PBP1s known to bind pheromone ligands, such as *M. brassicae* PBP1 (Maïbèche-Coisne et al., 1997). In addition, *Slit-PBP1* appeared to be enriched in the antennae of *S. littoralis* males, the sex for which pheromone reception is crucial for reproduction (Merlin, unpublished data). These preliminary data prompted us to study about this gene. Another gene, Slit-R2, is the homolog of the atypical receptor DOR83b known to be a chaperone for, and heterodimerization partner of, true functional olfactory receptors in *D. melanogaster* (Larsson et al., 2004). We also focused on another gene encoding an esterase, *Slit-Est* (Merlin et al., 2007), since proteins from this family are known to participate in rapid inactivation of acetate pheromone components in moth antennae (Ishida and Leal, 2005).

The products of these olfactory genes could be involved in the olfactory receptor neuron responses and therefore in the EAG. *Slit-Est* was expressed clearly in a circadian manner. Interestingly, this gene was maximally expressed at the beginning of the scotophase, a few hours prior to the maximal male behavioral response to pheromone (Silvegren et al., 2005). This delay may result in maximal enzyme activity along with increases in abundance of the esterase at a time when males are most active, participating in an efficient receptor deactivation process and therefore in maximal signal resolution when males are ready to fly toward the females. However, no information is yet available on the time necessary to generate active antennal esterases from their transcripts. A possible rhythm in the corresponding protein level has not been investigated in this study and could be tested with specific antibodies raised against this esterase. Whether such a rhythm in signal resolution capacities could be linked to the physiological or behavioral olfactory rhythms observed still remains to be determined. Contrary to *Slit-Est*, *Slit-PBP1* and *Slit-R2* do not exhibit circadian expression. Since their exact roles in Z9,E11-14:Ac reception in *S. littoralis* are not yet determined, it is possible that other PBPs or ORs not yet discovered and specific to this compound could be under circadian regulation. A more comprehensive view of olfactory gene expression rhythms could be achieved in *S. littoralis* by microarray studies on a representative pool of antennal genes and could lead to the discovery of additional clock-regulated olfactory candidates.

In this study, we extended the knowledge of antennal olfactory rhythms to a new insect order, Lepidoptera, by demonstrating in the moth *S. littoralis* the presence of a peripheral antennal clock along with a circadian rhythm in EAG amplitudes and in at least 1 antennal olfactory gene expression, *Slit-Est.* However, the links between the antennal clock, the EAG rhythm, and the behavioral rhythm observed are unclear at this point. Since moth antennae are carrying other sensory sensilla (mechano-, thermo-, and hygro-sensilla) in addition to olfactory sensilla, it could not be excluded that the antennal circadian clock may regulate other sensory modalities. Also, it will be necessary to study the relative autonomy of the antennal clock, since no paradigm has emerged from insect studies on the distribution and functional organization of circadian clocks. Experiments at both physiological and molecular levels performed on isolated antennae maintained in culture will be necessary to define whether the moth antennal clocks are autonomous or dependent on a central control and if the antennal clocks could be involved in the modulation of the EAG response level and/or olfactory gene expression rhythms.

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