Insect Biochemistry and Molecular Biology 112 (2019) 103185

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Insulin-like peptides involved in photoperiodism in the aphid *Acyrthosiphon pisum*

Miquel Barberà, Rubén Cañas-Cañas, David Martínez-Torres*

Institut de Biologia Integrativa de Sistemes, Parc Cientific Universitat de València, C/ Catedrático José Beltrán nº 2, 46980, Paterna, València, Spain

ARTICLE INFO

Keywords:
Aphid
Insulin
ILP
Photoperiodism
Diapause
In situ hybridisation
Quantification

ABSTRACT

Aphids were the first animals reported as photoperiodic as their life cycles are strongly determined by the photoperiod. During the favourable seasons (characterised by long days) aphid populations consist exclusively of viviparous parthenogenetic females (known as *virginoparae*). Shortening of the photoperiod in autumn is perceived by aphids as the signal that anticipates the harsh season, leading to a switch in the reproductive mode giving place to the sexual morphs (*oviparae* females and males) that mate and lay winter-resistant (diapause-like) eggs. The molecular and cellular basis governing the switch between the two reproductive modes are far from being understood. Classical experiments identified a group of neurosecretory cells in the *pars intercerebralis* of the aphid brain (the so called group I of neurosecretory cells) that were essential for the development of embryos as parthenogenetic females and were thus proposed to synthesise a parthenogenesis promoting substance that was termed "virginoparin". Since insulin-like peptides (ILPs) have been implicated in the control of diapause in other insects, we investigated their involvement in aphid photoperiodism. We compared the expression of two ILPs (ILP1 and ILP4) and an Insulin receptor coding genes in *A. pisum* aphids reared under long- and short-day conditions. The three genes showed higher expression in long-day reared aphids. In addition, we localised the site of expression of the two ILP genes in the aphid brain. Both genes were found to be expressed in the group I of neurosecretory cells. Altogether, our results suggest that ILP1 and ILP4 play an important role in the control of the aphid life-cycle by promoting the parthenogenetic development during long-day seasons while their repression by short days would activate the sexual development. Thus we propose these ILPs correspond to the so called "virginoparin" by early bibliography. A possible connection with the circadian system is also discussed.

1. Introduction

Aphids (Hemiptera: Aphididae) are small insects that feed on plant phloem sap and develop particularly complex life cycles that include several polyphenisms. One remarkable polyphenism shown by most aphid species consists in the presence of two alternative modes of reproduction (each with their associated morphs). During the favourable seasons, aphid populations consist of viviparously reproducing parthenogenetic females (also known as *virginoparae*) but, as the adverse season arrives, they shift to produce a sexually reproducing generation consisting of oviparous females (*oviparae*) and males that mate and lay cold-resistant (diapause-like) eggs to overcome the winter months. Inducing the switch to sexual reproduction in aphids is thus equivalent to induction of diapause in other insect species. Since the shortening of the photoperiod is the main cue governing the switch from parthenogenesis to sexual reproduction (although temperature may also subsidiarily participate), aphids constitute a paradigmatic example of photoperiodism (i.e. they have the ability to perceive and use day length –the photoperiod-as an anticipatory measure of the seasonal changes that are about to come to produce a response). In fact, aphids were the first animals to be reported as photoperiodic (Marcovitch, 1924). The pea aphid, *Acyrthosiphon pisum*, develops a typical, rather simplified, life cycle including the two above mentioned reproductive phases. Besides the previously described full cycle or holocycle, some aphids known as anholocyclic have lost the ability to respond to photoperiod shortening and reproduce by parthenogenesis all the year round (i.e. they never enter diapause) (Moran, 1992). In a way, anholocyclic aphids constitute natural photoperiodic mutants that can thus help to study photoperiodism in aphids. Although aphid life cycles are, in general, well described and the environmental factors responsible for the switches between alternative morphs have been known for long (especially in species like the pea aphid, that exhibit simpler cycles), little is known on the signalling cascade at the molecular level that activates the change from asexual to sexual reproduction (or that

* Corresponding author.
E-mail address: david.martinez@uv.es (D. Martínez-Torres).

https://doi.org/10.1016/j.ibmb.2019.103185
Received 17 April 2019; Received in revised form 28 June 2019; Accepted 6 July 2019
Available online 07 July 2019

prevents it during spring and summer). However, different studies have recently offered some insights into the molecular basis governing the process (Barberà et al., 2018, 2017; 2013; Cortés et al., 2010; Ishikawa et al., 2012; Le Trionnaire et al., 2009, 2008; Ramos et al., 2003; Tagu et al., 2005), especially after the pea aphid genome was made available (The International Aphid Genomics Consortium, 2010).

For some authors (Koštál, 2011), a yet unknown photoperiodic clock, or calendar, would be responsible for photoperiodism in aphids (and other insects). This clock would be composed of three main elements. First, a main photoperiod sensing site which in aphids would be localised in a particular region of the brain (Lees, 1964; Steel and Lees, 1977) rather than in the eyes as is the case in mammals and other insects (Numata et al., 2015; Saunders, 2002; Wood and Loudon, 2014). Second, the core of that clock, that would keep track of the number of days that the photoperiod is below a particular threshold, still awaits being definitely identified and characterised. While in vertebrates photoperiodic responses are controlled by the circadian clock, there is an still ongoing debate on its participation in insect seasonal responses, including the aphid switch between reproductive modes (Bradshaw and Holzapfel, 2010; Ikeno et al., 2010; Levy et al., 2018; Meuti et al., 2015; Mukai and Goto, 2016; Numata et al., 2015; Omura et al., 2016; Pavelka et al., 2003; Pegoraro et al., 2014). Previous work from our group has shown that cells expressing circadian clock genes in the aphid brain, known as clock neurons, are located in regions known to be essential for the photoperiodic response (Barberà et al., 2017; Steel, 1978; Steel and Lees, 1977). Third, the output mechanism of the putative photoperiodic clock would involve hormones and/or neuropeptides. Among these, it is worth noting that although melatonin is the main internal cue that controls circadian rhythms and also seasonal responses in vertebrates (Dardente et al., 2010; Wood and Loudon, 2014), its role is not so well established in insects (but see Barberà et al., [2018]). Instead, pigment dispersing factor (PDF) seems to be a widespread output of the circadian clock that could also be involved in photoperiodic responses in some insect species (Meuti et al., 2015; Shiga and Numata, 2009). An axis including the circadian clock, insect-type AA-NATs, melatonin and PTTH has been shown to regulate diapause in the moth *Antheraea pernyi* (Mohamed et al., 2014; Wang et al., 2015, 2013). In recent years a role of insulin-like peptides (ILPs) in the regulation of diverse functions including diapause has been shown in different arthropods, generally relating low ILP levels to diapause (Christie et al., 2016; Denlinger et al., 2012; Denlinger and Armbruster, 2014; Koštál et al., 2017; Matsunaga et al., 2016; Mizoguchi and Okamoto, 2013; Ragland and Keep, 2017; Schiesari et al., 2016; Sim and Denlinger, 2013; Vafopoulou and Steel, 2012a; Zheng et al., 2016). For instance, in *Culex pipiens* ILPs activate JH production eventually resuming ovary development after diapause (Sim and Denlinger, 2009). Moreover, interactions between the circadian clock and insulin and PTTH signalling pathways have been demonstrated (Vafopoulou et al., 2012; Vafopoulou and Steel, 2014). Thus it seems that rather diverse scenarios with different sets of actors are probably associated to the various types of seasonal responses observed in different insect species.

In aphids, a series of studies by Steel and Lees (Steel, 1978, 1976; 1977; Steel and Lees, 1977) allowed the identification of five groups of neurosecretory cells (NSC) in the *Megoura viciae* brain based on paraaldehyde fuchsin (PAF) staining, which is known to stain neurosecretory material containing proteins with disulphide bonds. Moreover, by ablating regions of the brain containing particular groups of these NSCs, the authors were able to identify the functions of two of them (Steel, 1978). The destruction of the group II of NSC affected moulting but had no effect on the photoperiodic response and therefore, it was suggested that these neurones synthesised prothoracicotropic hormone (PTTH) and bursicon. Indeed, in a previous report, we confirmed that PTTH is, as proposed, expressed in the group II of NSC (Barberà and Martínez-Torres, 2017). Most relevant, destruction through microcauterization of the group I of NSC located in the *pars intercerebralis* (medial, anterior *protocerebrum*) dramatically affected the photoperiodic response (Steel and Lees, 1977; Steel, 1976). When the group I of NSC was ablated, aphids reared under long day conditions that were thus expected to reproduce by parthenogenesis, switched to produce sexual morphs. These results prompted the authors to propose that group I NSCs in fact synthesised an unknown parthenogenesis-promoting factor that they named virginoparin (Steel, 1976). Until now, attempts by different groups to identify the nature of this virginoparin have not been successful. Recent transcriptomic and proteomic approaches on the aphid *A. pisum* (Huybrechts et al., 2010; Le Trionnaire et al., 2012, 2009) revealed the catalogue of neuropeptides and neurohormones encoded by the aphid genome. Interestingly, PDF (which is present in all other insect genomes sequenced to date) is absent in aphids. However, up to 10 genes coding Insulin Like Peptides (ILP) were identified in the aphid genome (Huybrechts et al., 2010). Moreover, these authors, based on previous reports (Le Trionnaire et al., 2009), suggest that the insulin pathway is inhibited by short photoperiod since a gene encoding an insulin receptor (InR) was downregulated in short-day reared aphids while a gene encoding an insulin degrading enzyme (IDE) was upregulated. It is thus probable that some of the ILPs present in the aphid genome play a role in the signalling pathway regulated by the photoperiod.

In order to shed some light on the participation of ILPs in the regulation of the aphid seasonal response, we investigated the expression of genes coding ILPs 1–4 in *A. pisum* and also of the putative ILP receptor. In particular, we compared the levels of expression of these genes in holocyclic and anholocyclic aphids reared under both long and short photoperiods. In addition, we investigated the sites of expression in the aphid brain of two of these ILP coding genes.

2. Materials and methods

2.1. Aphid strains

Acyrthosiphon pisum aphids of the LSR1 and the GR strains were used for most experiments in the present report. LSR1 is the pea aphid strain whose genome was firstly sequenced (The International Aphid Genomics Consortium, 2010). It is a holocyclic strain that responds typically to short photoperiods switching to sexual reproduction. On the other hand, the GR strain is anholocyclic as it does not respond to short photoperiods thus reproducing continuously by viviparous parthenogenesis, independently of the experienced photoperiod. Strains LSR1 and GR both come from aphids originally collected on alfalfa (*Medicago sativa)* in Ithaca, New York (USA) and Gallur (Spain), respectively. Both strains have been maintained in our lab on *Vicia fabae* for more than 5 years under long day (LD) photoperiod conditions (i.e. 16 h lights on and 8 h of darkness, or 16L:8D) at 18 °C. Strain LSR1 produces sexual females and males when reared under short day (SD) conditions (i.e. 12 h lights on and 12 h of darkness, or 12L:12D).

2.2. Acyrthosiphon pisum ILPs

To investigate their evolutionary relationships amino acid sequences of 10 previously identified pea aphid ILPs (Huybrechts et al., 2010) (Table S1) were aligned along with sequences from other insects from different orders and some vertebrate sequences used as outgroups (Fig. S1). Both sequence alignments and phylogenetic analysis were done using MEGA6 (Tamura et al., 2011). For phylogeny reconstruction, the Neighbour Joining algorithm on Poisson corrected distances was used. Node support was estimated by bootstrap using 500 replicates (Nei and Kumar, 2000).

2.3. Experimental characterisation of aphid ILP and InR transcripts

To experimentally validate gene models of relevant pea aphid ILP and their putative receptor (Insulin-like Receptor or InR) coding genes, total RNA was extracted from aphids of the above described aphid

strains using the Direct-Zol™ RNA MiniPrep (Zymo Research) following supplier's recommendations. Total RNA was quantified by spectrophotometry using a NanoDrop ND-1000 (Nanodrop Technologies) and stored at −70 °C until cDNA synthesis. 1–5 μg total RNA was reverse transcribed using an oligo $(dT)_{18}$ primer and the Superscript II reverse transcriptase (Invitrogen). Primers to amplify the selected aphid genes transcripts on cDNA were designed based on the predicted transcript models (Table S2). Amplified products were sequenced directly after purification (High Pure PCR Product Purification Kit, Roche) using designed primers (see Table S2).

2.4. Quantification of expression by RT-qPCR

Real time quantitative PCR (RT-qPCR) was used to quantify the expression of *ILP1* to *ILP4* and *InR* genes in *A. pisum* heads of the LSR1 and GR strains reared under LD and SD conditions as explained above using primers based on constitutive exons (see Table S2). Thus, four groups of aphids were analysed (LSR1 LD, LSR1 SD, GR LD and GR SD). For each of these groups, three biological replicates including 20 aphid heads were sampled at two time points: 6 h after lights went on, or *Zeitgeber* Time 6 (ZT6), and ZT18. All sampled aphids were immediately frozen in liquid nitrogen and kept at −70 °C until RNA extraction. RT-qPCR including three technical replicates per sample 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). The gene Rpl7 was used as an endogenous control of constitutive expression (Nakabachi et al., 2005). Differences in mean expression values between rearing photoperiods, strains and time points were tested for statistical significance using ANOVA. All statistical tests were performed with the SPSS Statistics package v22.0 software (IBM Corp).

2.5. Aphid central nervous system 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 × 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. 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. Pooled CNSs were then washed twice in methanol, and stored at −20 °C in tight sealed microcentrifuge tubes until the RNA *in situ* hybridisation (ISH) protocol was initiated.

2.6. Synthesis of DIG-labelled riboprobes and ISH

Sense and antisense RNA probes labelled with digoxigenin (DIG) were synthesised as previously described (Barberà et al., 2017; Barberà and Martínez-Torres, 2017). Antisense probes were used to localise specific mRNAs while sense probes were used as negative controls. Quantification of the probes was assessed by dot blot assays. Probes were obtained in this way to localise the expression of *ILP1* and *ILP4* genes in fixed aphid brains obtained as described above. Primers used to amplify the gene fragments used as probes are indicated in Table S2. *In situ* hybridisations and detection of transcripts were carried out also as previously described (Barberà et al., 2017; Barberà and Martínez-Torres, 2017). Fast Red/HNPP (Roche) was used as alkaline phosphatase (AP) substrate that produces a chromogenic red and fluorescent precipitate.

2.7. Microscopy and imaging

Whole-mount fluorescent preparations of the CNS were scanned with a confocal laser scanning microscope (FV1000, Olympus) using 20× and 40× 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. FastRed/HNPP substrate was detected with a 559 nm excitation laser. Images were processed and arranged with Fiji v1.50i software (Schindelin et al., 2012).

2.8. Quantification of ILPs fluorescence signal

Intensity of fluorescent signal in Insulin Producing Neurons (IPN) was measured to compare the expression of ILP1 in different strains and photoperiods. To avoid batch effects, brains were processed in a single experiment and hybridisations and image acquisition were performed together. Measurements were done in Z-axis image slices corresponding to the centre of each cell. Unspecific signal from trachea overlapping with cell specific signal was not included in the measurements. The intensity of the signal (ΔF) was calculated as the mean intensity of the area corresponding to the cell from which the mean background intensity of an equivalent area was subtracted.

3. Results

3.1. Characterisation of aphid ILP1, ILP4 and InR

Since we were most interested in identifying the nature of the neurosecretory material produced by group I of neurosecretory cells (NSCI, see Introduction), among the 10 ILP coding genes previously identified in the pea aphid genome, ILP1 to ILP4 were considered most relevant for the present work as they showed characteristics of insulin typically produced by endocrine cells or peptidergic neurons (i.e. cysteine residues that form disulphide bonds with the motif $CC(X)_3C(X)_8C$ and two convertase cleavage sites necessary to cut the C peptide to render the active hormone, Fig. 1) (Huybrechts et al., 2010). Moreover, ILP1 to ILP4 are the only aphid ILPs that constitute a strongly supported monophyletic group (Fig. S1) probably reflecting two duplication events of an ancestral form of ILP exclusive of the aphid lineage. Of these, we focused on ILP1 and ILP4 as they are especially abundant in aphid head specific RNAseq libraries available at AphidBase, while expression of ILP2 and ILP3 was negligible in aphid heads according to Aphidbase RNAseq data (see Table S1). Moreover, we failed to amplify ILP2 and ILP3 through PCR from aphid heads and bodies' total RNA from the two aphid strains analysed in the present report (see below).

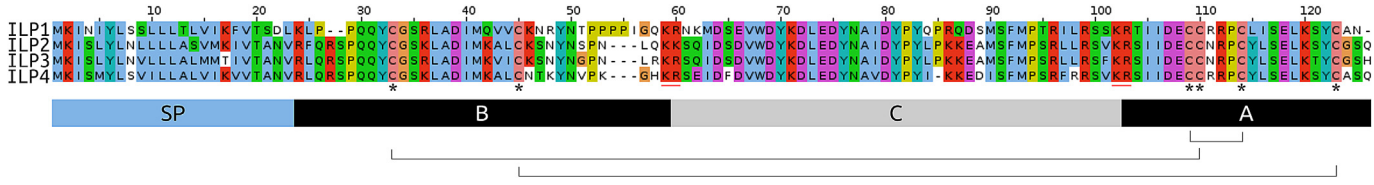

Fig. 1. Alignment of four insulin-like peptides (ILP1-ILP4) predicted in the *A. pisum* genome. Asterisks indicate the 6 conserved cysteines that participate in disulphide bridges (indicated by black lines). Boxes below the alignment indicate the predicted signal peptide (SP), and peptides A, B and C. Red bars indicate the conserved motifs for prohormone convertases cleavage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Firstly, we experimentally confirmed in our aphid strains the transcript predictions available for ILP1 and ILP4 (sequences were deposited in GenBank and given accessions numbers MK510954 and MK510958, respectively). In addition, we experimentally obtained the sequence of the insulin receptor (InR) in the two studied aphid strains. The sequences were basically coincidental with the transcript model present in AphidBase for this gene (Table S1) although six positions in the coding sequence were heterozygous for synonymous changes (sequences were deposited in GenBank with accession numbers MK510960 and MK510961).

3.2. Quantification of expression of ILP1, ILP4 and InR in aphid heads

We confirmed that expression of both ILP1 and ILP4 is almost negligible in aphid bodies compared with aphid heads (Fig. S2). We also confirmed a lack of expression in aphid heads or bodies of both ILP2 and ILP3 (not shown). These results confirmed our interest on the expression of both ILP1 and ILP4 genes. We then compared the expression of these two ILP coding genes, as well as the InR coding gene, in heads of two aphid strains reared under different photoperiod conditions and at two different time points to test whether expression of ILP and InR genes varied along the day (see Materials and Methods). The three genes showed similar patterns of expression (Fig. 2). All three genes showed a much higher expression at ZT6 than at ZT18 under LD conditions in holocyclic aphids while under SD conditions levels of expression were similarly lower at both ZTs. Focusing on ZT6, expression of ILP1 was about 10 times higher in aphids of the LSR1 (holocyclic) strain reared under LD than in aphids of the same strain reared under SD conditions ($p < 0.000$). Interestingly, no significant differences at ZT6 were observed between aphids of the GR (anholocyclic) strain reared under the same two photoperiods ($p = 0.83$) showing low levels of expression in both of them (comparable to the level observed for the holocyclic strain under SD) (see Fig. 2A). Similarly, expression of ILP4 at ZT6 was about 3 times higher in aphids of the holocyclic strain reared under LD than under SD conditions (p-value < 0.000). However, as for the ILP1 gene, no differences were observed at ZT6 between aphids of the anholocyclic strain ($p = 0.89$) which also showed similarly lower levels of expression under both photoperiods (see Fig. 2B). Finally, the analysis of InR also showed significant higher expression (almost 2.5 times) at ZT6 in holocyclic aphids kept under LD than under SD conditions ($p < 0.02$) and, as for the other two genes, no significant differences where observed between photoperiods in aphids of the anholocyclic strain ($p = 0.607$, see Fig. 2C) which, as before, showed lower expression.

At ZT18 all three genes showed similarly low levels of expression in aphids reared under both LD or SD conditions in both holocyclic and anholocyclic aphids (see Fig. 2).

3.3. In situ localisation of ILP gene expression

To identify the region of the aphid brain where the two studied ILP genes have their site of expression, we performed *in situ* localisation of their respective transcripts using specific RNA probes (see Materials and Methods). Apart from unspecific staining of trachea, which are also visible in the negative control preparations using sense probes (Fig. 3A) (see Materials and Methods), expression of ILP1 was evident in two groups of four neurons each symmetrically localised in both brain hemispheres in the medial anterior region of the *protocerebrum* known as *pars intercerebralis* (Fig. 3B). Similarly, expression of ILP4 was also observed in two groups of four cells each showing the same pattern and position as that already described for ILP1 (Fig. 3C). To confirm whether both ILP genes were in fact being expressed in the same neurons, a combined ISH simultaneously using probes against both ILP1 and ILP4 transcripts was carried out. A total of eight neurons showed positive signal exactly with the same pattern as that observed with each individual probe (i.e. no additional cells were detected) (Fig. 3D) which allowed us to conclude that ILP1 and ILP4 are co-expressed in the same cells. In addition, we also compared the localisation of ILP1 and ILP4 transcripts in brains obtained from holocyclic and anholocyclic aphids reared under both LD and SD conditions. The same two groups of four cells revealed positive signal in all cases (data not shown). To test whether the *in situ* results matched those obtained by RTqPCR, we compared the intensity of the ILP1 fluorescent signal emitted by these cells in preparations of brains from aphids of the holocyclic and anholocyclic strains reared under LD and SD photoperiods (Fig. 4). In agreement with RT-qPCR results, the fluorescent signal was significantly higher in brains of LD aphids of the holocyclic strain than in SD aphids of the same strain. Also in agreement with RTqPCR results, less signal intensity was observed in general for the anholocyclic strain and, although it was higher in LD brains, differences were non-significant ($p = 0.056$) (see Fig. 4).

4. Discussion

For many years it has been known that photoperiod is a key environmental factor controlling cyclical parthenogenesis in aphids (Marcovitch, 1924). A particular group of cells in the *pars intercerebralis* of the mother's brain (the so called group I of neurosecretory cells or NSCI) was shown to play an essential role in the control of the process by apparently secreting a parthenogenesis promoting substance that was named virginoparin (Steel, 1976). A region in the *pars lateralis*, adjacent to the region containing the NSCI, was also apparently involved in this process and was proposed to contain both the site for the photoperiod receptor and the putative photoperiodic clock that would transmit day length information to the NSCI which, in turn, would produce (or not) the above mentioned virginoparin. While this early work greatly contributed to the localisation and identification of some of the cellular elements in the cascade controlling the mode of reproduction in aphids, the nature of the molecular elements involved and, in particular, of the so called virginoparin has remained elusive.

In the present report, as part of a long lasting search for molecular elements in that cascade, we present results on three *Acyrthosiphon pisum* genes of the insulin signalling pathway: two genes encoding respectively insulin-like peptides 1 and 4 (ILP1 and ILP4) and a gene encoding an insulin receptor (InR). We have experimentally confirmed the transcript models for these previously identified genes (Huybrechts et al., 2010; Le Trionnaire et al., 2009) but, more important, we present results on the quantification of their expression under different photoperiods and on the localisation of the expression of the two ILP genes in the aphid brain that are compatible with both ILP1 and ILP4 coding genes being the long sought virginoparin proposed by Steel (1976) and thus with playing a substantial role in the control of the aphid life cycle.

4.1. Evolution of ILP genes in A. pisum

Identification of some genes in the insulin signalling pathway present in the genome of the aphid *Acyrthosiphon pisum* was already reported some years ago (Huybrechts et al., 2010). Putative relevance of this pathway for the regulation of the reproductive polyphenism had already been shown by Le Trionnaire et al. (2009) who reported that expression of two genes in the pathway (a gene coding for an insulin degrading enzyme and a gene coding for an insulin receptor) was regulated by the photoperiod. As already noticed by Huybrechts et al. (2010), 4 of the 10 identified insulin-like peptides (ILPs 1 to 4) corresponded to classical insulin molecules as shown in Fig. 1. Moreover, when the 10 insulin related peptides in the genome of the pea aphid described by these authors are aligned to investigate possible evolutionary relationships among them and with related sequences from other insects (see Fig. S1), it becomes clear the impossibility of establishing such relationships for most of them with the exception of ILP1 to 4. The latter four genes are the only aphid ILP genes grouped into a strongly supported monophyletic clade pointing to their origin after

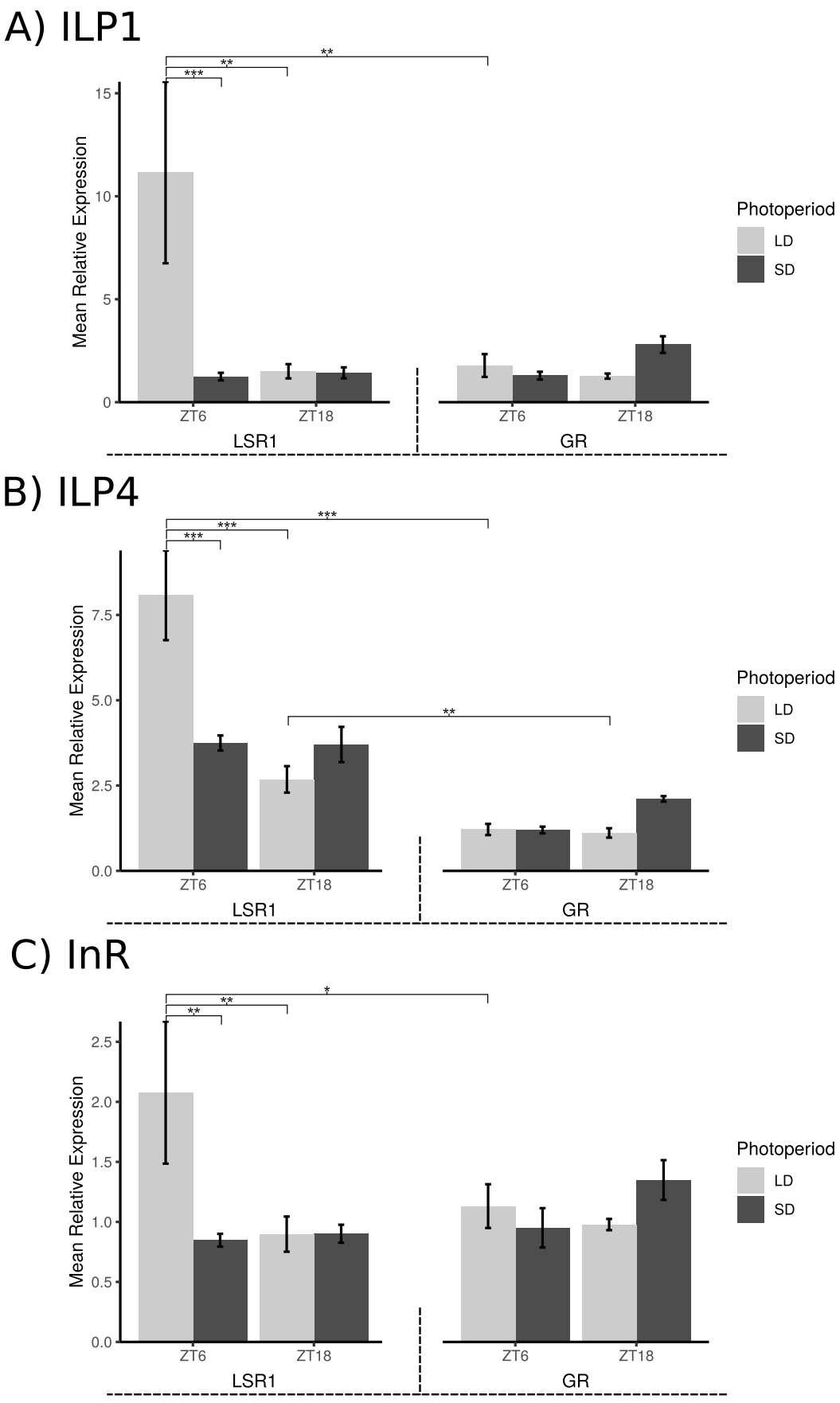

Fig. 2. Mean relative expression of ILP1, ILP4 and InR coding genes in *A. pisum* strains LSR1 (holocyclic) and GR (anholocyclic) reared under LD and SD photoperiods sampled at two different time points (ZT6 and ZT18), see Materials and Methods. Bars represent the mean relative expression ± SEM. Significant differences after ANOVA analysis are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.000$).

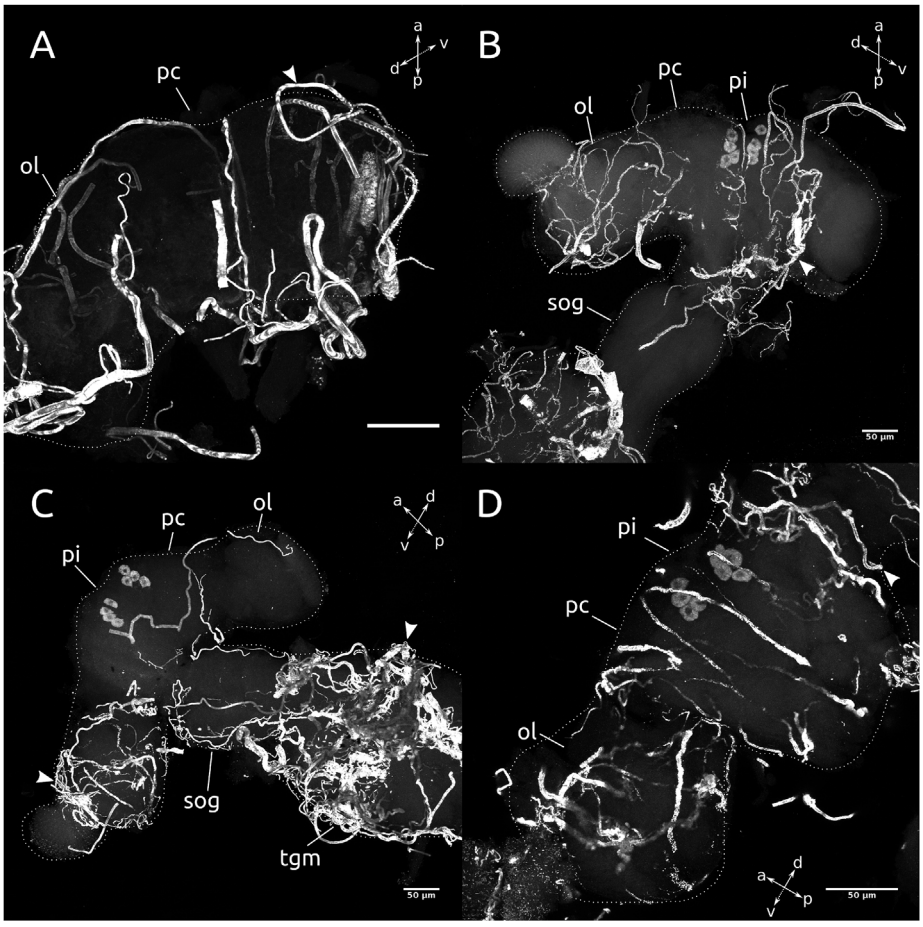

Fig. 3. Localisation of ILP1 and ILP4 transcripts in the central nervous system of adult aphids by *in situ* hybridisation. Images correspond to confocal Z-stacks. A) Control hybridisation representative of ILP1 and ILP4 sense probes. Note the absence of signal in *pars intercerebralis*. B) and C) Localisation of ILP1 and ILP4 transcripts respectively. Note in both B) and C) the presence of four neurons per hemisphere located at the *pars intercerebralis*. D) Combined hybridisation with both ILP1 and ILP4 probes used simultaneously. Note that the total number of insulin expressing neurons is eight, as with each single probe. ol, optic lobe; pc, *protocerebrum*; sog, suboesophageal ganglion; tgm, thoracic ganglionic mass; pi, *pars intercerebralis*. Bars = 50 µm. Unspecific tracheal staining is indicated by white arrowheads.

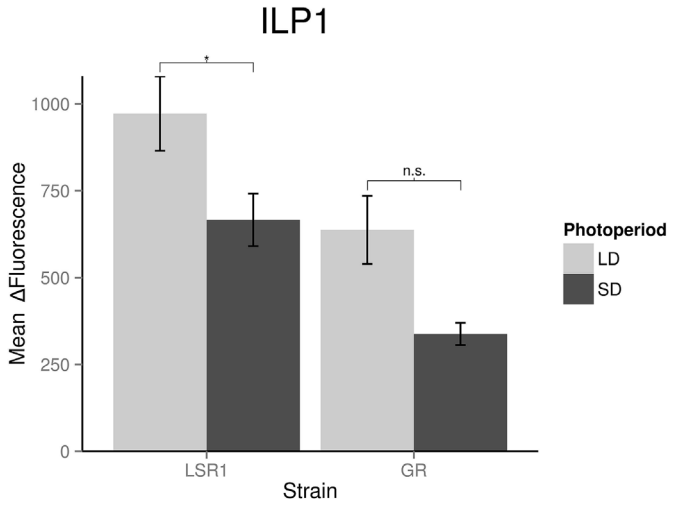

Fig. 4. Intensity of ILP1 fluorescent signal in Insulin Producing Neurons of *A. pisum* strains LSR1 (holocyclic) and GR (anholocyclic) reared under LD and SD photoperiods. Bars represent the mean ΔF ± SEM. Significant differences after ANOVA analysis are indicated by * ($p < 0.05$). Non-significant differences are indicated by n.s.

duplication events followed by a divergence process. This process would be specific of the aphid lineage as judged by the presence of three predicted orthologous genes in the genome of the aphid *Myzus persicae* (see Fig. S1). In this respect, it is worth recalling that lineage-specific gene duplications seem to be the rule rather than the exception in the genome of the pea aphid and some authors have proposed this could be related with the regulation of the different polyphenisms found in aphids including, among others, those associated with reproduction (i.e. parthenogenetic females vs. sexual morphs) (Shigenobu et al., 2010; The International Aphid Genomics Consortium, 2010).

4.2. Quantification of expression of insulin related genes

One of the main results from the present report is the higher expression observed for the ILP1 and ILP4 genes and for a gene coding a putative ILP receptor (InR) in aphids of a holocyclic strain reared under LD conditions at a particular moment of the day (i.e. ZT6; see Fig. 2). Shortening of the photoperiod resulted in reduced expression of these three genes in this strain. Our results on InR are coincident with results previously obtained by Le Trionnaire et al. (2012), who also found InR downregulated under SD conditions. This is most suggestive as it could be easily interpreted that high expression of genes coding ILP1 and/or ILP4 (and concomitantly of the gene coding for their receptor) would be signalling that days are long and thus somehow would promote the continuity of the parthenogenetic status. Contrarily, short days cause a repression of these genes (as observed in our survey under SD conditions) and thus reduce the levels of those molecules necessary to keep the parthenogenetic development active. In other words, low ILP1/ILP4 levels at some particular time point would signal the switch to sexual morph differentiation of embryos in holocyclic aphids. In addition, the fact that in the anholocyclic strain (GR) the length of the day did not affect the expression of these genes, provides further support for the suggested role of ILP1/ILP4 in transducing information on day length changes. Indeed, one would expect differences in the levels of any molecule that would act as an effector of the photoperiodic information in holocyclic aphids but no differences in aphids that are unable to respond to changes in photoperiod. However, the fact that levels of expression of genes coding for ILP1 and 4 (and also InR) are low in the

anholocyclic strain rather than high (see Fig. 2) may seem contradictory but, in our opinion, what is relevant is the ability to modify their expression levels in response to a change in the photoperiod rather than the particular basal levels of expression of those genes in a given strain. Alternatively, we can't discard that ILP1 and/or ILP4 are not the main signal leading to diapause but rather the consequence of photoperiodism affecting other (unknown) processes differently in different strains. Quantifying the expression of these genes in additional aphid strains will show whether there is intraspecific variation in the levels of expression of insulin related genes.

Although our report has shown that higher expression of both ILP genes and the InR gene under LD conditions in the holocyclic aphid strain occurs at a particular moment of the day (i.e. ZT6) (see Fig. 2), more data are need, including sampling aphids at several time points, to detect a possible rhythmicity in the expression of these genes. According to our results, a peak of expression of these genes in the morning of long days in the holocyclic strain would be the signal promoting the parthenogenetic development. Under short days, however, the absence of that signal would induce the sexual development in the same strain.

4.3. Site of expression of ILP1 and ILP4

Our second main result was the identification of the site of expression in *A. pisum* of the genes encoding both ILP1 and ILP4 in eight cells in the *pars intercerebralis*, similarly to what has been found in other insects (Cao et al., 2014; Mizoguchi and Okamoto, 2013). By their localisation, we propose those cells in fact correspond to the NSCI described by Steel and Lees (1977) in *M. viciae*. Also in support of this conclusion it is worth to recall here that the five groups of neurosecretory cells identified in the aphid brain (Steel, 1978, 1977; Steel and Lees, 1977), including NSCI, were revealed by paraldehyde fuchsin (PFA) staining which specifically stains proteins containing disulphide bridges and both ILP1 and ILP4 have the typical insulin structure containing 6 conserved cysteines that form typical disulphide bridges (see Fig. 1). Additional support for both ILP1 and ILP4 being the neurosecretory material synthesised by NSCI comes from the observation of progressive cytological changes in the neurosecretory material synthesised by NSCI during its axonal transport (Steel, 1977), akin, according to the author, to the progressive cleavage of a prohormone into a hormone. Such progressive changes would be expected for both ILPs as typical insulin hormones that are synthesised as prohormones and only after cleavage of the C-peptide render the active hormone. However, there is a discrepancy in the number of cells found in *A. pisum* expressing ILP1 and ILP4 (8 cells, 4 in each brain hemisphere) and the number of NSCI cells reported in *M. viciae* (10 cells, 5 per hemisphere). This difference could reflect a mere divergence between the two aphid species. Alternatively, assuming that both aphid species contain 10 NSCI neurons, then there would be at least two types of cells: eight cells producing ILPs, and two other cells producing another factor also containing disulphide bonds or receiving ILPs from the other cells. A third possibility would involve a change in the number of cells by brain remodelling, as it has been described for clock neurons in *Rhodnius prolixus* (Vafopoulou and Steel, 2012b).

4.4. Could ILP1 and ILP4 be the long sought virginoparin?

Putting together our previously discussed main results with Steel's proposal of a virginopare promoting substance produced by NSCI (Steel, 1976; see Introduction), it is evident that ILP1 and ILP4 are a secretory product of NSCI thus being good candidates to be the proposed substance. Both their localisation and their photoperiodic pattern of expression match what we would expect for any molecule candidate to be the virginoparin proposed by Steel (1976). Indeed, we have shown that genes coding for these two insulin-like peptides (ILP1 and ILP4) are expressed in the NSCI and we have demonstrated that their high expression under long day conditions at a particular moment switches to low levels when days become short. The high levels of ILP1 and ILP4 produced under LD would promote the continuity of the parthenogenetic development of embryos while a drop in the levels of ILP1 and ILP4 under SD would arrest the parthenogenetic development (and thus trigger a switch to sexual development of the embryos). The drop in the levels of these two ILPs under short days would thus be equivalent to a cauterisation of the cells producing them (the NSCI) (Steel and Lees, 1977; Steel, 1976). It is not straightforward, however, to understand why two similar molecules instead of one, but this could be related with having a redundant message or with fine tuning the process. To test if our proposal is correct, RNAi of ILP1/ILP4 on aphids reared under LD should result in their progeny (or a portion of it) switching to sexual morph development (which is the effect of NSCI cauterisation). According to their first proponents, the cytological evidence suggests that the neurosecretory product of NSCI is transported along their axons and delivered directly to the abdomen or, perhaps, to the reproductive system itself where they would exert their function by influencing the endocrine system of the developing progeny (Steel, 1978, 1977; Steel and Lees, 1977). If the identity of virginoparin, as we propose, coincides with that of ILP1 and ILP4, developing antibodies against these neuropeptides to perform IHC experiments will be of great value providing information on the levels of these neuropeptides under different photoperiod regimes and, more important, details on their pathway from their site of synthesis (the NSCI) to their delivery site, thus allowing to test whether they are directly transported to the embryos as proposed for virginoparin (Steel, 1977).

Since the sexual phase in aphids can be considered to some extent a sort of diapause (Shingleton et al., 2003), our observation of higher ILP expression under parthenogenesis-promoting LD conditions coincides with several reports on other insect species that show the involvement of ILPs as intermediates in the decision of entering or not into diapause. A correlation between upregulation of ILP expression and non-diapausing conditions has been observed, for instance, in *Drosophila* (Schiesari et al., 2016), *Helicoverpa armigera* (Zhang et al., 2017) and mosquito (Sim and Denlinger, 2013) among other examples in insects and also in *C. elegans* (Matsunaga et al., 2016). In these examples, down regulation of ILPs leads to diapause. This would be equivalent to the switch to sexuality that occurs in aphids after microcauterisation of NSCI (Steel, 1978; Steel and Lees, 1977) or after exposure to short days (which we have shown here results in reduced expression of ILP1 and ILP4 coding genes). Thus, similar to other organisms, a role of insulin signalling as intermediate in diapause initiation in response to short days (or in diapause prevention under long days) could be inferred in aphids. Moreover, it has been suggested in the mosquito *C. pipiens* that ILP-containing cells could be the site for integration of the photoperiodic signal, probably in connection with the circadian clock machinery (Sim and Denlinger, 2009). It may be relevant to note that some of these ILP coding genes are expressed in a circadian manner in some insects (Barber et al., 2016; Cong et al., 2015; He et al., 2017; Vafopoulou and Steel, 2014), which suggests a connection with the circadian clock. In this respect, it should be noted that we recently identified aphid clock neurons expressing genes *period* and *timeless* in the *pars lateralis* of the aphid brain (Barberà et al., 2017), in the vicinity of the region containing the NSCI (shown in the present report to express ILP1 and ILP4). It is worth to recall here that damaging the *pars lateralis* (Steel and Lees, 1977) resulted in a depletion of neurosecretory material in the NSCI, thus having similar effects to damaging the NSCI, that is, the production of sexual morphs under long day conditions. Thus, our identification of clock neurons in the *pars lateralis* would support the view of these clock neurons providing some input to the NSCI, probably by some neurotransmitter yet to be identified. This input would contain the information on day length needed to either keep ILP1/ILP4 synthesis on or switch it off (both scenarios are depicted in Fig. 5). Under long days, clock neurons (through some yet unknown neurotransmitter) would inform NSCI to continue with ILP1/

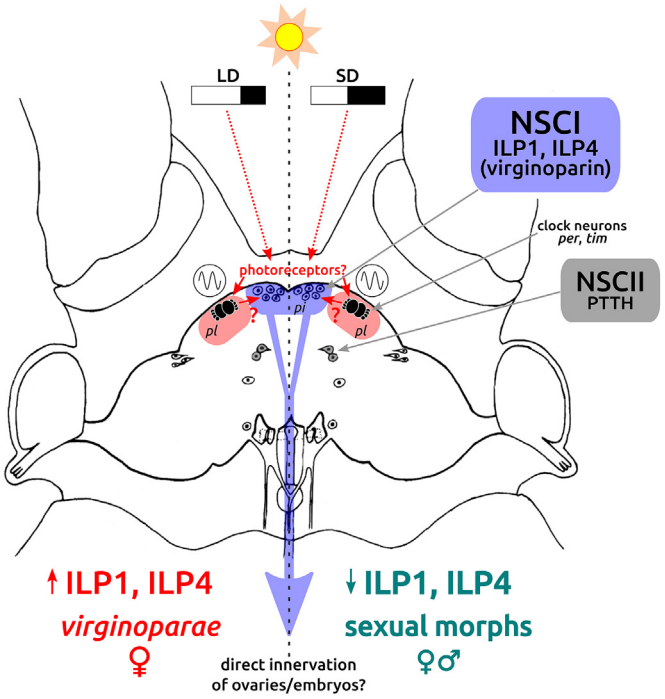

Fig. 5. Schematic representation of the aphid head and brain with indication of some of the elements discussed in the text likely involved in the control of the photoperiodic response and hypothetical scenarios occurring under LD and SD photoperiods. *Pars intercerebralis* (*pi*) is shown in purple background and *pars lateralis* (*pl*) in red background.

ILP4 synthesis and these peptides would be sent to the embryos to regulate their own endocrine system to develop as parthenogenetic females. Under short days, clock neurons would tell NSCI to drop the levels of these ILPs below some threshold which would signal the embryos to switch their endocrine system into the sexual development mode.

Although our results do not allow to infer a circadian synthesis of ILPs, we have shown a vast difference in transcript levels at two different time points under LD conditions in the holocyclic strain. More data are necessary in order to establish a possible connection between the circadian clock and the synthesis of these two ILPs. Looking at the effects on the expression of ILP1 and ILP4 genes of RNAi targeting *period* and/or *timeless* would probably be very informative on the hypothetical relationship between the circadian clock and ILP synthesis that ultimately would control the reproductive status. It would also be interesting to localise the site of expression of the InR since that could contribute to have a more complete picture of how these ILPs work once the message is delivered into the developing embryos. Studies on the effects of knocking down ILP1 and/or ILP4 genes through RNAi on the levels of embryonic JH or on the expression of some embryonic genes related with JH metabolism would be very valuable.

Formatting of funding resources

This work was supported by project CGL2015-68188-P (MINECO-FEDER).

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 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103185.

Table S1
Summary of accession numbers and other relevant data available at Aphidbase on the 10 ILPs[a] and the InR genes predicted in the *A. pisum* genome (Huybretchs et al., 2010; Le Trionnaire et al., 2009).

Name[a]	Aphidbase	Head[c]	Scaffold	NCBI predicted protein	Gene
IRP1	ACYPI53158	Yes	GL350634	XP_003247548	LOC100568938
IRP2	ACYPI54347	No	GL349870	XP_003244126	LOC100574788
IRP3	ACYPI45823	No	GL349647	XP_003240930	LOC100575361
IRP4	ACYPI003026	Yes	GL350062	XP_001949438	LOC100161832
IRP5	ACYPI004444	Yes	GL350239	XP_001949253	LOC100169635
IRP6	ACYPI56726	No	GL349640	XP_003240733	LOC100570058
IRP7	ACYPI21806	No	GL349624	XP_016656301	–
IRP8	ACYPI49277	No	GL350251		LOC100569550
IRP9	ACYPI42753	No	GL349941		–
IRP10	ACYPI52860	No	GL349805	XP_016658657	–
InR[b]	ACYPI009339	Yes	GL350260	XP_008185917	

[a] Although we use the term ILP (Insulin-Like Peptides), originally, these sequences were reported as Insulin Related Peptides (IRPs) (Huybretchs et al., 2010).
[b] Insulin-Like Receptor (Le Trionnaire et al., 2009).
[c] Presence of readings corresponding to that gene in data of RNA seq libraries obtained from aphid heads available at Aphidbase.

Table S2

Primers used for PCR, sequencing and probe synthesis.

Gene	Name	Sequence
ILP1	ILP1F1[b]	GACCAACAGCGGCTGACAATAATC
	ILP1F2	CACCTTTCAACGAACCACATCAC
	ILP1R1[b]	TAGTTAGAACTTTATTCAGCAAC
	ILP1QF[a]	ACATTCAATCAAGAACACAATG
	ILP1QR[a]	CTGCCAATCTAGAACCACAGTA
ILP2	ILP2F1	CAACGGTTGACAATCATCAGTTC
	ILP2F2	CAGTTCACCACAATTCTGCGACC
	ILP2R1	AAATAACTTACAATTAAGTCTAC
	ILP2QF[a]	CATTCATTCAAACAAGAATACAATG
	ILP2QR[a]	ACAGTATTGTTGAGGTGAACGTTG
ILP3	ILP3F1	TCATCAGTTCACCACAATTCTGT
	ILP3R1	GTCTGTATATTCGTACTACTGAC
	ILP3QF[a]	TTCATTCAAGCAAGAATACAATG
	ILP3QR[a]	ATGTCTGCCAATCTAGAACCACAG
ILP4	ILP4F1[b]	CCAACAACAACTGACGATCATCAG
	ILP4F2	ACCATATCCACACTTATTTGAAC
	ILP4R1[b]	CTATAATAATGGTTTATTCAGTGTC
	ILP4QF[a]	AGCAAGAATACCAACAATGAAG
	ILP4QR[a]	CATTGTACTTGGTATTGCATAGAGC
common to all ILPs	ILPRC	CTTCTAGGTCTTTGTAATCCCATAC
InR	InRF0	GCAGAGTGTAACGACGAAGAG
	InRF1	TATGTAAAAACATTTTGTTATAATG
	InRF2	CTTGTGTAATGGAATGTCCTCC
	InRF3	CCTAAAAGTGATACTTCACCAAAG
	InRF4	CAGAAGCAACTACTTATGATTTGG
	InRF5[ab]	AGAATGAAATTGTACCATGTGC
	InRF6	GGAAGAAGATGATGATGATGATG
	InRF7	GTAGTTCCTAGTAATCAGTTGAC
	InRF8	CGTGGTTGATAGCGACAAAATCTG
	InRF9	TCTGTCTGTAATTCGAGGACAAGAC
	InRF10	GGTATGATGCCAATCCGATGGATG
	InRF11	CTTGATGTTTCTGAAAATGGTATC
	InRR0	ACACATAACATAGACCCATCTCTG
	InRR1[b]	AGTTTGATCTAAAAAATTACTTCT
	InRR2	CTTGTTTGAGCTGTTGTCATTGC
	InRR3[a]	TGTGATACAACACCATATAGGCG
	InRR4	CTATTATTTGTAGGTTGTGCGAC
	InRR5	ACTTTACGACATGGTCCAGAAC
	InRR6	CTGTGTATAAGGTTTTAGTCCTG
	InRR7	GGTTTTCGGTAATGTTTTGGATTC

[a] Used for RT-qPCR.
[b] Used for probe synthesis.

References

Barber, A.F., Erion, R., Holmes, T.C., Sehgal, A., 2016. Circadian and feeding cues integrate to drive rhythms of physiology in Drosophila insulin-producing cells. Genes Dev. 30, 2596–2606. https://doi.org/10.1101/gad.288258.116.

Barberà, M., Collantes-Alegre, J.M., Martínez-Torres, D., 2017. Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid Acyrthosiphon pisum. Insect Biochem. Mol. Biol. 83, 54–67. https://doi.org/10.1016/j.ibmb.2017.02.006.

Barberà, M., Escrivá, L., Collantes-Alegre, J.M., Meca, G., Rosato, E., Martínez-Torres, D., 2018. Melatonin in the seasonal response of the aphid Acyrthosiphon pisum. Insect Sci. 00, 1–15. https://doi.org/10.1111/1744-7917.12652.

Barberà, M., Martínez-Torres, D., 2017. Identification of the prothoracicotropic hormone (Ptth) coding gene and localization of its site of expression in the pea aphid Acyrthosiphon pisum. Insect Mol. Biol. 26, 654–664. https://doi.org/10.1111/imb.12326.

Barberà, M., Mengual, B., Collantes-Alegre, J.M., Cortés, T., González, A., Martínez-Torres, D., 2013. Identification, characterization and analysis of expression of genes encoding arylalkylamine N-acetyltransferases in the pea aphid Acyrthosiphon pisum. Insect Mol. Biol. 22, 623–634. https://doi.org/10.1111/imb.12050.

Bradshaw, W.E., Holzapfel, C.M., 2010. Circadian clock genes, ovarian development and diapause. BMC Biol. 8, 115. https://doi.org/10.1186/1741-7007-8-115.

Cao, J., Ni, J., Ma, W., Shiu, V., Milla, L.A., Park, S., Spletter, M.L., Tang, S., Zhang, J., Wei, X., Kim, S.K., Scott, M.P., 2014. Insight into insulin secretion from transcriptome and genetic analysis of insulin-producing cells of Drosophila. Genetics 197, 175–192. https://doi.org/10.1534/genetics.113.160663.

Christie, A.E., Roncalli, V., Lenz, P.H., 2016. Diversity of insulin-like peptide signaling system proteins in Calanus finmarchicus (Crustacea; Copepoda) – possible contributors to seasonal pre-adult diapause. Gen. Comp. Endocrinol. 236, 157–173. https://doi.org/10.1016/j.ygcen.2016.07.016.

Cong, X., Wang, H., Liu, Z., He, C., An, C., Zhao, Z., 2015. Regulation of sleep by insulin-like peptide system in Drosophila melanogaster. Sleep 38, 1075–1083. https://doi.org/10.5665/sleep.4816.

Cortés, T., Ortiz-Rivas, B., Martínez-Torres, D., 2010. Identification and characterization of circadian clock genes in the pea aphid Acyrthosiphon pisum. Insect Mol. Biol. 19, 123–139. https://doi.org/10.1111/j.1365-2583.2009.00931.x.

Dardente, H., Wyse, C.A., Birnie, M.J., Dupré, S.M., Loudon, A.S.I., Lincoln, G.A., Hazlerigg, D.G., 2010. A molecular switch for photoperiod responsiveness in mammals. Curr. Biol. 20, 2193–2198. https://doi.org/10.1016/j.cub.2010.10.048.

Denlinger, D.L., Armbruster, P.A., 2014. Mosquito diapause. Annu. Rev. Entomol. 59, 73–93. https://doi.org/10.1146/annurev-ento-011613-162023.

Denlinger, D.L., Yocum, G.D., Rinehart, J.P., 2012. Hormonal control of diapause. In: Gilbert, L.I. (Ed.), Insect Endocrinology. Elsevier, pp. 430–463. https://doi.org/10.1016/B978-0-12-384749-2.10010-X.

He, Q., Wu, B., Price, J., Zhao, Z., 2017. Circadian rhythm neuropeptides in Drosophila: signals for normal circadian function and circadian neurodegenerative disease. Int. J. Mol. Sci. 18, 886. https://doi.org/10.3390/ijms18040886.

Huybrechts, J., Bonhomme, J., Minoli, S., Prunier-Leterme, N., Dombrovsky, A., Abdel-Latief, M., Robichon, A., Veenstra, J.A., Tagu, D., 2010. Neuropeptide and neurohormone precursors in the pea aphid, Acyrthosiphon pisum. Insect Mol. Biol. 19, 87–95. https://doi.org/10.1111/j.1365-2583.2009.00951.x.

Ikeno, T., Tanaka, S.I., Numata, H., Goto, S.G., 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol. 8, 116. https://doi.org/10.1186/1741-7007-8-116.

Ishikawa, A., Ogawa, K., Gotoh, H., Walsh, T.K., Tagu, D., Brisson, J. a, Rispe, C., Jaubert-Possamai, S., Kanbe, T., Tsubota, T., Shiotsuki, T., Miura, T., 2012. Juvenile hormone titre and related gene expression during the change of reproductive modes in the pea aphid. Insect Mol. Biol. 21, 49–60. https://doi.org/10.1111/j.1365-2583.2011.01111.x.

Koštál, V., 2011. Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity? J. Insect Physiol. 57, 538–556. https://doi.org/10.1016/j.jinsphys.2010.10.006.

Koštál, V., Štětina, T., Poupardin, R., Korbelová, J., Bruce, A.W., 2017. Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. Proc. Natl. Acad. Sci. 114, 8532–8537. https://doi.org/10.

1073/pnas.1707281114.
Le Trionnaire, G., Francis, F., Jaubert-Possamai, S., Bonhomme, J., De Pauw, E., Gauthier, J.-P., Haubruge, E., Legeai, F., Prunier-Leterme, N., Simon, J.-C., Tanguy, S., Tagu, D., 2009. Transcriptomic and proteomic analyses of seasonal photoperiodism in the pea aphid. BMC Genomics 10, 456. https://doi.org/10.1186/1471-2164-10-456.
Le Trionnaire, G., Hardie, J., Jaubert-Possamai, S., Simon, J.-C., Tagu, D., 2008. Shifting from clonal to sexual reproduction in aphids: physiological and developmental aspects. Biol. Cell 100, 441–451. https://doi.org/10.1042/BC20070135.
Le Trionnaire, G., Jaubert-Possamai, S., Bonhomme, J., Gauthier, J.-P., Guernec, G., Le Cam, A., Legeai, F., Monfort, J., Tagu, D., 2012. Transcriptomic profiling of the reproductive mode switch in the pea aphid in response to natural autumnal photoperiod. J. Insect Physiol. 58, 1517–1524. https://doi.org/10.1016/j.jinsphys.2012.07.009.
Lees, A.D., 1964. The location of the photoperiodic receptors in the aphid Megoura viciae buckton. J. Exp. Biol. 41, 119–133.
Levy, R., Kozak, G., Dopman, E., 2018. Non-pleiotropic coupling of daily and seasonal temporal isolation in the european corn borer. Genes (Basel). 9, 180. https://doi.org/10.3390/genes9040180.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
Marcovitch, S., 1924. The migration of the Aphididae and the appearance of sexual forms as affected by the relative length of daily exposure. J. Agric. Res. 27, 513–522.
Matsunaga, Y., Honda, Y., Honda, S., Iwasaki, T., Qadota, H., Benian, G.M., Kawano, T., 2016. Diapause is associated with a change in the polarity of secretion of insulin-like peptides. Nat. Commun. 7, 1–8. https://doi.org/10.1038/ncomms10573.
Meuti, M.E., Stone, M., Ikeno, T., Denlinger, D.L., 2015. Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, Culex pipiens. J. Exp. Biol. 218, 412–422. https://doi.org/10.1242/jeb.113233.
Mizoguchi, A., Okamoto, N., 2013. Insulin-like and IGF-like peptides in the silkmoth Bombyx mori: discovery, structure, secretion, and function. Front. Physiol. 4, 1–11. https://doi.org/10.3389/fphys.2013.00217.
Mohamed, A. a M., Wang, Q., Bembenek, J., Ichihara, N., Hiragaki, S., Suzuki, T., Takeda, M., 2014. N-acetyltransferase (nat) is a critical conjunct of photoperiodism between the circadian system and endocrine Axis in Antheraea pernyi. PLoS One 9, e92680. https://doi.org/10.1371/journal.pone.0092680.
Moran, N., 1992. The evolution of aphid life cycles. Annu. Rev. Entomol. 37, 321–348. https://doi.org/10.1146/annurev.ento.37.1.321.
Mukai, A., Goto, S.G., 2016. The clock gene period is essential for the photoperiodic response in the jewel wasp Nasonia vitripennis (Hymenoptera: pteromalidae). Appl. Entomol. Zool. 51, 185–194. https://doi.org/10.1007/s13355-015-0384-1.
Nakabachi, A., Shigenobu, S., Sakazume, N., Shiraki, T., Hayashizaki, Y., Carninci, P., et al., 2005. Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that harbors an endocellular mutualistic bacterium, *Buchnera*. Proc Natl Acad Sci USA 102, 5477–5482. https://doi.org/10.1073/pnas.0409034102.
Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford university press.
Numata, H., Miyazaki, Y., Ikeno, T., 2015. Common features in diverse insect clocks. Zool. Lett. 1, 10. https://doi.org/10.1186/s40851-014-0003-y.
Omura, S., Numata, H., Goto, S.G., 2016. Circadian clock regulates photoperiodic responses governed by distinct output pathways in the bean bug, Riptortus pedestris. Biol. Rhythm Res. 47, 937–945. https://doi.org/10.1080/09291016.2016.1212515.
Pavelka, J., Shimada, K., Kostal, V., 2003. Timeless: a link between fly's circadian and photoperiodic clocks? Eur. J. Entomol. 100, 255–265. https://doi.org/10.14411/eje.2003.041.
Pegoraro, M., Gesto, J.S., Kyriacou, C.P., Tauber, E., 2014. Role for circadian clock genes in seasonal timing: testing the bünning hypothesis. PLoS Genet. 10, e1004603. https://doi.org/10.1371/journal.pgen.1004603.
Ragland, G.J., Keep, E., 2017. Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. Physiol. Entomol. 42, 246–256. https://doi.org/10.1111/phen.12193.
Ramos, S., Moya, A., Martínez-Torres, D., 2003. Identification of a gene overexpressed in aphids reared under short photoperiod. Insect Biochem. Mol. Biol. 33, 289–298. https://doi.org/10.1016/S0965-1748(02)00243-6.
Saunders, D.S. David S., 2002. Insect Clocks. Elsevier.
Schiesari, L., Andreatta, G., Kyriacou, C.P., O'Connor, M.B., Costa, R., 2016. The insulin-like proteins dILPs-2/5 determine diapause inducibility in Drosophila. PLoS One 11, e0163680. https://doi.org/10.1371/journal.pone.0163680.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.
Shiga, S., Numata, H., 2009. Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, Protophormia terraenovae. J. Exp. Biol. 212, 867–877. https://doi.org/10.1242/jeb.027003.
Shigenobu, S., Bickel, R.D., Brisson, J. a., Butts, T., Chang, C.C., Christiaens, O., Davis, G.K., Duncan, E.J., Ferrier, D.E.K., Iga, M., Janssen, R., Lin, G.W., Lu, H.L., McGregor, a. P., Miura, T., Smagghe, G., Smith, J.M., Van Der Zee, M., Velarde, R. a., Wilson, M.J., Dearden, P.K., Stern, D.L., 2010. Comprehensive survey of developmental genes in the pea aphid, Acyrthosiphon pisum: frequent lineage-specific duplications and losses of developmental genes. Insect Mol. Biol. 19, 47–62. https://doi.org/10.1111/j.1365-2583.2009.00944.x.
Shingleton, A.W., Sisk, G.C., Stern, D.L., 2003. Diapause in the pea aphid (Acyrthosiphon pisum) is a slowing but not a cessation of development. BMC Dev. Biol. 3, 7. https://doi.org/10.1186/1471-213X-3-7.
Sim, C., Denlinger, D.L., 2013. Insulin signaling and the regulation of insect diapause. Front. Physiol. 4, 1–10. https://doi.org/10.3389/fphys.2013.00189.
Sim, C., Denlinger, D.L., 2009. A shut-down in expression of an insulin-like peptide, ILP-1, halts ovarian maturation during the overwintering diapause of the mosquito Culex pipiens. Insect Mol. Biol. 18, 325–332. https://doi.org/10.1111/j.1365-2583.2009.00872.x.
Steel, C.G., 1978. Some functions of identified neurosecretory cells in the brain of the aphid, Megoura viciae. Gen. Comp. Endocrinol. 34, 219–228. https://doi.org/10.1016/0016-6480(78)90213-7.
Steel, C.G., Lees, A.D., 1977. The role of neurosecretion in the photoperiodic control of polymorphism in the aphid Megoura viciae. J. Exp. Biol. 67, 117–135.
Steel, C.G.H., 1976. Neurosecretory control of polymorphism in aphids. In: Phase and Caste Determination in Insects. Elsevier, pp. 117–130. https://doi.org/10.1016/B978-0-08-021256-2.50015-7.
Steel, C.G.H.G., 1977. The neurosecretory system in the aphid Megoura viciae, with reference to unusual features associated with long distance transport of neurosecretion. Gen. Comp. Endocrinol. 31, 307–322. https://doi.org/10.1016/0016-6480(77)90095-8.
Tagu, D., Sabater-Muñoz, B., Simon, J.-C., 2005. Deciphering reproductive polyphenism in aphids. Invertebr. Reprod. Dev. 48, 71–80. https://doi.org/10.1080/07924259.2005.9652172.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. https://doi.org/10.1093/molbev/msr121.
The International Aphid Genomics Consortium, 2010. Genome sequence of the pea aphid Acyrthosiphon pisum. PLoS Biol. 8, e1000313. https://doi.org/10.1371/journal.pbio.1000313.
Vafopoulou, X., Cardinal-Aucoin, M., Steel, C.G.H., 2012. Rhythmic release of prothoracicotropic hormone from the brain of an adult insect during egg development. Comp. Biochem. Physiol. Mol. Integr. Physiol. 161, 193–200. https://doi.org/10.1016/j.cbpa.2011.10.026.
Vafopoulou, X., Steel, C.G.H., 2014. Synergistic induction of the clock protein PERIOD by insulin-like peptide and prothoracicotropic hormone in Rhodnius prolixus (Hemiptera): implications for convergence of hormone signaling pathways. Front. Physiol. 5, 1–12. https://doi.org/10.3389/fphys.2014.00041.
Vafopoulou, X., Steel, C.G.H., 2012a. Insulin-like and testis ecdysiotropin neuropeptides are regulated by the circadian timing system in the brain during larval-adult development in the insect Rhodnius prolixus (Hemiptera). Gen. Comp. Endocrinol. 179, 277–288. https://doi.org/10.1016/j.ygcen.2012.08.018.
Vafopoulou, X., Steel, C.G.H., 2012b. Metamorphosis of a clock: remodeling of the circadian timing system in the brain of Rhodnius prolixus (Hemiptera) during larval-adult development. J. Comp. Neurol. 520, 1146–1164. https://doi.org/10.1002/cne.22743.
Wang, Q., Egi, Y., Takeda, M., Oishi, K., Sakamoto, K., 2015. Melatonin pathway transmits information to terminate pupal diapause in the Chinese oak silkmoth Antheraea pernyi and through reciprocated inhibition of dopamine pathway functions as a photoperiodic counter. Entomol. Sci. 18, 74–84. https://doi.org/10.1111/ens.12083.
Wang, Q., Mohamed, A. a M., Takeda, M., 2013. Serotonin receptor B may lock the gate of PTTH release/synthesis in the Chinese silk moth, Antheraea pernyi; a diapause initiation/maintenance mechanism? PLoS One 8, 1–13. https://doi.org/10.1371/journal.pone.0079381.
Wood, S., Loudon, A., 2014. Clocks for all seasons: unwinding the roles and mechanisms of circadian and interval timers in the hypothalamus and pituitary. J. Endocrinol. 222, R39–R59. https://doi.org/10.1530/JOE-14-0141.
Zhang, X.-S., Wang, T., Lin, X.-W., Denlinger, D.L., Xu, W.-H., 2017. Reactive oxygen species extend insect life span using components of the insulin-signaling pathway. Proc. Natl. Acad. Sci. U. S. A 201711042. https://doi.org/10.1073/pnas.1711042114.
Zheng, X., Gong, Y., Kumar, D., Chen, F., Kuan, S., Liang, Z., Hu, X., Cao, G., Xue, R., Gong, C., 2016. Effect of BBX-B8 overexpression on development, body weight, silk protein synthesis and egg diapause of Bombyx mori. Transgenic Res. 25, 507–516. https://doi.org/10.1007/s11248-016-9947-3.