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Analysis of the circadian clock gene *period* in the sheep blow fly *Lucilia cuprina*

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Summary

We have isolated a homologue of the *period* (*per*) gene from the Australian sheep blow fly, *Lucilia cuprina*, as part of a comparative approach to the analysis of dipteran circadian systems. Sequence analysis of the 4 kb *per* cDNA revealed the conservation of three functional domains, namely the PAS dimerization motif, and the nuclear and cytoplasmic localization domains. A fourth domain, the threonine–glycine (TG) repeat region, is also conserved in *L. cuprina per* but has been severely truncated. No length variation was found in the TG repeat of *L. cuprina* or *L. sericata* collected from several different latitudinal zones. Expression analysis indicated a diel oscillation in *per* mRNA in LD 12:12 with a period of 24 h and a peak at Zt 12. PER-immunoreactive protein oscillations were also demonstrated, with peak immunoreactivity lagging approximately 3 h behind peak mRNA levels. These results show the existence of a *Drosophila*-like circadian system in a calliphorid fly. They also provide evidence for the conservation of *per* function across the Diptera, and confirm the relevance of the *Drosophila* system as a model for fly circadian rhythms.

1. Introduction

Circadian rhythms derive from a temperature-compensated endogenous oscillator which in nature is entrained on a daily basis by light and temperature cycles (Saunders, 1977). This timing system, which mediates rhythmic output to various elements of behaviour and physiology, arguably confers a selective advantage as it allows the prediction of rhythmic environmental phenomena, thus enabling organisms to prepare themselves physiologically and behaviourally for the onset of biologically important events.

The genetic control of endogenous rhythmicity has been studied extensively in *Drosophila melanogaster* and is known to rely on the action of at least five central clock genes: *period*, *timeless* (Sehgal *et al.*, 1994; Myers *et al.*, 1995; Gekakais *et al.*, 1995), *dClock*, *dBmal1* (*Cyc*) (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998) and *doubletime* (Price *et al.*, 1998). The products of these genes interact to generate a time-delayed negative feedback loop which results in the circadian expression of *per* and *tim* mRNAs and proteins, and subsequently rhythmic behaviour and physiology.

period (*per*) was the first circadian clock gene to be identified (Konopka & Benzer, 1971) and cloned (Bargiello *et al.*, 1984; Reddy *et al.*, 1984). The *per* mRNA along with the PER protein oscillate with a period equal to that of the behavioural rhythm (Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Hardin *et al.*, 1990), and *per's* function is necessary for the rhythmic expression of many processes in the insect life cycle from egg hatching (Sauman *et al.*, 1996), eclosion and adult activity patterns (Konopka & Benzer, 1971) through to ultradian courtship song rhythms (Kyriacou *et al.*, 1993; Konopka *et al.*, 1996).

There are at least three motifs in *D. melanogaster* PER known to be important in the correct functioning of the clock. These comprise a PAS dimerization domain, which encodes two highly degenerate 51 amino acid repeats and which mediates the dimerization of PER protein to its heterodimeric partner TIM (Huang *et al.*, 1993; Gekakais *et al.*, 1995), a nuclear localization signal (NLS), which mediates

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nuclear entry of the PER–TIM complex (Baylies *et al*., 1993; Vosshall *et al*., 1994), and a cytoplasmic localization signal (CLS) involved in the retention of PER in the cytoplasm until nuclear entry is appropriate (Saez & Young, 1996). The presence of the PER–TIM complex in the nucleus results in altered transcription of their genes thus completing the feedback loop.

A fourth region, the threonine–glycine (TG) repeat region, has been proposed to provide thermal stability of the circadian phenotype since the rescue of arrhythmic per^0 flies with a *per* gene lacking the repeat produces a rhythmic fly lacking temperature compenzation (Ewer *et al*., 1990). A North–South latitudinal cline in TG repeat length in European *D. melanogaster* supports the hypothesis that repeat length is involved in adaptation to different thermal environments (Costa *et al*., 1992; Sawyer *et al*., 1997). It has also been shown that coevolving regions flanking the TG repeat are involved in the temperature compenzation of the circadian clock (Peixoto *et al*., 1993, 1998; Nielsen *et al*., 1994) especially in non-drosophilid species which show a stable TG repeat length (Nielsen *et al*., 1994).

Isolation of full-length *per* homologues from a number of species including *Drosophila* (Colot *et al*., 1988), the giant silkmoth *Antheraea pernyi* (Reppert *et al*., 1994) and mammals (Tei *et al*., 1997; Sun *et al*., 1997; Zylka *et al*., 1998*a*), together with the isolation of partial *per* fragments from numerous other species (Reppert *et al*., 1994; Nielsen *et al*.,1994; Regier *et al*., 1998), has illustrated the wide degree of conservation of this central clock component within the Metazoa. Despite its evolutionary maintenance, *period* shows surprisingly high levels of sequence divergence between species (Regier *et al*., 1998). The subtle functioning of these homologues is also amazingly diverse. In *Drosophila*, cycling of the *per* and *tim* mRNAs and proteins and the nuclear entry of the PER–TIM dimer are essential for the generation of a circadian rhythm. However, in the eight lateral neuron (LN) cells of the lepidopteran brain, oscillations of a sense and an antisense *per* mRNA circumvent the need for nuclear entry of the PER protein (Sauman *et al*., 1996). The functional diversity of *per* is further illustrated by the murine system. Mice possess three *per* homologues which respond differentially to light at particular times of the circadian cycle and which dimerize to each other in addition to mouse Tim (Sangoram *et al*., 1998; Zylka *et al*., 1998*a*, *b*).

Given the diverse structure and function of *per* in different lineages, we have examined whether the *Drosophila* model for *per* function is relevant to other dipterans by analysing *per* in the Australian sheep blow fly *Lucilia cuprina*.

L. cuprina is a calliphorid dipteran whose circadian rhythms have been analysed extensively. Many behavioural patterns in its life cycle have been shown to be controlled by the circadian clock, from the timing of larval exodus (Smith *et al*., 1981) through to eclosion and adult activity (Smith, 1983, 1987). A naturally occurring clock mutant (*ary*) which results in the arrhythmicity of eclosion and adult activity patterns has been mapped to chromosome V in *L. cuprina* (Smith, 1987). The well-described circadian system of *L. cuprina*, in addition to the availability of extensive genetic resources (Weller & Foster, 1993), makes it a useful comparative model to analyse *period* gene structure and expression in the Diptera. To these ends we have isolated the full-length *per* cDNA from *L. cuprina*, and have conducted expression analyses both at the mRNA and protein levels.

2. Materials and methods

(i) *Fly rearing*

A mixed laboratory strain of *L. cuprina* originally obtained from AgResearch, Wallaceville, New Zealand was cultured in LD 12:12 (25 °C). Adult flies were supplied with sugar and water, and protein requirements were satisfied with slices of sheep liver. Liver was also used to egg flies. Eggs laid on liver were transferred to a culturing medium (consisting of 50% minced liver and 50% cat food). Post-feeding larvae were transferred to containers filled with Vermiculite and were left to pupate and eclose.

(ii) *cDNA isolation*

Total RNA was extracted from 10 fly heads in 1 ml of Trizol Reagent (Life Technologies) according to the manufacturer's instructions, and was subsequently precipitated using 0·5 ml isopropanol. After washing with 75% ethanol, RNA was resuspended in 20 μl of DEPC-treated H_2O and DNase treated for 15 min using amplification grade DNAse I (Life Technologies). Following denaturation at 70 °C for 10 min, 5 μl of RNA was reverse transcribed for 1 h at 42 °C using M-MLV reverse transcriptase (Life Technologies) according to the manufacturer's instructions and primed with 10 μM oligodT_{15}.

Degenerate oligonucleotide primers (PerF1 and PerR2) used for the amplification of a *per* cDNA fragment from *L. cuprina* were designed using consensus data from 10 different published insect *per* sequences, and the unpublished sequence of house fly (courtesy of Dr A. Piccin and Prof. C. P. Kyriacou, University of Leicester). The sequences of PerF1 (GGN MGN WSN TTY ATH GAY TTY GTN CA) and PerR2 (TTN TCR TTR TAR TTN ARY TGR TTR TA) correspond to amino acid positions 271–279 and 613–701 of the *D. melanogaster* PER protein sequence.

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Table 1. *Geographic location and latitude of samples of* L. cuprina *and* L. sericata *used for the sequence analysis of the TG repeat region of* per

Site (*L. cuprina*)	Latitude	Site (*L. sericata*)	Latitude
Baton Rouge (USA)	30° 30′ N	Vancouver (Canada)	49° 15′ N
Kluang (Malaysia)	3° 0′ N	Bloemfontein (SA)	29° 6′ S
Bloemfontein (SA)	29° 6′ S	Canberra (Australia)	35° 15′ S
Canberra (Australia)	35° 15′ S	Wallaceville (NZ)	41° 7′ S
Wanganui (NZ)	39° 56′ S	Rakaia (NZ)	43° 45′ S

SA, South Africa; NZ, New Zealand.

Hotstart PCR was used to amplify fragments from 1 μl of head cDNA in a 50 μl reaction mix containing 50 mM-KCl, 10 mM-Tris-Cl (pH 8·3), 1·5 mM-$MgCl_2$, 50 pmol of each primer, 100 μM dNTP and 1 U Ampli*Taq* DNA polymerase (Perkin Elmer). Amplification was conducted in a Hybaid Omn-E thermal cycler under the following conditions: 94 °C 3 min; 30 cycles of 94 °C 30 s, 45 °C 30 s, 72 °C 1 min. Amplicons were tested for *per*-homology by Southern transfer onto positively charged nylon membrane (Hybond N+). The membrane was probed with a fragment of house fly *per* (a gift from Dr A. Piccin and Prof. C. P. Kyriacou, University of Leicester) which had been random primed with [α^{32}P] dCTP (using the Life Technologies random prime kit). Prehybridization and hybridization were conducted at 45 °C in 0·25 M Na_2HPO_4 (pH 7·2) containing 7% SDS and 1mM EDTA. Membranes were washed twice in 2 × SSC, 0·1% SDS (45 °C, 30 min) and twice in 1 × SSC, 0·1% SDS (45 °C, 30 min) before exposure to X-ray film at −80 °C for 48 h. Autoradiographs were subsequently developed to visualize *per*-reactivity.

Positively hybridizing fragments were purified from a 1% agarose TAE gel using 'GlassMax' (Life Technologies) and were cloned into pGEM-T (Promega). Plasmid DNA was purified using a modified alkaline lysis method (Feliciello & Chinali, 1993) and sequenced using PRISM Dye Deoxy Terminator Cycle Sequencing (Applied Biosystems) on a 373A automated sequencer (Applied Biosystems).

The *per* fragment isolated above was radiolabelled and used to probe a random-primed *L. cuprina* adult head λ GT10 cDNA library (a gift from Dr P. East, CSIRO). Prehybridization and hybridization were conducted at 55 °C in 0·25 M-Na_2HPO_4 (pH 7·2) containing 5% SDS. Inserts from positive recombinant phage clones were subcloned into pUC 18 and sequenced as above by primer walking.

3′ RACE was employed to isolate the remaining 3′ region of the *L. cuprina per* cDNA according to Ma *et al*. (1994). RNA isolation and reverse transcription were conducted as above with the exception that a modified oligo-dT primer R_oR_idT (ATC GAT GGT CGA CGC ATG CGG ATC CAA AGC TTG AAT TCG AGC TCT TTT TTT TTT TTT TTT) (including two specific priming sites) was used for the reverse transcription. PCR was conducted as above using a primer designed to the 3′ end of the *per* cDNA clone and a reverse primer specific for one of the synthetic priming sites generated on the end of the cDNA by the modified oligo-dT primer. Thermal cycling was carried out using the following program: 94 °C 3 min; 35 cycles of 94 °C 15 s, 60 °C 30 s, 72 °C 1 min. Amplicons were purified using a 'Qiaquick' PCR purification kit (Qiagen) and were cloned as above. Positive transformants were initially screened by their restriction profile, and Southern hybridization with an overlapping *per* cDNA fragment. Positively hybridizing clones were then sequenced as above.

(iii) *Geographic analysis of TG repeat length*

PCR of an 733 bp fragment of *per* was conducted using genomic DNA samples of *L. cuprina* and the sibling species *L. sericata* collected from eight different geographic locations (Table 1).

PCR was conducted using the following cycling conditions: 94 °C 3 min; 35 cycles of 94 °C 15 s, 55 °C 30 s, 72 °C 1 min. Amplicons were sequenced directly as above and the sequences aligned using Sequencher (Gene Codes Corp.). Phylogenetic analysis was conducted using PAUP 3·1 (Swofford, 1993).

(iv) *Quantitative competitive RT-PCR*

Primers QPerF1 (TGG AAT ACC AAT AGC CGA ATC ACG C), QPerR2 (CAA TAC CGA CAC TGC TGC ACT ACT C) and QComp (TGG AAT ACC AAT AGC CGA ATC ACG CGA TTG ACA TTC CGC GAA GCA CC) were designed to enable the amplification of a *per* fragment and a *per* competitor. Amplification of a *per* cDNA fragment with QPerF1 and R2 produced an amplicon of 359 bp. Amplification with the QPerF1/QComp combination

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produced a shorter amplicon (the competitor) of 238 bp which contained identical priming sites to the QPerF1/R2 product. Both these fragments were cloned into pGEM-T as described above and were sequenced to check their integrity and orientation. The plasmids were then linearized and a sense RNA strand of each fragment was transcribed using a Promega *in vitro* transcription kit. RNA was DNase treated using Promega RQ1 RNase-free DNaseI, purified by phenol/chloroform extraction, and ethanol precipitated. Purified RNA was resuspended in DEPC-treated H_2O and quantified using a Genequant RNA/DNA Calculator (Pharmacia).

Quantitative competitive RT-PCR was undertaken using the standard curve methodology of Tsai & Wiltbank (1996). To generate a standard curve, RT-PCR was conducted using constant amounts of competitor RNA (10 pg) and serial dilutions of *in vitro* transcribed native template between 100 pg and 0·5 pg. The specific primer QPerR2 was used to prime the reverse transcription as opposed to oligo-dT. The PCR conditions used were as follows: 94 °C 3 min; 35 cycles of 94 °C 15 s, 65 °C 30 s, 72 °C 1 min. Gel images were quantified using Scion image (NIH) and data plotted using Microsoft Excel 7.

Experimental samples of 10 fly heads were taken every 2 h for 48 h from flies held in LD 12:12. Total RNA was extracted and quantified and 5 μg was used in each RT-PCR with 10 pg of competitor. Samples were visualized and quantified as above. Relative band intensities were converted into pg of *per* mRNA per μg of total RNA using the standard curve measurements.

(v) *Northern dot blots*

A 291 bp fragment of *L. cuprina* RP49 cDNA was isolated using redundant PCR and primers RP49F1 (CAC CAG TCG GAT CGN TAT GCC) and RP49R2 (GAC AGC TGC TTG GCN CGN TC) designed to a consensus RP49 sequence from *D. acanthoptera*, *D. subobscura* and *D. pseudoobscura*. The fragment was cloned, sequenced, linearized and transcribed as an antisense DIG-labelled probe using a Promega *in vitro* transcription kit and DIG RNA labelling mix (Boehringer Mannheim). This probe, along with sense and antisense DIG-labelled *per* probes transcribed from the QPerF1/R2 fragment, were used to probe dot blots of 20 μg total RNA. Prehybridization and hybridization were conducted using DIG-EasyHybe (Boehringer Manheim) at 60 °C. Subsequent washes were conducted according to the instructions in the DIG protocols handbook (Boehringer Manheim) and the reaction was visualized using a chemiluminescant system (Boehringer Manheim).

(vi) *Western blotting*

Samples of 10 fly heads were taken at 3 h intervals from flies held in LD 12:12 and frozen in liquid N_2. Heads were subsequently homogenized in 50 mM-Tris-Cl (pH 7·5), 10% glycerol, 5 mM magnesium acetate, 0·2 mM-EDTA containing 1 mM-DTT, 1mM-PMSF, 0·1% leupeptin and 0·1% aprotinin (Persichetti *et al.*, 1993). Protein quantification was conducted using the Bradford assay (Bradford, 1976), and 40 μg of each sample was resolved on an 8% SDS-polyacrylamide gel. Following separation and semi-dry Western blotting, the membrane was blocked for 1·5 h in 1 × PBS 0·1% Tween 20 (PBS-T) and incubated for 1·5 h with the primary PER antibody PER no. 107 (a gift from Dr L. Saez, Rockefeller University) diluted 1:500 in PBS-T. Incubation with a secondary HRP-linked anti rabbit IgG antibody (Amershsam), diluted 1:1000 in PBS-T was conducted for 1 h and positive immunoreactivity was visualized using an enhanced chemiluminescent system (Amersham).

3. Results

(i) per *encodes an inferred protein of 1037 amino acids*

Isolation of a 3996 bp *L. cuprina per* cDNA (GenBank accession number Y19108) encoding an inferred protein of 1037 amino acids (MW 110 kDa) was achieved in three steps. Isolation of an initial 1·2 kb fragment (corresponding to nucleotide positions 813–2103 of *D. melanogaster per*) using degenerate PCR facilitated the screening of a *L. cuprina* adult head cDNA library. Screening of this library detected a 2·8 kb *per* cDNA which extended from the initiation methionine through to nucleotide 2316 (corresponding to nucleotide position 2811 in *D. melanogaster*). The remaining 800 bp of coding sequence and 346 bp of 3′ untranslated region were obtained using further redundant PCR and 3′ RACE. The entire *L. cuprina per* cDNA (Fig. 1) was constructed by joining these overlapping fragments.

The sequence shown in Fig. 1 includes 425 bp of 5′ untranscribed sequence; however, the CAP sequence is not evident at the extreme 5′ end. The initiation ATG codon is embedded in a good Kozak consensus sequence (CAAAATGGAA). There is a predicted polyadenylation signal from nucleotides 3397–3403 (Fig. 1). Codon and nucleotide composition in both coding (59%) and non-coding (72%) regions are biased towards A and Ts.

The overall identity of the inferred PER protein to *Musca domestica*, *D. melanogaster* and *Antheraea pernyi* PER is 84%, 73% and 37% respectively (unpublished *Musca domestica* sequence donated by Dr A. Piccin and Prof. C. P. Kyriacou, University of Leicester). However, within the PAS domain and the

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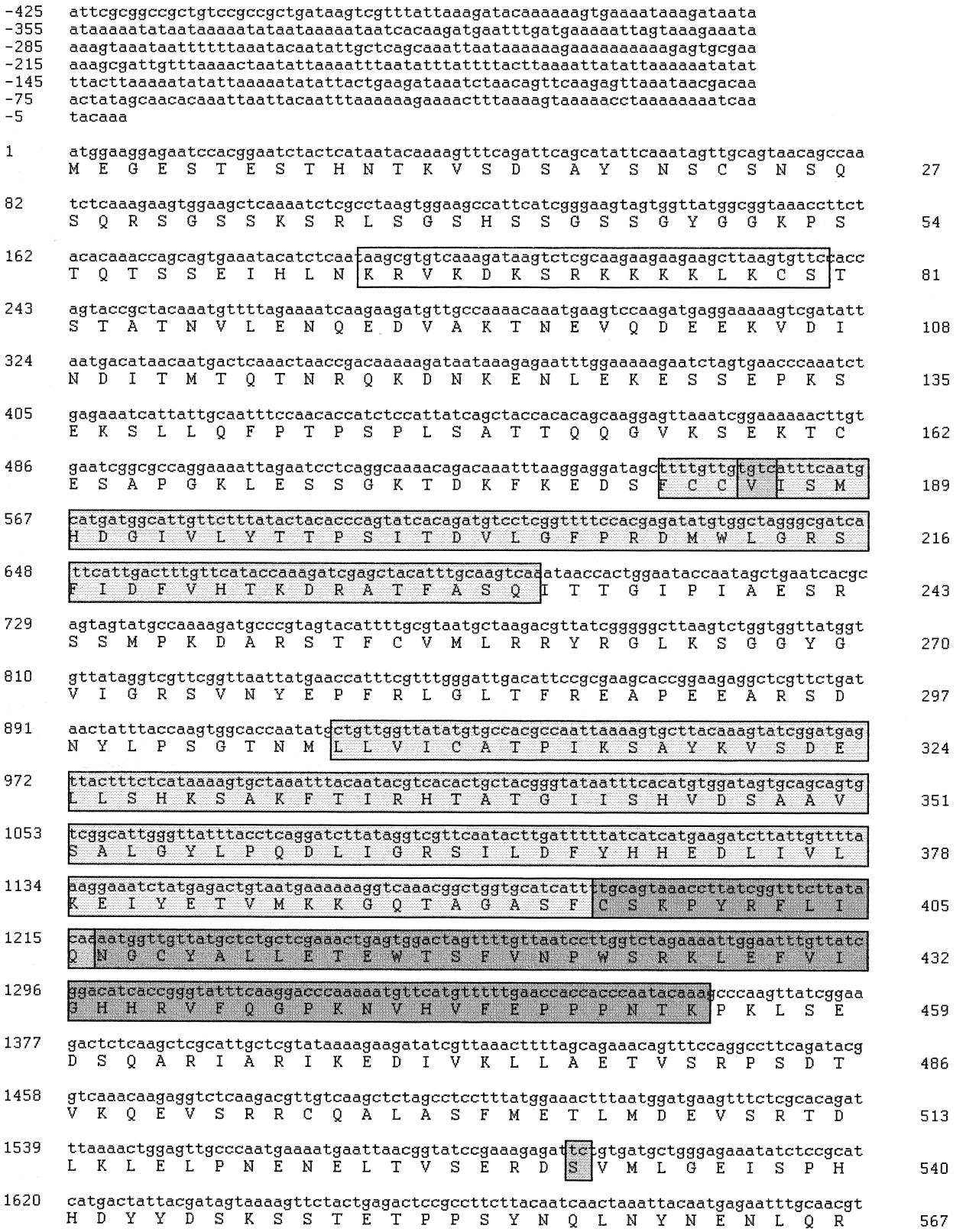

Fig. 1. For legend see page 262.

cytoplasmic and nuclear localization domains, this homology is greatly increased (Table 2). The amino acids present in the positions of the three *period* mutations are identical to those in wild-type *D. melanogaster* (Fig. 1). The TG repeat region is short, consisting of only two complete repeats.

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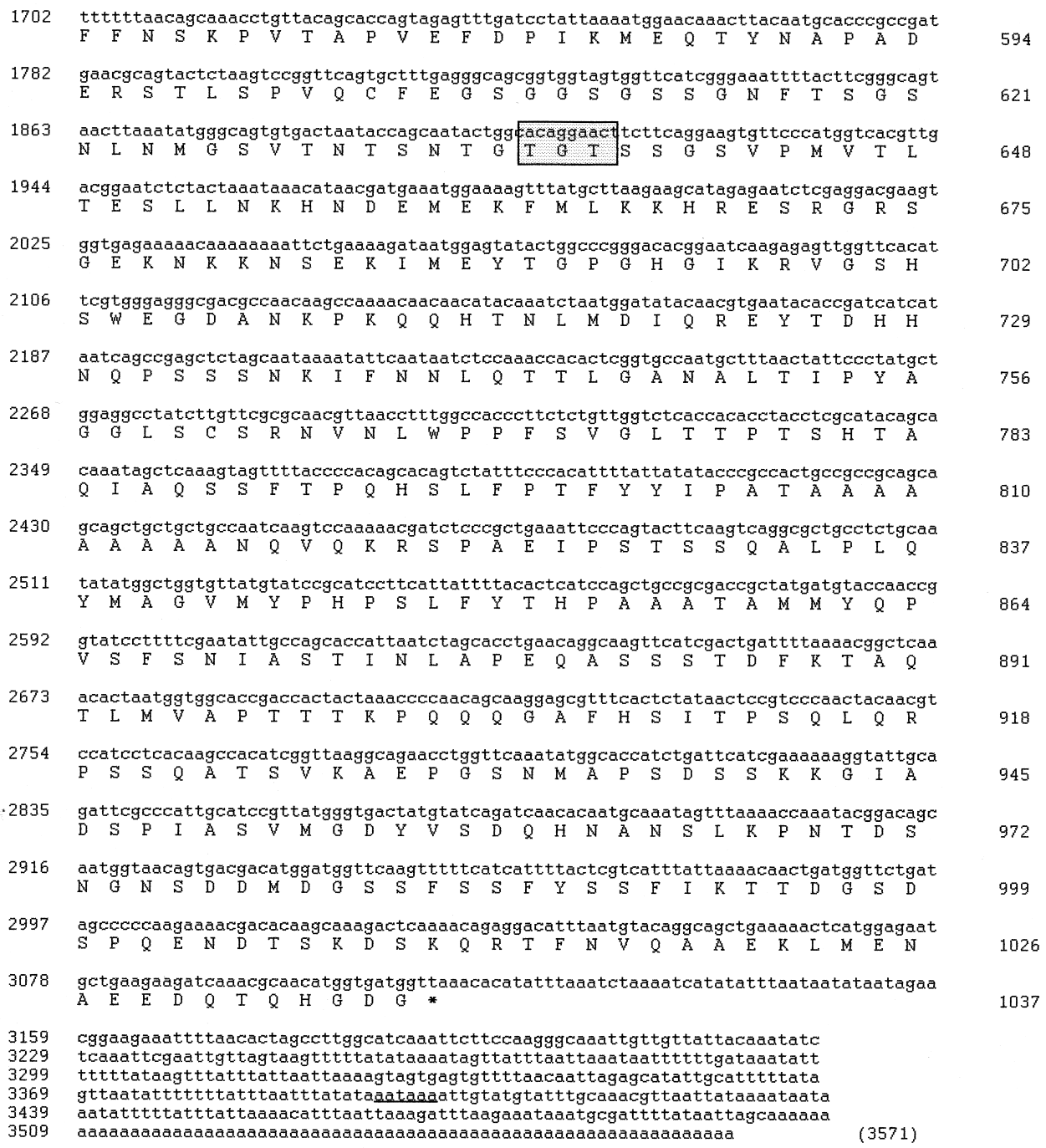

Fig. 1. Nucleotide and inferred amino acid sequence of *L. cuprina per* cDNA (Genbank accession number Y19108). Motifs corresponding to *D. melanogaster* functional domains are highlighted: □, nuclear localization signal (aa 65–80); ▨, PAS A domain (aa 183–232); □, PAS B domain (aa 307–396); ▩, cytoplasmic localization signal (aa 394–454); ▨, sites of *period* mutations in *D. melanogaster*: (I) Per^{L} mutation (aa 186), (II) per^{0} mutation and (III) per^{s} mutation. Nucleotide numbering is on the left and starts at the adenine of the predicted ATG start of translation. Amino acid numbering is on the right. The predicted stop of translation is marked with an asterisk and the predicted polyadenylation signal is underlined.

(ii) *TG repeat length does not vary with latitude in* L. cuprina *or* L. sericata

No polymorphism in TG repeat length was found across any of the samples analysed. All the flies analysed maintained the two complete repeat form (TGTGT) as shown in Fig. 1. However, two amino acid encoding polymorphisms were detected in the 733 bp fragment spanning the repeat. All five *L. sericata* strains contained an alanine at amino acid 595 while all *L. cuprina* strains contained a glutamic acid at this position. The *L. cuprina* strain LS2

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Table 2. *Identity between the PAS A and B, NLS and CLS functional domains of* L. cuprina, *and those of* D. melanogaster *and* M. domestica

Species	Total (%)	NLD (%)	CLD (%)	PAS A (%)	PAS B (%)
M. domestica	84	87	85	98	90
D. melanogaster	73	80	85	96	89

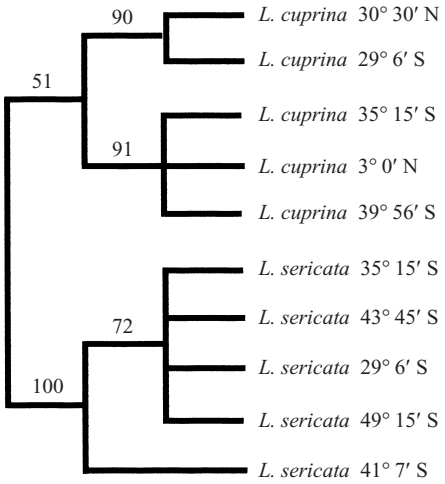

Fig. 2. Cladogram generated from the consensus of an exhaustive PAUP search. The sequence used to construct the tree was obtained from analysis of a 733 bp genomic DNA fragment of *L. cuprina* and *L. sericata per* spanning the TG repeat region. Bootstrap values are indicated on those branches which have greater than 50% support. Branches with less than 50% support have been collapsed.

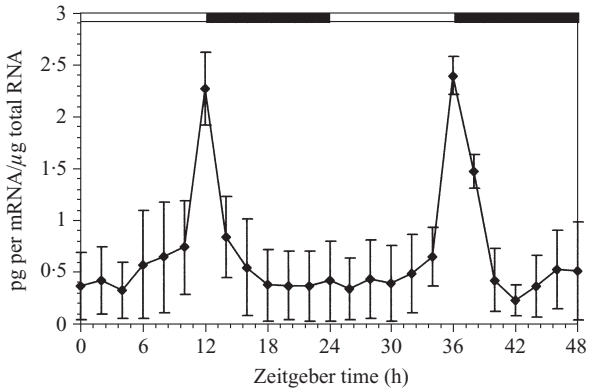

Fig. 4. Mean *per* mRNA levels of samples measured during a 48 h period. Values are means of three separate RT-PCR experiments and units given are picograms of *per* mRNA per microgram of total RNA. A light regime of LD 12:12 (lights on Zt0 and Zt 24 lights off Zt 12 and Zt 36) is indicated by the black and white bar.

(Canberra) was heterozygous for threonine and isoleucine at amino acid position 726. All other strains had a threonine at this position. Phylogenetic analysis of the entire 733 bp fragment showed a clear branching of the two species of *Lucilia* but failed to show any correlation between sequence of regions flanking the TG repeat and latitude of collection of the flies (Fig. 2).

(iii) L. cuprina per *mRNA and protein levels oscillate 3 h out of phase*

A clear diel rhythm of *per* mRNA levels was shown in LD 12:12 using quantitative competitive RT-PCR (Figs. 3 and 4). *per* mRNA oscillations exhibited a period of 24 h and a peak at Zt 12 (the light–dark transition) (Fig. 4). While diel oscillations were detected in *per* mRNA using quantitative RT-PCR and northern dot-blots, no oscillation was detected in RP49 levels by northern dot blot analysis (data not shown). Probing of northern blots with a sense *per* RNA probe failed to detect the expression of an antisense RNA strand (data not shown).

The rhythm in *per* mRNA expression was mirrored by a diel rhythm in PER protein-like immunoreactivity. The levels of a 110 kDa PER-immunoreactive band identified in western blots was found to oscillate in LD 12:12 with a delayed phase angle relative to *per* mRNA oscillations. Peak levels were detected at Zt 15 (3 h after the peak *per* mRNA levels) (Fig. 5).

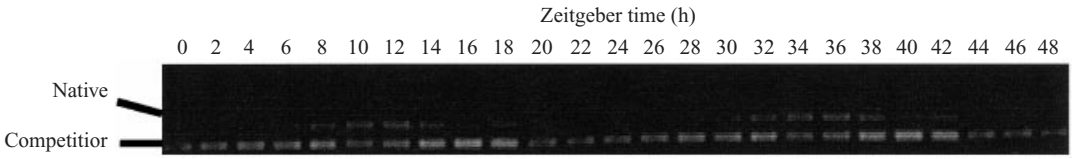

Fig. 3. Quantitative RT-PCR of *per* mRNA resolved on a 1% agarose gel. Samples were taken at 2 h intervals in LD 12:12 (lights on at Zt0 and Zt 24, lights off at Zt 12 and Zt 36). The larger 359 bp band is native *per* template, while the smaller 238 bp band is the competitor.

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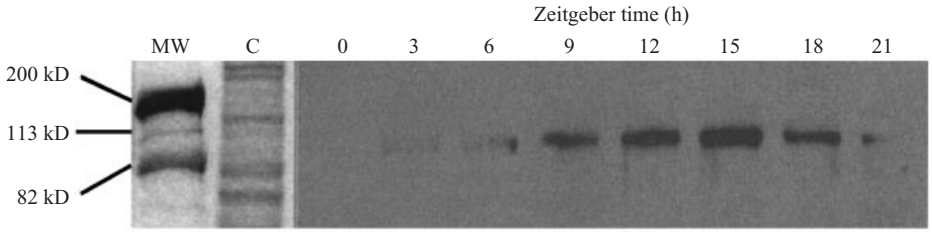

Fig. 5. Autoradiograph of western blot (ECL) of Per-lir levels in *L. cuprina* maintained in LD 12:12. Samples taken every 3 h were resolved on an 8% SDS acrylamide gel and Westerns probed with Per no. 107 polyclonal antibody (a gift from L. Saez). The reactive band detected has an estimated molecular weight of approximately 110 kDa. Lanes 1 and 2 show a Coomassie-stained molecular weight marker (BioRad) and a total protein sample. Lanes 3–10 indicate the amount of Per-lir in samples taken from the time points noted.

4. Discussion

per is a central clock gene involved in the generation of the circadian feedback loop and has been extensively investigated in *D. melanogaster*. We have isolated an entire *per* cDNA homologue from a representative of a second dipteran family: the sheep blow fly *Lucilia cuprina* (Calliphoridae). The predicted protein of 1037 amino acids is 73% identical to *Drosophila* PER. This is within the range of comparisons of other nuclear genes: 73% for the LcEcR ecdysone receptor gene (Hannan & Hill, 1997), 74% for the notch homologue (Chen *et al*., 1998), 64% for the E3 gene (Newcomb *et al*., 1997) and 78% for the white gene (Garcia *et al*., 1996). As with other *L. cuprina* genes, the levels of AT bias are high (Garcia *et al*., 1996; Newcomb *et al*., 1997), thus preventing informative nucleotide comparisons from being made with other flies such as *Drosophila*.

In contrast to the Diptera, lepidopteran *per* has been noted for its extremely rapid rate of evolution (Schmid and Tautz, 1997; Regier *et al*., 1998). Within the PAS domain lepidopteran *per* has evolved 5 to 40 times more rapidly than other nuclear genes (Regier *et al*., 1998). The difference in rates of *per* evolution between the Diptera and the Lepidoptera cannot be attributed to an evolutionary time scale, as molecular clock evidence shows that the radiation of the higher flies and the moths both occurred during the Cretaceous period (Beverley & Wilson, 1984).

The presence of particular regions of conservation and variability in the *per* coding sequence first mentioned by Colot *et al*. (1988) was evident in *L. cuprina*, and many of the conserved regions were associated with functional domains (Table 1).

Possession of a PAS domain and in many cases a TG repeat are hallmarks of a clock gene. PAS domains and TG repeats have been found in clock genes from a wide range of species, from *Drosophila* through to the bread mould *Neurospora crassa* (Citri *et al*., 1987; Kay, 1997; Linden & Macino, 1997), and the analysis of these regions may provide evidence for the evolutionary origins of circadian clocks (Kay, 1997). Particularly high conservation of the PAS, CLD and NLD domains between *D. melanogaster* and *L. cuprina* suggests that the *L. cuprina* feedback loop may function in a similar manner to that of *Drosophila*, and we may predict the existence of a *timeless* homologue in the sheep blow fly. The high degree of sequence divergence of *per* within the Lepidoptera may reflect the different functional nature of the circadian feedback loop in moths.

The two-repeat form of the TG motif evident in *L. cuprina* is characteristic of all non-drosophilid flies, which have been shown to maintain this stable repeat length (Nielsen *et al*., 1994). Despite the different habitat ranges and upper lethal limits of *L. cuprina* and *L. sericata*, the lack of variation in TG repeat length, and the lack of correlation of flanking sequences with latitude of strain collection (Fig. 2), provide no evidence for the involvement of this region in temperature compensation of the circadian clock in these species (as it has done in *Drosophila*). The lack of variation in this region does not, however, preclude its involvement in temperature compensation. Similarly it does not rule out the involvement of other regions such as the PAS domain in conferring temperature compenzation (Huang *et al*., 1995; Curtin *et al*., 1995).

L. cuprina maintains a 22 h free-running period in constant conditions (Smith, 1983) compared with the 24 h period of *Drosophila*. The amino acids which give rise to changes in period length when altered in *Drosophila* have been conserved in the wild-type form of *L. cuprina per* (Fig. 1). This suggests that the shorter free-running period in *Lucilia* is attributable either to the action of a different amino acid mutation in *per*, or to a difference in another functional element of the *L. cuprina* feedback loop.

In *Drosophila*, *per* was localized by mapping three free-running period mutations to the left arm of the X chromosome (Konopka & Benzer, 1971). The mapping of *L. cuprina per* using clones isolated in the present work has shown *per* to occupy a position homologous to that of *per* in the *Drosophila* genome (P. Batterham, personal communication 1998). This

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mapping also indicates that the *ary* mutation, which maps to chromosome V (3L equivalent in *Drosophila*) and has severe effects on the circadian phenotype (Smith, 1987), is either a *Lucilia* homologue of another clock element or a novel clock gene. There also remains the possibility that *ary* corresponds to the arrhythmic XR allele of *D. pseudoobscura* (Smith, 1987).

The expression studies conducted here demonstrate that both *per* mRNA and protein levels oscillate in a diel manner. This provides further evidence that the *per* homologue in *Lucilia* is a functional element of the circadian feedback loop. The expression pattern of *per* mRNA in *L. cuprina* is remarkably similar to that of *D. melanogaster*, with peak levels maintaining comparable phase angles in both species (mRNA levels peak at Zt 12 in *L. cuprina* and Zt 13 in *D. melanogaster*). As in *D. melanogaster*, peak protein levels lag significantly behind mRNA levels; however, the phase angle of PER protein oscillations in *Lucilia* is advanced with respect to the *Drosophila* system (peak PER-lir levels occur at Zt 15 in *L. cuprina* compared with Zt19–20 in *Drosophila*). It may be possible that the shortened phase angle is an artefact of the entrainment of the free-running period to 24 h by the light cycle, as has been noted in per^0 mutants (Marrus *et al*., 1996); however, this shortened phase angle may also reflect the protracted 22 h behavioural circadian cycle in *Lucilia*. The observation of a shorter phase angle in *Lucilia* may have significant implications for the *L. cuprina* feedback loop, as the kinetics of transcription and translation are such that the approximately 3 h delay observed in *Lucilia* does not require an independent delay mechanism, whereas the 4–6 h delay observed in *Drosophila* does (U. Schibler, personal communication 1998).

Despite the diversity of *per* expression in the insect species previously investigated, the pattern in *Lucilia* corresponds closely with that of *Drosophila*. In addition to these oscillations, no antisense RNA has been detected, thus eliminating the possibility of cycling sense and antisense mRNA strands as is found in Lepidoptera. The implications of these data for the use of *Drosophila* as a fly model are important. As this is the first system which appears to mirror *Drosophila* closely, these findings enhance the relevance of the *Drosophila* model. Therefore, the *Drosophila* system may be the rule rather than the exception, at least in the Diptera.

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