Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrtosiphon pisum*.

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Abstract

Aphids are typical photoperiodic insects that switch from viviparous parthenogenetic reproduction typical of long day seasons to oviparous sexual reproduction triggered by the shortening of photoperiod in autumn yielding an overwintering egg in which an embryonic diapause takes place. While the involvement of the circadian clock genes in photoperiodism in mammals is well established, there is still some controversy on their participation in insects. The availability of the genome of the pea aphid *Acyrtosiphon pisum* places this species as an excellent model to investigate the involvement of the circadian system in the aphid seasonal response. In the present report, we have advanced in the characterization of the circadian clock genes and showed that these genes display extensive alternative splicing. Moreover, the expression of circadian clock genes, analysed at different moments of the day, showed a robust cycling of central clock genes *period* and *timeless*. Furthermore, the rhythmic expression of these genes was shown to be rapidly dampened under DD (continuous darkness conditions), thus supporting the model of a seasonal response based on a heavily dampened circadian oscillator. Additionally, increased expression of some of the circadian clock genes under short-day conditions suggest their involvement in the induction of the aphid seasonal response. Finally, *in situ* localisation of transcripts of genes *period* and *timeless* in the aphid brain revealed the site of clock neurons for the first time in aphids. Two groups of clock cells were identified: the Dorsal Neurons (DN) and the Lateral Neurons (LN), both in the *protocerebrum*.

Keywords

Aphids, life-cycle, circadian clock genes, photoperiodism, RT-qPCR, *in situ* hybridization.

1. Introduction

Biological rhythms represent adaptations found widespread in living organisms which coordinate the temporal patterns of internal physiology and behaviour with external, predictable rhythmic changes that occur in the environment. Most prominent, rhythms that specifically adapt organisms to the day/night cycles are known as circadian rhythms and those developed to deal with yearly-based changes (seasons in temperate regions of the planet) are referred to as annual (mainly seasonal) rhythms.

Circadian rhythms consist of endogenous (but entrainable) oscillations in organismal activities that have a periodicity of approximately 24 h and are found across the tree of life, from cyanobacteria and fungi to plants and animals (Dibner et al., 2010; Harmer et al., 2001; Wood and Loudon, 2014).

Circadian rhythms are the outcome of a circadian clock, a mechanism conserved in a wide range of organisms that includes the interaction of several regulatory proteins in a network of negative and positive feedback loops (Doherty and Kay, 2010). The different components of the central circadian clock can be organised into three categories: input elements that provide external information (typically light but also temperature) to entrain the system (Dibner et al., 2010), core elements that generate the endogenous oscillation and can be entrained by external cues (*Zeitgebers*) perceived by the former elements, and output elements that convey the clock-generated oscillation to the rest of the organism via hormones, neuropeptides and other means (Saunders, 2002).

The basic clock functional structure is explained by a transcription/translation feedback loop model (TTL) consisting of two feedback loops—one negative and a second one positive—whose elements are highly conserved in animals (Doherty and Kay, 2010; Özkaya and Rosato, 2012). Also some of the cis elements these transcription factors bind to seem to be highly conserved. For instance, E-boxes (consensus sequence CACGTG) are recognised by the heterodimeric clock transcriptional activator CLOCK:CYCLE in *Drosophila*, or

CLOCK:BMAL in mammals (Özkaya and Rosato, 2012; Yoshitane et al., 2014). However, some transcription factors have been described in *Drosophila* that are not homologous to any mammal counterpart. This is the case of VRILLE and PDP1, that constitute a second transcriptional feedback loop in *Drosophila* (Hardin, 2005), which recognise specific V/P boxes (consensus sequence TTATGTAA) in the promoter of gene *Clock*, the former inhibiting and the latter promoting, respectively, its transcription (Ozkaya y rosato). Although the circadian system of *Drosophila* has been the most well studied, the increasing availability of genomes from other insects has favoured the analysis of other insect circadian clocks. The most noteworthy difference between drosophilids and the rest of insects is the presence in the latter of a gene orthologous to the mammalian cryptochrome that is absent in *Drosophila* (Yuan et al., 2007). In fact, depending on the composition on cryptochrome genes, three types of circadian systems have been defined in insects (Yuan et al., 2007). Although the architecture of circadian clock cells varies between species of insects it appears that neurons important for circadian rhythms are generally found in a group of specialised neurons located in the region between the *protocerebrum* and the optic lobes (Numata et al., 2015).

Photoperiodism has been defined as “the use of changes in day length on an annual basis to regulate seasonal behavioural or physiological processes”(Dunlap et al., 2004). A wide range of organisms, mainly higher plants and animals living in terrestrial environments, use this “noise-free” information to control various seasonally appropriate switches in their metabolism, most of which have a clear functional significance or survival value. Photoperiodism was first discovered in plants (Garner and Allard, 1920), and shortly followed by the discovery of photoperiodism in animals, particularly in the vetch aphid *Megoura viciae* (Marcovitch, 1924). Indeed, insects, being poikilotherms, make their development prone to be directly influenced by the impact of the low temperatures of winter or, in phytophagous insects, indirectly by the effects of winter on their host plants (Saunders, 2002). In general,

insects have developed more or less complex life cycles in which they synchronise the appropriate phase of their life cycles with the season they are specialised for. Different insects overcome the adverse season following alternative strategies such as a programmed migration (Reppert et al., 2016), diapause (Denlinger and Armbruster, 2014; Omura et al., 2016) or developing specific morphs (Saunders, 2009; Simon et al., 2010; Tagu et al., 2005). In plants and mammals, the circadian clock and the photoperiodic response have been shown to be linked (Dardente et al., 2010; Imaizumi, 2010). Similar to what is known in vertebrates, a role for the circadian clock in insect photoperiodism has been proposed by several groups (Denlinger and Armbruster, 2016; Ikeno et al., 2010; Omura et al., 2016; Pegoraro et al., 2014; Reppert et al., 2016). However, the implication of the circadian clockwork in the insect photoperiodic response is not yet fully understood and some authors strongly put this proposal into question (Bradshaw and Holzapfel, 2010). In fact, the vetch aphid has classically been seen as a paradigmatic example of a non-circadian based model for the seasonal response known as the “Hourglass Model” (Lees, 1973). Nonetheless, the current model for the seasonal response of aphids involves a heavily dampened circadian oscillator in photoperiodic time measurement (Hardie and Vaz Nunes, 2001). However, in recent years, evidence for the involvement of circadian genes in the seasonal response in different insect species have been accumulating. RNAi experiments on circadian genes have shown to have an effect on both circadian rhythms and seasonal responses in the dipteran *Chymomyza costata* (Pavelka et al., 2003), the orthopteran *Modicogryllus siamensis* (Sakamoto et al., 2009), the hemipteran *Riptortus pedestris* (Ikeno et al., 2010; Omura et al., 2016), the lepidopteran *Antheraea pernyi* (Mohamed et al., 2014) and the dipteran *Culex pipiens* (Meuti et al., 2015). In addition, mutant alleles of circadian clock genes in *Drosophila* have been shown to correlate with differential seasonal responses (Pegoraro et al., 2014; Sandrelli et al., 2007). However, currently there is no consensus on the involvement of the circadian

system in insect photoperiodism (Bradshaw and Holzapfel, 2010; Dolezel, 2015; Goto, 2013; Košťál, 2011; Meuti and Denlinger, 2013; Numata et al., 2015).

Aphids are paradigmatic photoperiodic insects that have a strong seasonal response (Hardie and Vaz Nunes, 2001; Marcovitch, 1924). This response is characterised by the switch from viviparous asexual reproduction (parthenogenesis), typical of spring and summer, to the production of sexual morphs that, eventually, will produce a winter resistant egg in which the embryonic diapause takes place. In addition to the previously described cycle characteristic of holocyclic lineages, there are aphid anholocyclic species or lineages that have lost the ability to produce sexual morphs. The availability of the pea aphid *Acyrtosiphon pisum* genome sequence (The International Aphid Genomics Consortium, 2010) allowed the search for the pea aphid genes orthologous of those that constitute the circadian clock of *Drosophila melanogaster* and other insects. The main aphid clock genes that would participate in the two feedback loops (see above) were identified in our group (Cortés et al., 2010). By comparing the set of circadian clock genes identified in the pea aphid with those of other insects, the pea aphid circadian clock was classified in the ancestral type (both *Drosophila*-type and mammal-type cryptochromes are present) (Yuan et al., 2007). Despite extensive bibliography on the seasonal response of aphids, there is very little information on the molecular basis responsible and on the location of the putative photoperiodic clock elements and whether it would overlap with localisation of circadian clock elements. Studying the molecular bases of the pea aphid seasonal response will undoubtedly allow to gain knowledge on the molecular mechanisms that control aphid life cycles in general but also to learn on how diapause is regulated in insects. Microcauterisation experiments revealed that a region in the dorsal anterior *protocerebrum* encompassing *pars intercerebralis* and *pars lateralis*, was required for the production of parthenogenetic females in aphids reared under LD (Long Day, 16L:8D) conditions (Steel, 1978). Thus, it has long

been assumed that, as a result of the putative photoperiodic clock activity, specialised aphid cells in the *pars intercerebralis* of the *protocerebrum* known as group I of NSC (neurosecretory cells) would be responsible for sending the message to the developing embryos to determine their developmental fate, either as parthenogenetic embryos or as sexual eggs (Steel and Lees, 1977). In the present report, we have focused in taking a closer look at the circadian clock genes to analyse their putative role in aphid photoperiodism.

Characterisation of clock genes included a search for alternatively spliced transcripts. In addition, we analysed the rhythmic expression of circadian clock genes in holocyclic and anholocyclic strains in the context of photoperiodism. Finally, the anatomical location of clock neurons was revealed for the first time in aphids by *in situ* localising transcripts of central clock genes *period* (*per*) and *timeless* (*tim*).

2. Materials and methods

2.1. Aphid rearing and induction of seasonal response

Two strains of the pea aphid *Acyrtosiphon pisum* were used in the experiments: York Red 2 (YR2) and Gallur Rojo (GR), respectively sampled in York (UK) and Gallur (Spain). The first is our holocyclic reference strain and displays a typical seasonal response (i.e. shortening of photoperiod induces the seasonal response in autumn triggering the development of sexual morphs). The latter is our anholocyclic reference strain which develops by parthenogenesis irrespective of photoperiodic conditions. Both strains have been kept in our laboratory under LD (Long Day, 16L:8D) at 18°C as parthenogenetic clones on *Vicia faba* for more than 10 years.

Synchronised aphids at the third larval stage (L3) were split in two groups. One group, referred to as LD G0, remained under LD conditions. The second group was transferred to SD (Short Day, 12L:12D) conditions, thereby named SD G0. The next generation of aphids born from G0 individuals were named G1, which were kept under the same photoperiodic conditions as their respective mothers. Adult LD and SD aphids of the G1 generation were collected the day

after final moult by snap-freezing them in liquid nitrogen and stored at -70°C until RNA extraction. The induction of the seasonal response was confirmed by allowing 10-15 SD G1 aphids to give birth to the next generation (SD G2) for 7 days. These SD G2 aphids were allowed to reach adulthood and then inspected for the presence of males and sexual females. As expected, G1 aphids of the holocyclic strain (YR2) produced sexual morphs under SD photoperiod while aphids of the anholocyclic strain (GR) only produced parthenogenetic females. More specifically, 10 individual G1 aphids of the holocyclic strain produced a total of 178 sexual females and 6 males during the 7 days analysed. Contrarily, none of the aphids born from the 15 G1 aphids analysed of the GR strain during the first 7 days after onset of parturition was classified as sexual female or male.

2.2. Experimental characterisation of transcripts

To experimentally obtain gene transcripts, total RNA was extracted from heads of frozen individuals of the above-described aphid groups using the TRI Reagent® Solution (Ambion) following the manufacturer's protocol. Total RNA was quantified by spectrophotometry using a NanoDrop ND-1000 (Nanodrop Technologies, Inc.) and stored at -70 °C until cDNA synthesis. 1 µg total RNA was reverse transcribed using an oligo (dT)₁₈ primer and PrimeScript™ Reverse Transcriptase (Takara Bio Inc.). Primers for the identified genes (Table S1) were designed based on the predicted transcript models to amplify them through PCR on aphid cDNA. Amplified products were usually sequenced directly after purification (High Pure PCR Product Purification Kit, Roche) using adequate primers (Table S1).

2.3. Analysis of expression by RT-qPCR

RT-qPCR was used to quantify the expression of the six circadian clock genes in *A. pisum* individuals of the YR2 and GR strains reared under LD and SD conditions as explained above using primers based on constitutive exons shown in Table S1. Thus, four groups of aphids were analysed (YR2 LD, YR2 SD, GR LD and GR SD). For each of these groups, insects were sampled at

six different moments along the day/night cycle starting 3 h after lights went on or *Zeitgeber Time* 3 (ZT3) and collected at 6 h intervals (ZT9, ZT15, ZT21, and ZT3 and ZT9 in the second day). The self-sustainability of the rhythm of expression of genes *period* and *timeless* was evaluated in a separate set of aphids of the YR2 strain reared under LD conditions that was transferred to DD (continuous darkness) after reaching adulthood. These were sampled at 12 different moments along three consecutive subjective day/night cycles starting at CT3 and collected at 6 h intervals (i.e. CT3, CT9, CT15 and CT21 followed by the equivalent CTs for the 2nd and 3rd cycles). All samples were immediately frozen in liquid nitrogen and kept at -70 °C until RNA extraction. RT-qPCR was performed as described in Barberà et al. (2013). Relative expression for each sample was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) on values normalized to a reference sample placed in all experiments (inter-run calibrator). Differences in mean expression values between the variables strains, rearing photoperiods and moments of the day/night cycle were tested for statistical significance using ANOVA. In addition, homogeneity of variances was assessed by Levene tests. HSD Tukey and Bonferroni *post hoc* tests were applied when variances were homogeneous, and Games-Howell *post hoc* analyses when variances were not homogeneous. All statistical tests were performed with the SPSS Statistics package v22.0 software (IBM Corp).

When ANOVA tests revealed significant differences in expression between ZTs (or CTs in the DD experiment), a COSINOR analysis (Refinetti et al., 2007) was performed to test for circadian rhythmicity and to obtain the parameters defining the rhythm (MESOR, amplitude and acrophase).

2.4. RNA probe synthesis

RNA probes labelled with digoxigenin (DIG) were synthesised by a modification of the method described by Wülbeck and Helfrich-Förster (2007). First, we amplified cDNA regions of 737 bp and 789 bp for genes *period* and *timeless*, respectively. PCR products were purified by ammonium precipitation

and the amplicon size was checked by electrophoresis on agarose gels. Next, the purified PCR product was cloned into pGEM®-T easy vector (Promega) and used to transform DH5α *Escherichia coli* competent cells (Life Technologies). Then, colony PCR was used to screen for transformants with the appropriate inserts and those that revealed positive, were purified and sequenced from both extremes using SP6 and T7 primers to know the orientation of the insert. Subsequently, minipreps of selected colonies were performed to obtain high amounts of purified insert-containing plasmids using High Pure Plasmid Isolation Kit (Roche). The templates for the synthesis of riboprobes were obtained by simply doing a PCR with SP6 and T7 primers (see Table S1) on the corresponding purified plasmid and purifying the DNA fragments with the High Pure PCR Product Purification Kit (Roche). The purified PCR products were then quantified by spectrophotometry using a NanoDrop ND-1000 (Nanodrop Technologies) and used as a template for RNA transcription. Taking into account the insert orientation, purified templates were used to synthesise DIG-labelled sense and antisense riboprobes with RNAPol SP6 or RNAPol T7 (both from Roche). Sense probes were used as negative controls. Antisense probes were used to localise gene specific mRNAs. Transcription and DIG-labelling were carried out in 0.2 ml microcentrifuge tubes following the manufacturer's protocol (Roche). DIG-labelled probes were purified with mini QuickSpin RNA columns (Roche) following the manufacturer's instructions, diluted 50% with deionised formamide and stored at -20°C until use.

2.5. Aphid fixation and dissection

Insects were fixed in PFAT-DMSO (4% paraformaldehyde in 1X PBS, 0.1% TritonX-100, 5% DMSO v/v) over night at 4°C. Fixed individuals were washed 3 x 10 min in PBSTx (TritonX-100 0.1% in 1X PBS). Subsequently, dissections were carried out in glass block dishes on ice in cold PBSTx. The head capsules were carefully opened with tweezers and the central nervous systems (CNS), including brain and ganglia, were dissected out and pooled in

ice cold methanol immediately after dissection, washed twice in methanol, and stored at -20°C in tight sealed microcentrifuge tubes until ISH protocol was initiated.

2.6. Localization of transcripts by RNA in situ hybridisation

Localisation of transcripts was carried out using DIG-labelled RNA probes (see above). Fixed CNSs (see above) stored in 100% methanol were gradually rehydrated in PBTw (0.1% Tween 20 in RNase free 1X PBS), washed in PBTw, and treated with proteinase K (3 µg/ml) for 3 min at room temperature and 1 h on ice. Proteinase digestion was blocked with 2 mg/ml glycine. Next, CNSs were refixed in PFATw (4% para-formaldehyde in 1X PBS, 0.1% Tween 20) for 20 min and washed 5 times in RNase free PBTw for 5 min each. Then, the samples were incubated for 5 min in a 1:1 mix of PBTw and pre-hybridisation buffer (preHB; 5x SSC (saline sodium citrate), 50% formamide, and 0.1% Tween 20). Blocking was carried out by incubating for 2 h on a water bath at 65°C in hybridisation buffer (HB; preHB plus 100 µg/ml heparin, 100 µg/ml salmon sperm DNA and 500 µg/ml yeast tRNA, pH 6.0). Next, the blocked CNSs were hybridised by incubating with HB plus the corresponding probe overnight at 65°C in a water bath. Non-specifically hybridised probe was removed with the following washes at 65°C: two washes of 15 min in preHB, three washes of 15 min each in increasing mixes of preHB:2x SSC with 0.1% Tween 20 (3:2, 1:1, 2:3), a single wash of 15 min in 2x SSC with 0.1% Tween 20, followed by two washes of 15 min in 0.2x SSC with 0.1% Tween 20. At this point, the following washes were carried out at room temperature: three washes of 10 min each in increasing mixes of 0.2x SSC with 0.1% Tween 20: PBTw transition (3:2, 1:1, 2:3), and three washes of 5 min in PBTw. Next, CNSs were blocked by incubation for 2 h in PBTw supplemented with 1% Blocking Reagent (Roche) at room temperature with gentle shaking. Afterwards, the blocking buffer was replaced with fresh blocking buffer plus 1:1000 AP-conjugated anti-DIG Fab fragments (Roche) and incubated overnight at 4°C with gentle shaking. Two different AP substrates were used to

detect hybridised probes: NBT/BCIP that produce a blue/purple precipitate and Fast Red/HNPP that produces a chromogenic red and fluorescent precipitate (both from Roche). Both substrates were used following the manufacturer's instructions. The reaction was stopped by washing three times for 5 min in PBTw. Then, the tissues were immersed in increasing concentrations of glycerol (25%, 50%, 75% and 100%). Finally, the CNS were mounted on microscope glass slides and sealed with nail polish.

2.7. Microscopy and imaging

Bright field images of CNSs were obtained with a DS-Ri1 CCD camera (Nikon) mounted on an Eclipse E800 microscope (Nikon). Images of whole-mount fluorescent preparations of the CNS were obtained with a confocal laser scanning microscope (FV1000, Olympus) using 20x and 40x objectives. All brains were scanned with a scanning speed of 400 or 200 Hz, a pinhole of 1 Airy unit, a step size of 1.0–0.4 μm , and a line average of 2–4. Kalman filter was activated to remove noise signal. Preparations of transcripts by ISH using FastRed/HNPP as AP substrate were imaged with a 559 nm excitation laser. Images were processed and arranged with Fiji v1.50i software.

3. Results

3.1. Characterisation of gene transcripts and identification of putative regulatory elements

Transcripts of genes at the core of the pea aphid circadian clock were PCR amplified using different primer combinations (Table S1) and screened for splicing variants in aphids of the holocyclic (YR2) and the anholocyclic (GR) strains reared under summer-like long day (LD, 16L:8D) and winter-like short day (SD, 12L:12D) photoperiods (see Materials and Methods). Sequences of the experimentally obtained transcripts for the YR2 strain were deposited at the NCBI database (Table S2). Putative homologues of the pea aphid circadian clock genes were also searched for and identified in the genome of the aphid *Myzus persicae* (Table S3). Several splicing variants were identified for the

different pea aphid clock genes (Fig. S1). However, no association of specific transcript variants to a particular aphid strain or photoperiod could be identified.

For gene *per*, the splicing variant called *per-A* coincided with the transcript previously described (Cortés et al., 2010). Variant *per-B* included an in-frame extra exon (exon 4) upstream the conserved PAS A domain increasing the predicted protein in 56 amino acids (Fig. S1A). The predicted transcript and the gene structure (including hypothetical alternative splicing variants) were conserved in the identified *Myzus persicae per* homologue (MYZPE13164_O_v1_000089220).

In a previous work, the transcript of the gene homologous to the *Drosophila* gene *timeless (tim)* could not be fully characterised (Cortés et al., 2010). In the present work, previously missed regions at the N-terminal and C-terminal ends were experimentally obtained, thus completing the aphid's gene CDS (Fig. S1B). The pea aphid *tim* transcript consisted, including the CDS and UTRs, of 18 exons that were distributed in three different scaffolds in the pea aphid genome database. The first exon, found in scaffold GL349835 contained only UTR, although it was not annotated in the database. Exons comprising the gene CDS (exons 2 to 17) were located in scaffold GL349627 and were annotated as ACYPI36439 transcript prediction. Last coding exon 17 was also found in scaffold GL350105 along with the 3' UTR-containing exon 18 but no annotation was available. A total of five splicing variants were experimentally obtained although so called variants *tim-A* and *tim-B* were most abundant as judged by the intensity of the PCR-amplified bands and were the ones deposited in the database. It is noteworthy that proteins predicted from transcripts *tim-A* and *tim-B* differed only by the presence in the latter of exon 11 that codes for the NLS (nuclear localisation signal) (Fig. S1B). An equivalent putative CDS was found in a single scaffold of the *M. persicae* genome (MYZPE13164_O_v1_000088930). *M. persicae tim* transcript sequence and predicted exon-intron structure was generally conserved with respect to that of

A. pisum.

The amplification of the CDS of the aphid gene homologue to the *Drosophila* gene *Clock* (*Clk*) resulted in a sequence that could be arranged in 14 exons and three transcript variants named *Clk*-A, *Clk*-B and *Clk*-D (Fig. S1C). Variant *Clk*-A encoded a transcript with the conserved regions previously described by Cortés et al. (2010). Variant *Clk*-B included an intronic sequence that introduced a stop codon at the end of exon 2 (Fig. S1C). However, the presence of a putative alternative initial methionine downstream could still allow translation initiation preserving the conserved domains described for this gene, including the bHLH (basic helix-loop-helix). The variant *Clk*-D differs from *Clk*-A in that the region encoded by exon 11 is absent, although this did not interrupt the ORF. A putative orthologue of *Clk* was identified in *M. persicae* (MYZPE13164_O_v1_000050320), in which the presence of splicing signals suggests that an alternative splicing of exon 11 is also possible as in *A. pisum*. Since no conserved domains were identified in exon 11, no putative functionality could be associated to this alternative splicing (Fig. S1C).

The amplification of aphid transcripts homologous to the *Drosophila* gene *cycle* (*cyc*) revealed four main splicing variants that we named *cyc*-A, *cyc*-B, *cyc*-C and *cyc*-F (Fig. S1D). Of these, the splicing variant *cyc*-A corresponded to the previously described transcript (Cortés et al., 2010). The predicted protein encoded by variant *cyc*-B was similar to that predicted from transcript *cyc*-A except for the presence in the former of an extra 31 amino acids region encoded by the alternative exon 4 (Fig. S1D). No conserved domains were found within the extended region. Transcript variant *cyc*-C lacked exons 3 to 8 which resulted in a stop codon in exon 9 which putatively would produce a truncated protein. However, use of an alternative Met at the end of exon 9 would still produce a protein that would lack the conserved domains bHLH, the NLS, the NES (Nuclear Export Signal) and most of the PAS A (Fig. S1D). The search and analysis of the *M. persicae* putative orthologous *cycle* gene (MYZPE13164_O_v1_000093520) showed that the structure of exons was

conserved among both aphid species.

The pea aphid homologue of the *Drosophila* gene *PAR-domain protein 1* (*Pdp1*) was amplified yielding two transcript variants (Fig. S1E) that coincided with those previously described (Cortés et al., 2010). Both variants encoded similar proteins, except that *Pdp1*-B lacked 21 bp at the end of exon 4 encoding the heptapeptide motif VKKSRKQ. Conservation of a Q-rich domain present in Diptera and Hymenoptera seems to be low in the aphid predicted PDP1 protein, and the A-rich domain specific of *Drosophila* is apparently absent in the aphid. A search in the *M. persicae* genome revealed the presence of a putative orthologous *Pdp1* gene (MYZPE13164_O_v1_000172150), which included the two alternative donor sites at conserved positions, thus putatively allowing alternative splicing of an equivalent region of 21 bp. This suggests that the splicing variants differing in the presence or absence of the heptapeptide motif in the encoded protein mentioned above would also be present in *M. persicae*.

The amplification of the aphid homologue to the *Drosophila* gene *vri* (*vri*) produced two new splicing variants (Fig. S1F) in addition to that already described by Cortés et al. (2010). The new splicing variants differ from the previously sequenced transcript in that both contain a shorter exon 3 and lack exon 4. The two newly described transcript variants use different donor sites at the end of exon 3 and different acceptor sites on exon 5 which keep the same ORF in the latter exon. Thus *vri*-A contains an extra peptide that corresponds to the 5' region of exon 5. The variability in the C terminal region did not affect the conserved domains described for this gene (Fig. S1F). At the amino acid level, the new models contain the already described BRLZ and G/S domains described for *Drosophila* (Cyran et al., 2003). The putative aphid VRI would also contain two Q-rich domains that could be specific of aphids (Fig. S1F). For the different clock genes, the genomic region upstream the putative initial methionine was screened for elements described to be relevant in circadian regulation such as E-boxes and V/P-boxes (Fig. S2). Although several of these

elements were putatively identified in all clock genes analysed, comparisons between corresponding sequences from the two aphid species *A. pisum* and *M. persicae* revealed a high degree of conservation only for genes *per*, *cyc*, *Pdp1* and, to a certain degree, for *vri*. The genomic regions upstream of *per* exons in *A. pisum* revealed a group of four canonical E-boxes, distributed in two pairs that were located approximately 5.4 kb upstream the putative initial methionine (Fig. S2A). The number of putative E-boxes, their distribution and their distance to the first exon in *M. persicae* gene *per* was very similar (Fig. S2A). Although the three scaffolds of the pea aphid genome that contained fragments of gene *tim* included E-box and V/P-box elements, their number and position were not conserved between the two aphid species (Fig. S2B). The search in upstream regions of *A. pisum* and *M. persicae* *cyc* gene sequences revealed V/P-boxes in both species and one E-box in *A. pisum* (Fig. S1D). Furthermore, the presence of V/P-boxes in the intronic region downstream of exon 1 of *cyc* was particularly conserved between *A. pisum* and *M. persicae* (Fig. S2D). The search for regulatory elements in the *Pdp1* gene included genomic regions upstream and downstream exon 1 (Fig. S2E). It is noteworthy the finding of two E-box elements upstream and three E-boxes in the intron between exons 1 and 2, conserved in number and distribution in both aphid species. Additionally, a V/P-box was found only in the *M. persicae* upstream region (Fig. S2E). The search of regulatory elements in gene *vri* revealed two E-boxes upstream exon 1 and three E-boxes in the intron between exons 1 and 2 in *A. pisum* (Fig. S2F). Even though the number of E-boxes was conserved between *A. pisum* and *M. persicae*, their distribution along the genomic sequence differed. In addition, a V/P-box was also found in the intronic region in both species.

3.2. Quantification of expression of aphid clock genes

Visual inspection of expression profiles (Fig. 1) revealed a similar pattern of expression in genes *per* and *tim* that clearly differed between LD and SD aphids of the YR2 strain. A similar pattern for both genes, close to that

observed in YR2 aphids grown under LD, was also observed in aphids of the GR strain but no differences were observed between aphids reared under LD and SD photoperiods for gene *per* and only minor differences were observed for gene *tim* (Fig. 1). Apart from differences in expression at particular ZTs for some genes (Fig. 1), significant circadian rhythms were only detected for genes *per* and *tim* in YR2 aphids reared under LD (Table S4). For both genes, the peak of expression (acrophase) occurred at the end of the photophase (ca. ZT13). In general, expression of core clock genes was higher at any ZT in aphids reared under SD photoperiod in the strain YR2, except for *vri* and *Pdp1* (Fig. 1). Contrarily, only *Pdp1* and *vri* showed higher expression in SD for the GR strain.

When mean expression levels were compared between aphids of both strains reared under different photoperiods (Fig. 2), the four genes involved in the negative feedback loop of the circadian clock (*per*, *tim*, *Clk* and *cyc*) showed significant higher expression in aphids of the holocyclic YR2 strain reared under SD than in aphids reared under LD. Contrarily, expression of these genes remained constant between both photoperiods studied in anholocyclic GR aphids (Fig. 2). Of the two genes included in the second, positive feedback loop of the aphid circadian clock, *Pdp1* showed higher expression in SD aphids in both strains, while *vri* only expressed differently between photoperiods in the GR strain (Fig. 2).

In order to confirm the self-sustainability of the circadian rhythmicity detected for genes *per* and *tim* their expression in YR2 aphids reared under DD (constant darkness) conditions (Fig. 3, see Materials and Methods). The expression pattern of these genes during the first subjective day/night cycle was similar to that previously seen in LD (Fig. 1), showing a peak of expression at the end of the subjective photophase and a trough at the end of subjective scotophase. However, this rhythm was almost lost in the subsequent second and third cycles pointing to a quick dampening with heavily diminished amplitude for both genes (Fig. 3). In fact, the COSINOR analysis of

per and *tim* expression in DD did not show a significant rhythm in either of these genes. However, when the COSINOR test was restricted to CTs corresponding to the first subjective day/night cycle in DD (Table S4), a significant rhythm for *tim* with a peak of expression similar to that of LD was detected, but with an amplitude an order of magnitude lower (Fig. 3).

3.3. Localisation of *per* and *tim* transcripts in the aphid brain

The antisense probe against the aphid *per* homologue successfully located its transcripts in CNSs obtained from adult aphids of the YR2 aphid strain (Fig. 4). The specificity of the probe was confirmed by the lack of signal in the hybridisations carried out without probe (not shown) or using a *per* sense probe (Fig. 4B), apart from the typical tracheal background staining. *per* transcripts were only detected in the aphid brain (Fig. 4C). Particularly, *per* expression was routinely located in three cell clusters that were named dorsal neurons (DN), lateral neurons (LN) and lamina neurons (LaN) respectively (Fig. 4C), following the nomenclature used in other insects originally adopted from *Drosophila melanogaster* (Helfrich-Förster, 2002). The DNs expressing *per* were located in the most anterior dorsal region of each brain hemisphere, also known as *pars lateralis* (Fig. 4C-D), within the deeper layers of the protocerebral soma cortex, close to the underlying neuropil. *per* transcript detection in whole mount preparations did not allow to clearly distinguish the number of cells in the DN cluster, although it seemed to consist of six to eight neurons with small soma (Fig. 4D). The LN cluster was located in the boundary between the optic lobe and the *protocerebrum* (Fig. 4C and E-G), within or near the dorsal anterior region described as *lobula*. Generally, the LN group consisted of four neurons found anteriorly in the cortex surrounding the *lobula*. A detailed inspection of these neurons revealed that the LN cluster could be subdivided in two groups designated vLN (ventral lateral neurons) and dLN (dorsal lateral neurons). The vLN consisted of three cells located in the lower layers of the soma cortex, close to the underlying neuropil (Fig. 4E). The single dLN was found in intermediate layers of the soma cortex, in a region located

dorsally and distally respect to the vLN (Fig. 4F). The third group of neurons expressing *per* was found in the posterior region of the *lamina*, the most outer region of the optic lobe, thereby named LaN (Fig. 4C). A maximum of four cells expressing *per* were found in this group.

In addition to the localisation of *per*, localisation of *tim* transcripts in the CNS of *A. pisum* was also investigated using the corresponding antisense probe on YR2 aphids (Fig. 5). The specificity of the probe was confirmed by the absence of signal in brains hybridised with the sense probe (Fig. 5A), apart from the unspecific staining routinely seen in the tracheae (see above). Detectable levels of *tim* expression were found exclusively in the brain, in two of the regions previously described for *per in situ* hybridisation (Fig. 5). Therefore, the nomenclature used to describe *tim* expressing neurons followed that used for *per* (see above). Two groups of neurons were found to express *tim* (Fig. 5B) that were designated dorsal neurons (DNs) and lateral neurons (LNs) respectively. The DNs expressing *tim* were found in the antero-dorsal region of the *protocerebrum* known as *pars lateralis* (Fig. 5B-C). This group consisted of two distinguishable types of cells: a subgroup consisting of two clearly defined neurons with large soma, expressing high levels of *tim*, named large DN (l-DN), and another subgroup of six to eight cells with small soma and lower *tim* expression adjacent to the l-DN that were named small DN (s-DN) (Fig. 5C). The LN cluster of *tim* expressing neurons was located in the region between the *protocerebrum* and the optic lobe (Fig. 5B and D-F). In this case, *tim* staining also revealed two subgroups of LN in a pattern similar to that described for *per* expression (see above). The first subgroup consisted of a single *tim* LN located dorsally and slightly lateral respect to the rest of the LN cluster that was named dorsal lateral neuron (dLN) (Fig. 5D and F). The rest of the LN expressing *tim*, usually three to four neurons, were found ventro-medially with respect to the dLN and were named ventral lateral neurons (vLN) (Fig. 5E and F). Contrarily to *per* localisation, no detectable expression of *tim* was found in the LaN. Although localization of *per* and *tim*

expression in the aphid brain was first investigated in the holocyclic reference strain YR2 under conditions of LD, we also investigated the expression of these genes in the anholocyclic strain (i.e. strain GR) and found that both *per* and *tim* genes expressed in the same groups of neurons described above for the YR2 strain (Fig. 6).

The similarity in the arrangement of *per* and *tim* expressing neurons led us to check the possibility that both genes are being co-expressed in the same brain neuron clusters. As we had available DIG-labelled probes for both genes, we used them in combination to simultaneously detect both transcripts in the CNSs of the pea aphid (Fig. 7, also compare with preparations in which the single probes were used shown in Fig. 4 and Fig. 5). The comparison of the number of cells between the single probe and the combined probe hybridisations showed no more than eight s-DN and two l-DN (Fig. 7A). The number of s-DN expressing *per* or *tim* individually were the same than the number of neurons in the combined probe approach (Fig. 7A). Therefore, it could be assumed that *per* and *tim* are being coexpressed in the s-DN. Since no detectable expression of *per* in the l-DN was observed, it is suggested that only *tim* would be expressed in these cells. With respect to the LN cluster (Fig. 7B-C), the same number of neurons could be identified in individual and *per* + *tim* simultaneous hybridisations, indicating that *per* and *tim* were coexpressed in the LNs. All these observations are summarised in Fig. 7D.

Finally, we checked the correspondence between our RT-qPCR results for *per* and *tim* genes and the *in situ* detection of its transcripts. In order to do so, we performed *in situ* hybridization experiments to localize *per* and *tim* expression in aphid brains from adult aphids sampled at ZT15 and ZT21 time points, representative of a peak and a trough of gene expression respectively (see above). Both *per* and *tim* transcripts were detected in DN and LN in aphid brains frozen at ZT15 while no signal was detected for both genes in brains frozen at ZT21 (Fig. 8).

4. Discussion

In the present report we have presented results that provide new insights into the characterization of the aphid circadian system and on its possible role (or of some of its elements) in aphid photoperiodism.

4.1. Gene characterisation

In order to determine a possible involvement of the circadian clock machinery in the aphid seasonal response, it is necessary to first understand the regulation and diversity of its elements. In this respect we have experimentally characterized the whole CDS of 6 aphid clock genes homologous of those that constitute the two core feedback loops that control the circadian clock in *Drosophila*. In addition we have identified orthologues of these genes in the genome of a second aphid species (*Myzus persicae*) available at http://bipaa.genouest.org/is/aphidbase/myzus_persicae/ and showed the high degree of conservation of these genes in both aphid species. Interestingly, we observed that, in *A. pisum*, all clock genes analysed exhibit a more or less extensive alternative splicing, although no correlation between the presence of specific transcript variants and aphid strain or photoperiod conditions (i.e. LD and SD) could be established. Some of the transcript variants for some genes might generate truncated proteins lacking some of the conserved domains essential for its functionality within the circadian clock (e.g. see *Clk* and *cyc* splicing variants in Fig. 1 C and D, respectively). Moreover, the presence of conserved alternative splicing sites in the *M. persicae* corresponding sequences, allows prediction of equivalent alternative transcripts for most variants in clock genes of this aphid species. In addition to splicing variants for clock genes, here we provide the characterization of a complete CDS for the aphid gene *timeless* that was only partially characterized in a previous report (Cortes 2010). In this respect, it is worth to compare *tim-A* and *tim-B* variants (Fig. 1 B), which differ in the inclusion or not of the NLS-containing exon, which could be associated with a possible regulation of the translocation to the nucleus of the transcription factor TIM, as it has been shown in *Drosophila* (Saez and Young, 1996). Moreover, the experimental characterisation of *vri*

transcripts in *A. pisum* resulted in an ORF that differed from that previously defined for this gene (Cortés et al., 2010) and from predictions available in Aphidbase. Also interesting, the two alternative splicing variants described here for *Pdp1*, leading to two versions of a protein differing in the presence of a heptapeptide seem to be conserved across insects since an equivalent splicing leading to equivalent PDP1 proteins have also been described in *Drosophila* (Reddy et al., 2000). Precise quantification and knocking down specific splicing variants of circadian clock genes will shed light on the functional relevance, if any, of the extensive alternative splicing observed for most clock genes in the present report and remains to be investigated. We do not discard, however, the possibility that the splicing machinery in aphids has a high failure rate and that (some of) the observed variants result from an inaccurate splicing process.

4.2. Quantification of clock gene expression

Most relevant for insect circadian rhythm studies, our present results demonstrated a robust circadian rhythmicity at the mRNA level for genes *per* and *tim* with a maximum of expression at dusk (Fig. 1 and Fig. 8) thus confirming previous results (Cortés et al., 2010). Since, differently from *Drosophila*, but similar to other insects, aphids have a mammal-type cryptochrome (CRY-m or CRY2) (Cortés et al., 2010), it is likely that it would participate along with PER and TIM as a repressor of the CLK:CYC heterodimer thus relieving their own transcription. Similar expression patterns have been described for *per* and *tim* homologues (and also for *cry-m*) in several other insect species from different orders, including *Drosophila* and other Diptera (Gentile et al., 2009; Muguruma et al., 2010) as well as representatives of Hymenoptera (Bertossa et al., 2014; Ingram et al., 2012; Mukai and Goto, 2016; Rubin et al., 2006), Orthoptera (Moriyama et al., 2009; Sakamoto et al., 2009), Blattodea (Werckenthin et al., 2012) and Zygentoma (Kamae and Tomioka, 2012), among others.

On the other hand, while the present data do not show a clear rhythm in the

expression of *cyc*, *Pdp1* or *vri*, previous results obtained on pea aphid heads (Cortés et al., 2010) exhibited a circadian rhythm with a peak at dawn of *cyc* and *vri* transcripts, that is, in antiphase with *per* and *tim*. A peak of expression of *cyc*, or of its functional equivalent in *Drosophila*, *Clk* (Özkaya and Rosato, 2012), in antiphase with *per*, *tim* and *cry-m*, has been reported in other insect species (Gentile et al., 2009; Ingram et al., 2012; Rubin et al., 2006). Thus, at least for gene *cyc*, it is possible that its expression oscillates with very low amplitude, making it difficult to distinguish a circadian rhythm from background changes in expression (i.e. noise). The absence or difficulty to detect a circadian rhythm at the mRNA level in clock genes is not unprecedented in other insect species. In fact, a lack of a robust mRNA rhythm was observed for some of the circadian clock genes of *Riptortus pedestris* (Ikeno et al., 2008). Moreover, a circadian rhythm at the protein level as seen in *Rhodnius prolixus* (Vafoopoulou et al., 2010) or at the level of post translational modifications (Yu et al., 2006) cannot be ruled out and should be investigated. A remarkable trait of the aphid circadian clock derived from our present work is the lower expression and the rapid dampening of *per* and *tim* expression under DD (continuous darkness, 0L:24D), in which the rhythm is notably lost already on the second subjective day (Fig. 3). The low expression in DD can be tentatively explained by the absence of TIM degradation induced by light. In continuous darkness, the destabilisation of the putative complex PER:TIM:CRY-m would be averted and consequently, PER:TIM:CRY-m would continuously repress CLK:CYC activation of *per* and *tim* transcription. Hence the characteristic self-sustainability of circadian clock genes (Özkaya and Rosato, 2012) would be, at least, questionable in the pea aphid. In this respect, it is interesting that aphids are the only insects in which JETLAG is absent, but still retained CRY-d (or CRY1) and TIM (Cortés et al., 2010). Consequently, the yet unknown mechanism of degradation of TIM triggered by light is likely different from the one described in *Drosophila* (Özkaya and Rosato, 2012) and probably specific of aphids. However, it is important to recall here the work by (Hardie and Vaz

Nunes, 2001), in which a dampened circadian oscillator model for the seasonal response of aphids is suggested as an alternative to the classical view of an hourglass-like system. Thus, the quick dampening in the rhythm of expression of *per* and *tim* under DD shown in Fig. 3 supports this model. A heavily dampened oscillator model has also been proposed to replace the hourglass model in the mite *Tetranychus urticae* (Saunders, 2010). Even though there are very few studies on circadian rhythms in aphids, available data suggest that most of their activity would take place during the photophase (Jeon et al., 2003; Joschinski et al., 2016; Narayandas and Alyokhin, 2006). In order to confirm a role of genes *per* and *tim* in the diurnal rhythmicity of aphids, it would be necessary to analyse the effect in the described rhythms of knocking down the expression of these genes (e.g. through RNAi).

The absence of a circadian rhythm in *Pdp1* expression (Fig. 1) contrasts with previous reports in which *Pdp1* was found to oscillate (Cortés et al., 2010). This disagreement may be consequence of the lower resolution in sampling of our data. Regarding expression of *vri*, even though we did not observe a statistically significant rhythm, both present (Fig. 1) and previous data (Cortés et al., 2010) confirm a peak of expression late at night. The different profiles of expression of *vri* and *Pdp1* displayed in the pea aphid contrast with the synchronous increase of *vri* and *Pdp1* transcripts seen in *Drosophila* (Cyran et al., 2003), mosquitoes (Gentile et al., 2009) and the fire ant *Solenopsis invicta* (Ingram et al., 2012). Regarding the putative role of *Pdp1* as an activator of *Clk* and *cyc* expression, it is significant the similarity of *Pdp1*, *Clk* and *cyc* expression profiles (Fig. 1). In this respect, the distribution of regulatory elements in the upstream regions of circadian clock genes agrees with some of the putative roles as part of the oscillatory mechanism. This is shown by the presence of conserved E-box elements in *per* and *Pdp1* (Fig. S1 A and E, respectively) that would agree with their transcription activation by the heterodimer CLK:CYC, as seen for other insects (Kobelková et al., 2010; Mohamed et al., 2014; Reppert et al., 2016; Yu et al., 2006). Our finding that

V/P-boxes were conserved between the two aphid species in *cyc* but not in *Clk* (Fig. S2) points to a *cyc*-centred circadian clock in aphids. This hypothesis is further confirmed by the presence of the transactivation domain (T) in CYC instead of CLK (Fig. S1), as is generally seen in non-drosophilid insects (Ingram et al., 2012; Rubin et al., 2006).

4.3. Clock genes and photoperiodism

The study of photoperiodism in insects has been dominated by the controversy on whether the circadian clock plays a central role in the regulation of the seasonal response or if, contrarily, it is controlled by an hourglass-like system in which no circadian oscillator is involved (Dolezel, 2015; Košťál, 2011; Saunders, 2010). However, evidence of participation of circadian clock genes in photoperiodic responses have been accumulating in recent years in different insect species (Ikeno et al., 2010; Meuti et al., 2015; Mohamed et al., 2014; Omura et al., 2016; Pavelka et al., 2003; Pegoraro et al., 2014; Sakamoto et al., 2009). Nevertheless, some authors claim that those results should be taken cautiously, as the possibility of a pleiotropic effect (i.e. a role of a particular circadian clock gene independent of the circadian clock itself) could lead to misleading conclusions (Bradshaw and Holzapfel, 2010). In this respect, recent experiments in *Drosophila* analysed the effect of certain mutants of circadian clock genes in the seasonal response showing the involvement of clock genes as a module in photoperiodic time measurement, rather than being a pleiotropic effect of a single gene (Pegoraro et al., 2014). Aiming at elucidating a putative participation of circadian clock elements in the seasonal response of the pea aphid, expression of clock genes was compared between groups of aphids reared under two alternative photoperiods simulating summer and winter conditions (LD and SD photoperiods, respectively). In addition, we compared the expression of clock genes in two aphid strains, one exhibiting a robust photoperiodic response and a second strain that lacks this ability. Therefore, any clock gene showing different patterns of expression between photoperiods and/or strains could be

considered as a candidate to further studying its involvement in the photoperiodic response in aphids. Our results show that changes in expression between LD and SD photoperiods are observed mainly in YR2 but not in GR aphids (Fig. 2), pointing towards a participation of at least some of the circadian genes in the seasonal response in *A. pisum*. The fact that genes *per*, *tim*, *Clk* and *cyc* display this differential regulation depending on the photoperiod support their coordinated involvement in the negative feedback loop of the circadian clock. In other insects, several studies have shown an effect of diapause inducing photoperiod (usually SD) in the regulation of circadian genes. The influence of the switch to winter photoperiod includes phase shifts in circadian clock genes (Iwai et al., 2006; Kobelková et al., 2010; Stehlík et al., 2008; Werckenthin et al., 2012) and an increase of circadian clock genes expression under SD respect to LD (Bajgar et al., 2013; Doležel et al., 2005; Hodkova et al., 2003; Sakamoto et al., 2009; Syrová et al., 2003). Some authors also compared mutant strains that do not enter diapause under winter photoperiod and found that the increase in expression of circadian clock genes under SD did not occur in the non diapausing mutant (Syrová et al., 2003), as seen in the pea aphid GR strain for genes *per*, *tim* *Clk* and *cyc* (Fig. 2). However, in other insects such as *Riptortus pedestris* (Ikeno et al., 2008), *Protophormia terranova* (Muguruma et al., 2010), *Nasonia vitripennis* (Mukai and Goto, 2016), *Bombyx mori* (Iwai et al., 2006) and *Culex pipiens* (Meuti et al., 2015), the change in photoperiodic conditions did not affect the overall transcript levels of circadian clock genes but rather their circadian phase. The effect of knocking down circadian clock genes in photoperiodism has been reported in several insects (Ikeno et al., 2010; Meuti et al., 2015; Mukai and Goto, 2016; Omura et al., 2016; Pavelka et al., 2003). In all these species, knocking down of the negative elements of the circadian clock (i.e. *per*, *tim* or *cry-m*) averted diapause in SD conditions, while knocking down the positive elements induced diapause in LD conditions. Assuming that circadian genes participate in the seasonal response of the pea aphid and that their functions

are conserved in insects, knocking down (e. g through RNAi) the expression of genes *per* and *tim* in aphids reared in winter-like SD conditions would avert the seasonal response. Contrarily, knocking down *Clk* or *cyc* expression would induce the sexual phase in aphids reared under summer-like LD photoperiod.

4.4. Localisation of the central circadian clock in the aphid brain

Our results on the localisation of clock gene transcripts allow defining the places where the central circadian clock may be located in aphids. Our *in situ* hybridisation (ISH) experiments against *per* and *tim* transcripts revealed two regions of the aphid brain that expressed these two circadian genes: the DN (Dorsal Neurons) cluster located in the cortex of the *pars lateralis*—anterodorsal— of the *protocerebrum*, and the LN (Lateral Neurons) cluster located in the anterior cortex of the optic *lobula* (Fig. 4, Fig. 5 and Fig. 7). The localisation of both *per* and *tim* transcripts in the antero-dorsal *protocerebrum* and the regions between the *protocerebrum* and the *lobula* was similar to that found in other insects. A recent review by (Numata et al., 2015 and references within) showed that the latter regions are usually associated with control of circadian rhythms in flies, cockroaches, the hemipterans *Rhodnius prolixus* and *Riptortus pedestris* (Ikeno et al., 2014; Vafopoulou et al., 2010; Vafopoulou and Steel, 2012), and crickets. The role of the *per* expressing neurons described as LaN in the outer layers of the optic lobe needs to be further investigated.

It should be noted that, interestingly, the DN cluster is located in the *pars lateralis*, a region previously found to be essential for the photoperiodic response (Steel, 1978; Steel and Lees, 1977). Indeed, microcauterisation experiments revealed that this region was essential for the development of parthenogenetic females under LD conditions. Furthermore, it was shown that the *pars lateralis* controlled the group I of NSC (neurosecretory cells) in *pars intercerebralis* (Steel and Lees, 1977). This group I of NSC contained an unknown factor named “virginoparin” that promoted the production of parthenogenetic females under LD photoperiod. Hence, the effect of the

ablation of certain neurons in the *pars lateralis* on the mode of reproduction could be consequence of the elimination of the DNs expressing *per* and *tim*, suggesting an involvement of these genes in the aphid photoperiodic response. In other insects, clock neurons equivalent to the aphid LN are known to participate in the photoperiodic response. For instance, in the hemipteran *Rhodnius prolixus*, a group of neurons homologous to the LN that express PER and PDF (Pigment Dispersing Factor), a known circadian clock output, are known to regulate circadian secretion of PTTH (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012). In *Plautia stali*, neurons located in *pars lateralis* and *pars intercerebralis* of the *protocerebrum* are known to inhibit JH secretion from the *corpus allatum* (Matsumoto et al., 2013), although no localisation of circadian clock genes in these regions has been published so far. In *Riptortus pedestris*, removal of a group of neurons in the optic lobe medulla that expressed PDF, averted diapause under winter-like SD photoperiod (Ikeno et al., 2014). However, RNAi against *Pdf* did not affect the seasonal response of *R. pedestris* concluding that, even though PDF was not essential for diapause, the neurons containing PDF were essential. Therefore, it seems that regions in insect brains known to express circadian clock genes, i.e. the *pars lateralis* in the dorsal *protocerebrum* and regions between the *protocerebrum* and the optic lobes, are usually involved in regulation of circadian rhythms and photoperiodism (Dolezel, 2015; Numata et al., 2015).

As it has been achieved in other insects such as *Drosophila* (Helfrich-Förster et al., 2007) and cockroaches (Wen and Lee, 2008), it would be interesting to explore the detection of the proteins PER and TIM —and other circadian clock proteins— in the pea aphid would allow to identify axonic processes between different regions of the brain, including putative interactions among clock neurons and between them and the group I of NSC in *pars intercerebralis* as proposed by Steel and Lees (1977). Moreover, antibody detection would allow evaluation of a putative circadian rhythm in protein levels individually for each clock neuron, as well as its subcellular location. Finally, subcellular localisation

of the transcription factors PER and TIM has been found to be an important step in circadian clock regulation (Meyer et al., 2006). Detection of these proteins with antibodies would allow exploring if this circadian feature is conserved in aphids.

The present report provides new advances in the characterisation of the aphid circadian clock genes. We have also confirmed some characteristics of the circadian clock such as the rhythmic expression of *per* and *tim*. Most importantly, we described for the first time the localisation of the clock neurons in the aphid brain classified in three groups: DN, LN and LaN. In addition, we suggest that the DN in *pars lateralis* probably regulate the secretion of “virginoparin” from group I of NSC in *pars intercerebralis*. Therefore, at least some elements of the aphid circadian clock would participate in the regulation of the seasonal response. Nonetheless, in order to confirm this, further investigations on this set of genes would be needed, especially involving the application of RNAi and antibody detection experiments.

Acknowledgements

Myzus persicae DNA sequence data were downloaded from AphidBase, <http://www.aphidbase.com/>. Funding for *Myzus persicae* clone G006 genomic sequencing was provided by USDA-NIFA award 2010-65105-20558. Funding for *Myzus persicae* clone O genomic sequencing was provided by The Genome Analyses Centre (TGAC) Capacity and Capability Challenge programme project CCC-15 and BB/J004553/1 from the Biotechnology and Biological Sciences Research Council (BBSRC) and the John Innes Foundation. The facilities at the Genomics, Microscopy and Greenhouse services at the SCSIE (Universitat de València) were used for sequencing, microscopy image analysis and for growing *Vicia faba* plants, respectively.

Formatting of funding resources

This work was supported by project CGL2015-68188-P. M.B. and J.M.C.A. enjoyed PhD fellowships (BES-2009-026077 and BES-2012-052837, respectively) from the Spanish MICINN and MINECO respectively.

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FIGURE LEGENDS

Figure 1. Expression profiles of the circadian clock core genes obtained for aphids of the holocyclic strain YR2 (left) and aphids of the anholocyclic strain GR (right). The expression results from aphids reared under LD (Long Day, 16L:8D) are represented by black circles and those of SD (Short Day, 12L:12D) by black triangles. The mean relative expression of three replicates $\pm$ SEM are plotted at 6 h intervals over 1.5 days. Significant differences in expression at different time points are indicated in bold and italic letters for LD and SD photoperiods, respectively. The dark phase of the photoperiods is indicated as grey background ranging from ZT12 to ZT24 in SD and ZT16 to ZT24 in LD. The red curves represent the circadian rhythm estimated by the COSINOR software for those genes showing a significant circadian rhythm (see Table S4).

Figure 2. Comparison of levels of expression of the circadian clock genes between aphid strains and rearing photoperiods. Bars represent the mean relative expression calculated for all ZT within a strain and photoperiod $\pm$ SEM. Letters on top of each bar represent the subgroup significance (p -value < 0.05) of the ANOVA test comparing the variable photoperiod for each gene.

Figure 3. Expression profiles of circadian clock genes *period* and *timeless* along three consecutive days under DD. Mean relative expression values of three replicates $\pm$ SEM are plotted at 6 h intervals over three days. The subjective dark phase of the photoperiods is represented as dark grey. The red curves represent the circadian rhythm estimated by the COSINOR software for those genes with a significant ($p < 0,05$) circadian rhythm (see TableS4).

Figure 4. *In situ* localisation of *per* transcripts in brains of adult individuals of *A. pisum*. Section B shows an *in situ* hybridisation revealed with NBT/BCIP substrate; sections C-D show *in situ* hybridisations revealed with Fast

Red/HNPP substrate. A) Dorsal view of a dissected central nervous system (CNS) including the brain (BR), the suboesophageal ganglion (SOG), the thoracic ganglionic mass (TGM). The neuromeres corresponding to the SOG and the TGM are respectively indicated with numbers (S1, S2, T1, T2 and T3). B) Control hybridisation with *per* sense probe. C) Z-stack confocal image overview of the localisation of *per* transcripts in the aphid brain including the DN, the LN and the LaN. D) Z-stack confocal image of the most dorsal slices showing a detail of the DN localisation. E) Detail of the image stack shown in C indicating the distribution of dLN in red. F) Detail of the image stack shown in C indicating the distribution of vLNs in yellow. G) merge of E and F. Scale bars = 50 μ m.

Figure 5. *In situ* localisation of *tim* transcripts in brains of adult *A. pisum* aphids. Section A shows an *in situ* hybridisation revealed with NBT/BCIP substrate; sections B-F show *in situ* hybridisations revealed with Fast Red/HNPP substrate. A) Control hybridisation with *tim* sense probe. B) Z-stack confocal image showing an overview of the localisation of *tim* transcripts in the aphid brain including the DN and the LN. C) Z-stack confocal image of the dorsal region showing the two subgroups of DN: the l-DN and the s-DN. D) Detail of the image stack shown in B indicating the distribution of the dLN in red. E) Detail of the image stack shown in B indicating the distribution of vLNs in yellow. F) merge of D and E. Scale bars = 50 μ m.

Figure 6. *In situ* localisation of *period* (left column) and *timeless* (right column) transcripts in brains of adult *A. pisum* of the holocyclic YR2 (upper row) and anholocyclic GR (lower row) strains. Black and white arrowheads indicate DN and LN, respectively. Note the absence of differences in the distribution of transcripts for each gene between both strains.

Figure 7. Combined *in situ* localisation of *per* and *tim* transcripts in brains of

adult *A. pisum* aphids. A) Z-stack confocal image showing the l-DN and the s-DN. B) Z-stack confocal image showing in the dLN. C) Z-stack confocal image showing in the vLN. Scale bars = 50 μ m. D) Drawing of an aphid brain summarising the types of neurons expressing *per* and *tim* as inferred from *in situ* hybridisations. Meaning of letters are as in Fig. 4 and Fig. 5 (see also Results).

Figure 8. Light microscope images showing localisation of *per* and *tim* transcripts in aphids of the YR2 strain reared under LD conditions at two moments of the day/night cycle. A) Localisation of *per* transcripts in aphid brains collected at ZT15 that corresponds to the peak of *per* expression. Left, detail of DN. Right, detail of LN. B) Localisation of *tim* transcripts in aphid brains collected at ZT15 that corresponds to the peak of *tim* expression. Left, detail of DN. Right, detail of LN. C) Image indicating the absence of localisation of *per* and *tim* transcripts in aphid brains collected at ZT21 that corresponds to the trough of *per* and *tim* expression. D) Graphical representation of RT-qPCR results of *per* and *tim* genes at different timepoints for YR2 aphids reared in LD conditions. DN (dorsal neurons), LN (lateral neurons). Scale bars = 50 μ m.

period

timeless

YR2

GR

● *period* ● *timeless* ● *period + timeless*