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The clock gene *period* in the medfly *Ceratitis capitata*

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Summary

We have isolated the clock gene *period* (*per*) from the medfly *Ceratitis capitata*, one of the most economically important insect pest species. The overall pattern of conserved, non-conserved and functional domains that are observed within dipteran and lepidopteran *per* orthologues is preserved within the coding sequence. Expression analysis from fly heads revealed a daily oscillation in *per* mRNA in both light : dark cycles and in constant darkness. However PER protein levels from head extracts did not show any significant evidence for cycling in either of these two conditions. When the *Ceratitis per* transgene under the control of the *Drosophila per* promoter and 3′UTR was introduced into *Drosophila per*-null mutant hosts, the transformants revealed a low level of rescue of behavioural rhythmicity. Nevertheless, the behaviour of the rhythmic transformants showed some similarities to that of *Ceratitis*, suggesting that *Ceratitis per* carries species-specific information that can evidently affect the *Drosophila* host's downstream rhythmic behaviour.

1. Introduction

In *Drosophila*, the clock gene *period* (*per*) plays a key role in the generation and maintenance of a number of biological rhythms, including the circadian periodicity of locomotor activity and adult emergence from the pupal case (Hall, 2003). *Drosophila per* is expressed rhythmically at both mRNA and protein levels (Siwicki *et al*., 1988; Hardin *et al*., 1990; Edery *et al*., 1994) and the protein also shows a temporal regulation in its subcellular localization (Shafer *et al*., 2002). PER is phosphorylated by DOUBLETIME (DBT), the *Drosophila* homologue of human Casein Kinase 1ε (Kloss *et al*., 1998; Price *et al*., 1998), and this phosphorylation targets the protein for degradation. PER degradation is prevented by the formation of a complex with TIMELESS (TIM), another key component of the *Drosophila* circadian clock (Hall, 2003). The DBT/PER/TIM complex enters the nucleus, where it represses the positive regulation of the dCLK/CYC dimer on *per* and *tim* transcription (Hall, 2003). Recent findings have demonstrated that PER monomers alone can efficiently inhibit dCLK/CYC transcriptional activation (Rothenfluh *et al*., 2000) and PER mediated inhibition of dCLK/CYC is enhanced but not dependent on TIM (Weber & Kay, 2003).

The comparison of *per* coding regions from *D. melanogaster*, *D. pseudoobscura* and *D. virilis* reveals that the translation products consist of interspersed blocks of six conserved (c1–c6) and five non-conserved (n1–n5) regions (Colot *et al*., 1988). The longest conserved region (c2) includes those sites to which per^{L}, per^{S} and per^{01} mutations have been mapped (Baylies *et al*., 1987; Yu *et al*., 1987). In addition it encompasses the PAS domain, which includes two degenerate 51 amino acid direct repeats, interspersed by more than 100 residues. PAS regions are protein–protein dimerization domains (Crews *et al*., 1988) and are particularly prevalent in proteins that act as environmental sensors via light, oxygen

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and redox potential (Ponting & Aravind, 1997; Gu *et al*., 2000). The conserved region 2 (c2) also includes the cytoplasmic localization domain (CLD) which is C-terminal to PAS, and is involved in the retention of PER in the cytoplasm (Saez & Young, 1996). The non-conserved region n2 includes, in *D. melanogaster*, the Thr-Gly repeat, which shows dramatic sequence and length variation among dipterans and is known to be under selection (Costa & Kyriacou, 1998).

Orthologues of *Drosophila per* have been cloned in several dipterans, including the housefly *Musca domestica* (Piccin *et al*., 2000), the sheep blow fly *Lucilia cuprina* (Warman *et al*., 2000), the melon fly *Bactrocera cucurbitae* (Miyatake *et al*., 2002) and two related species, the Queensland fruit fly *B. tryoni* and its sibling *B. neohumeralis* (An *et al*., 2002). In addition, the giant silkmoth *Antheraea pernyi* has been the focus of several studies within Lepidoptera (Reppert *et al*., 1994; Chang *et al*., 2003). Here we describe the isolation and characterization of the clock gene *period* from the medfly *Ceratitis capitata*. The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) is one of the world's most destructive fruit pests. The species originated in the Mediterranean region of Europe and North Africa and is now known to be established in Florida, California, Argentina, Bermuda, Brazil, Costa Rica, Hawaii, Uruguay, Western Australia, many countries in Europe and Africa, and in Middle Eastern countries in the Mediterranean area. Because of its worldwide distribution, its ability to tolerate cooler climates and its wide range of hosts, it is considered the most economically important fruit fly species.

2. Methods

(i) *Fly strains*

All *Drosophila melanogaster* strains were reared in 12 h : 12 h light : dark cycles (12 : 12 LD, where Zeitgeber time (ZT)0 corresponds to lights-on and ZT12 to lights-off) at 23 °C. *Drosophila* and *Ceratitis* were fed on standard sugar medium. *C. capitata* (ISPRA strain) were provided at the pupal stage by Prof. G. Gasperi (Department of Animal Biology, University of Pavia, Italy).

(ii) *Identifying* Ceratitis per

Genomic DNA was extracted from adult *Ceratitis* as in Strauss (1994), partially digested with *Sau3A*I and cloned in Lambda EMBL3/*BamH*I, using the Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. A Thr-Gly encoding fragment from *Ceratitis* (amino acids 552 to 657) was used to screen $1{\cdot}3 \times 10^5$ pfu of the EMBL3 *Ceratitis* library and the largest clone identified, approximately 20 kb, was subcloned into pBluescript and partially sequenced. A 14 kb region corresponding to *C. capitata period* was identified, but lacked the 5′ end. By chromosome walking, an approximately 15 kb positive clone was isolated, subcloned and partially sequenced.

(iii) *Sequence comparison and phylogenetic analysis*

Multiple sequence alignment was performed with the ClustalW software suite (Thompson *et al*., 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al*., 2001), the 'Number of Differences' model to calculate distance and the UPGMA phylogeny inference method.

(iv) *RNA isolation and* Ceratitis per *cDNA isolation*

Fifteen adult heads were collected at ZT12, ZT14 and ZT16 and mixed in a single sample. Total RNA was then prepared using the TRIZOL reagent (Life Technologies) according to the manufacturer's instructions. Subsequently, cDNA was prepared with SUPERSCRIPT II RNase H^- Reverse Transcriptase (GIBCO BRL) according to the manufacturer's instructions.

Ceratitis per coding sequence was obtained as two amplified fragments: (i) a 1·8 kb 5′ fragment, using the 5′ primer 5′-CAGCATATATTTCCATTG-3′ (positions 306–324) and the 3′ primer 5′-TTCTTCA-TTGG**GTCGAC**C-3′ (positions 2095–2119; boldface represents the unique internal *Sal*I restriction site); (ii) a 1·45 kb 3′ fragment, with the 5′ primer 5′-G**GTCGAC**CCAATGAAGAA-3′ (positions 2095–2119; boldface represents the unique internal *Sal* I restriction site), and the 3′ primer 5′-CATGTA-TGTATGTACATATGTATGTATGC-3′ (positions 3470–3498). The primer positions refer to the *C. capitata per* sequence reported in Fig. 1. All the PCR reactions were performed with a high-fidelity DNA polymerase (Expand High Fidelity PCR System; Roche) in order to minimize PCR-induced errors. The two fragments were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced in order to ensure the absence of PCR mutations. Subsequently, in a three-way ligation reaction they were joined at the *Sal*I site in the pBluescript vector, in order to produce an approximately 3·1 kb fragment containing the whole *C. capitata period* cDNA.

(v) *Construction of the* C. capitata *and* D. melanogaster *transgene* MC1

The *C. capitata per* construct p*MC1* for P-element transformation was prepared by joining the 7·3 kb

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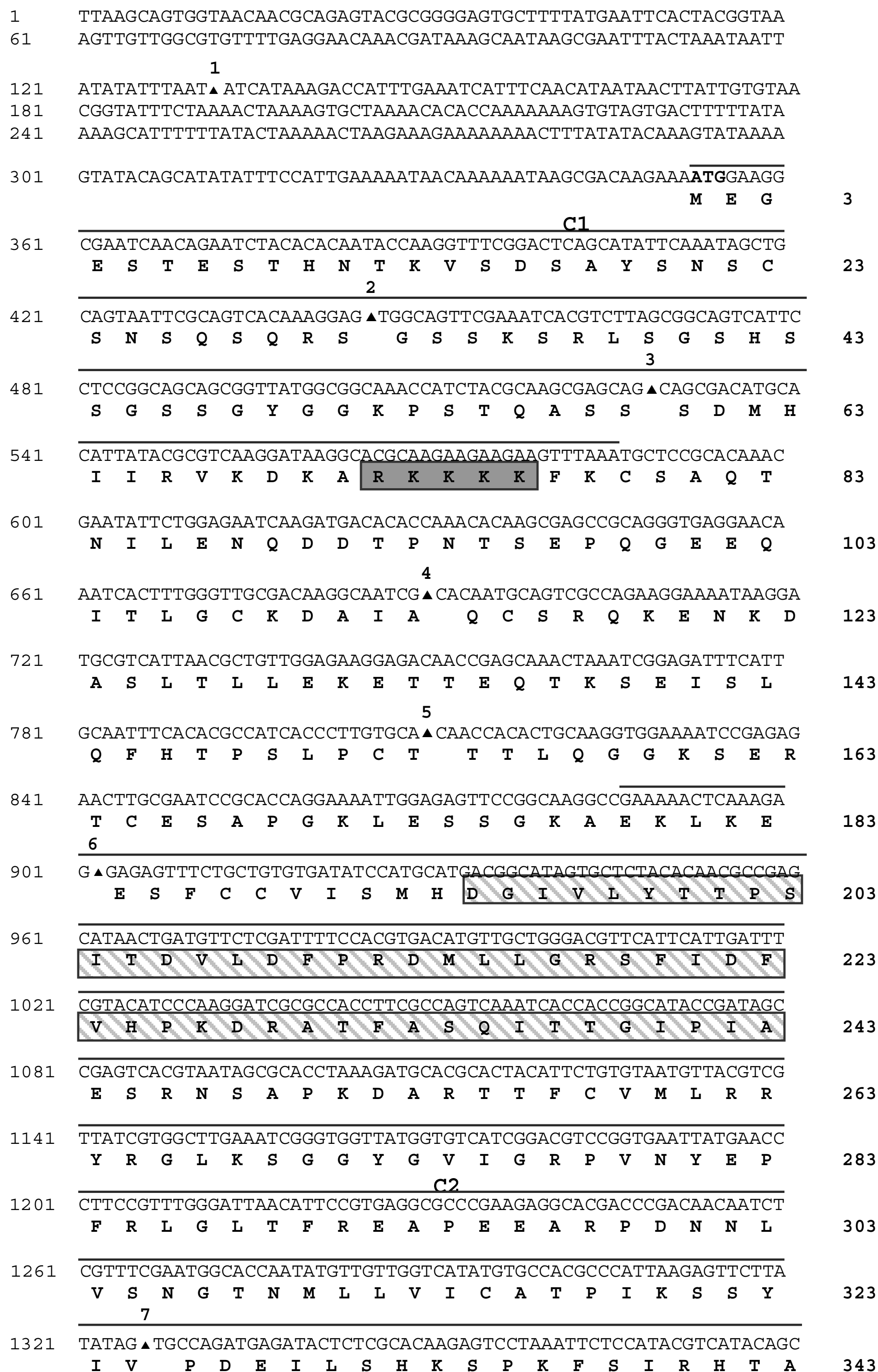

Fig. 1. (Cont.)

D. melanogaster per 5′ regulatory region and the 3 kb 3′UTR to the coding sequence of the *Ceratitis* gene, using a series of cloning steps.

First step. The approximately 7 kb *D. melanogaster per BamH*I–*Xba*I fragment, which contains the 5′ flanking DNA, the first, untranslated exon and the first large intron, was extracted from the 13·2 genomic clone (Petersen *et al*., 1988; Peixoto *et al*., 1998) and subcloned in pBluescriptKS. The immediately downstream 330 bp *Xba*I–*Spe*I fragment, was mutagenized via PCR, by using the 5′ primer 5′-GAATTG-**ACTAGT**AGTGAATCG-3′ (positions 49436–49457 in the GenBank AE003425 sequence; the *Spe*I site is in boldface) and the 3′ primer 5′-CAGTTGG-TTG**TCTAGA**GCGAG-3′ (54335–54356 in the GenBank AE003425; the *Xba*I site is in boldface), in order

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1381 GACGGGAATCATTTCGCACGTGGACAGCGCTGCGGTATCAACATTGGGTTATTTACCACA
      T  G  I  I  S  H  V  D  S  A  A  V  S  T  L  G  Y  L  P  Q    363

1441 AGATCTTATTGGACGTTCAATATTTGACTTTTATCATCCGGAAGATCTTATGGTATTGAA
      D  L  I  G  R  S  I  F  D  F  Y  H  P  E  D  L  M  V  L  K    383

1501 GGAAATTTACGAAACCGTCATGAAGAAGGGACAAACAGCGGGCGCTTCTTTTTGCAGCAA
      E  I  Y  E  T  V  M  K  K  G  Q  T  A  G  A  S  F  C  S  K    403

1561 ACCGTATCGGTTCCTCATACAAAATGGCTGCTACATATTATTGGAGACGGAGTGGACAAG
      P  Y  R  F  L  I  Q  N  G  C  Y  I  L  L  E  T  E  W  T  S    423

1621 TTTTGTGAATCCATGGTCGCGTAAGCTGGAGTTTGTTATCGGACACCATAGAGTGTTTCA
      F  V  N  P  W  S  R  K  L  E  F  V  I  G  H  H  R  V  F  Q    443
         8
1681 GG▲GTCCAAAACTTTGTAATGTTTTCGATACGGCACCGGCAAACAAACCAAAACTTTCAGA
      G  P  K  L  C  N  V  F  D  T  A  P  A  N  K  P  K  L  S  D    463

1741 TGAGGTACGTAGTCGAAATATACGCATTAAAGACGAAATACTCAAACTGCTGGAAGAGTC
      E  V  R  S  R  N  I  R  I  K  D  E  I  L  K  L  L  E  E  S    483

1801 CATATCGCGGCCTTCGGACACGGTAAAGCAAGAGGTGTCACGCCGTTGTCAAGCGTTAGC
      I  S  R  P  S  D  T  V  K  Q  E  V  S  R  R  C  Q  A  L  A    503

1861 TTCTTTCATGGAAACTCTCATGGACGAGGTGACACGCACTGATCTCAAATTAGAACTACC
      S  F  M  E  T  L  M  D  E  V  T  R  T  D  L  K  L  E  L  P    523

1921 TCATGAGAATGAGCTGACCGTCTCGGAGCGAGATTCGGTGATGTTGGGCAAGATCTCACC
      H  E  N  E  L  T  V  S  E  R  D  S  V  M  L  G  K  I  S  P    543

1981 ACATCACGATTACTATGATAGCAAAAGTTCGACGGAAACACCGCCGAGCTACAATCAACT
      H  H  D  Y  Y  D  S  K  S  S  T  E  T  P  P  S  Y  N  Q  L    563

2041 GAATTATAATGAGAATTTGCAGCGCTTTTTTAACAGCAAGCCTGTTACCGCGCCGGTCGA
      N  Y  N  E  N  L  Q  R  F  F  N  S  K  P  V  T  A  P  V  E    583

2101 GGTCGACCCAATGAAGAATGAAGAGTCTTACAGCATATCGGCTGACGCACGTAACACGCT
      V  D  P  M  K  N  E  E  S  Y  S  I  S  A  D  A  R  N  T  L    603

2161 CAGTCCGGTGCAGTGTTTCGAGGGTAGTGGCGGCAGTGGGTCCTCGGGAAATTTTACATC
      S  P  V  Q  C  F  E  G  S  G  G  S  G  S  S  G  N  F  T  S    623

2221 TGGCAGCCACATACACATGAGTAGTATTACGAACACCAGTAACGCCGGCACCGGCACCTC
      G  S  H  I  H  M  S  S  I  T  N  T  S  N  A  G  T  G  T  S    643

2281 ATCGGGTAGTGCACAGTTGGTCACATTGACCGAGTCGCTGTTGAATAAACATAACGATGA
      S  G  S  A  Q  L  V  T  L  T  E  S  L  L  N  K  H  N  D  E    663

2341 AATGGAGAAATTCATGCTGAAGAAACATCGCGAGTCTCGCGGTCGTTGCGGTGAGAAGAG
      M  E  K  F  M  L  K  K  H  R  E  S  R  G  R  C  G  E  K  S    683
                                 C3
2401 TAAAAAGGCCACAGAAAAGGTAATGGAGTATAGCGGGCCGGGTCATGGCCTCAAACGGGG
      K  K  A  T  E  K  V  M  E  Y  S  G  P  G  H  G  L  K  R  G    703

2461 CGGCTCACATTCTTGGGAAGGTGATGCCAACAAACCGAAACATCAACACACGAACGTAAT
      G  S  H  S  W  E  G  D  A  N  K  P  K  H  Q  H  T  N  V  M    723

2521 GGACGCGCAACGTGACTACGCTGATCATCACAATATGGCGGTGAGCGCGAGTGGCAAAGC
      D  A  Q  R  D  Y  A  D  H  H  N  M  A  V  S  A  S  G  K  A    743
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Fig. 1. (Cont.)

to convert it to a 330 bp *Spe*I–*Xba*I fragment. This fragment was subcloned in the pCRII-TOPO vector (Invitrogen) and automatically sequenced to check for errors. Subsequently, it was extracted and fused to the approximately 7 kb upstream *D. melanogaster per* region in pBluescriptKS, via the compatible *Xba*I/*Spe*I junction, obtaining a 7·3 kb 5′ *per* fragment.

Second step. The 3·1 kb *C. capitata per* cDNA was then extracted as a *Spe*I–*Xba*I fragment and subcloned downstream to the 7·3 kb *D. melanogaster* 5′ *per* region in the pBluescript vector using the compatible *Spe*I/*Xba*I junction, giving rise to a 10·4 kb chimeric fragment.

Third step. The approximately 3 kb *D. melanogaster* 3′UTR *per* region was amplified using the 13·2 genomic clone as template and introducing the *Not*I and *Sac*I restriction sites at the 5′ and 3′ termini respectively, with the following primers: 5′ primer

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2581 TTACGGCTCGTTGCAAACAACTCTGGGTGAGACAGCCTTTGCTAGTTCTTATGCTGCGGC
      Y  G  S  L  Q  T  T  L  G  E  T  A  F  A  S  S  Y  A  A  A     763
                                      C4
2641 GGGTATATCTTGCACGCGCAATGTCAATCTCTGGCCACCCTTCTCCGTGGGCATCAATAC
      G  I  S  C  T  R  N  V  N  L  W  P  P  F  S  V  G  I  N  T     783

2701 GTCGCACACGTCGCAAACTGTGGCGCAAGCCGGTTTCACACCGCAACACAGCATATTCCC
      S  H  T  S  Q  T  V  A  Q  A  G  F  T  P  Q  H  S  I  F  P     803

2761 GACATTCTACTACATACCAGCCACAGCGGCGGCGGCTGCAGCAGCCGCAACACAAGTTCA
      T  F  Y  Y  I  P  A  T  A  A  A  A  A  A  A  T  Q  V  Q     823

2821 GACCAAGCCCAACATGGCGGACATGCCGAGCACTTCGGCTCAAGCGCTGCCGCTTCAATA
      T  K  P  N  M  A  D  M  P  S  T  S  A  Q  A  L  P  L  Q  Y     843

2881 TATGACTGGTGTTATGTATCCGCATCCATCACTATTTTATACACACCCCGCGACGGCTAT
      M  T  G  V  M  Y  P  H  P  S  L  F  Y  T  H  P  A  T  A  M     863
                                      C5
2941 GATGTACCAGCCCATGTCATTTTCAAATATGTCCAACTCGTTGACGCTGTCGGAGCAGGC
      M  Y  Q  P  M  S  F  S  N  M  S  N  S  L  T  L  S  E  Q  A     883
                                 9
3001 GAGCAGTTCGAATGCTTTCAAAACG▲AATCAACCGGTCATGTTGGCTCCAACGCCCACGAA
      S  S  S  N  A  F  K  T   N  Q  P  V  M  L  A  P  T  P  T  K     903

3061 GACGCAAGGCGCTTTCCACTCGATTACACCTGCGCAGCTGCAACGCCCATCGTCACAGGC
      T  Q  G  A  F  H  S  I  T  P  A  Q  L  Q  R  P  S  S  Q  A     923
                                           10
3121 GACGTCGGTGAAAGCTGAACCCGGCTCTAATATG▲GCGCCTTCGGATTCATCGAAAAAGGG
      T  S  V  K  A  E  P  G  S  N  M   A  P  S  D  S  S  K  K  G     943

3181 TATTGCGGACTCACCGATTTCATCCGTTTTAGGGGATTATGTGTCGGATCAACACAATCC
      I  A  D  S  P  I  S  S  V  L  G  D  Y  V  S  D  Q  H  N  P     963
             11                                 C6
3241 TATGGATCTGAAG▲CCCAACAGTGATAGCAATGCTAACAGCGACGACATGGATGGTTCCAG
      M  D  L  K   P  N  S  D  S  N  A  N  S  D  D  M  D  G  S  S     983

3301 CTTTTCATCTTTTTACTCCTCATTTATAAAAACCACAGACGGATCGGATAGTCCTCAGGA
      F  S  S  F  Y  S  S  F  I  K  T  T  D  G  S  D  S  P  Q  E    1003
                                           12
3361 GAATGATAAGGAAGGGAAGCACCGAAAATATAAA▲GTACAAACCGACTCAAAGTCAATGGA
      N  D  K  E  G  K  H  R  K  Y  K   V  Q  T  D  S  K  S  M  D    1023

3421 TAATGCTGAAGAAGACCAAACGTTACATGGTGATGGTTAAATATTGTTTGCATACATACA
      N  A  E  E  D  Q  T  L  H  G  D  G  *                          1035

3481 TATGTACATACATACATGCATATTTATTTGTACGTGTACGTGTGTGAAACGTGAGTTGCA
3541 CATCATTGCAAGCGATGAGACAAGTTTTGTGGCAACGTGACGATCAGCAAACGGAATTTT
3601 TATTTACAAACTAAACCCCAAACACACAAGTCCACTTTGGCTTAGCATTAAACACGACAA
3661 AAAGTGTAAGTGTAGCATTCAAAATCACGTTGGCAAACAGCTATTTTTGAACTTTAATTT
3721 ACCTTATTCTCTCTATGCTTTAGATTAGCGCAAGGTATTGTCGCTGGCATGCAATAAAAT
3761 AAATGCTGAAAAAAAATACATTTTACAATTGGCTACGTAACAATTGTGACGACGTTGTGG
3841 TTATGAACCCCCACTTTTTTAACGGACGTAGTCTTTCTGTGTAACACAGATTTTTTTGAT
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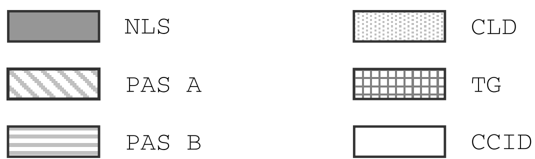

Fig. 1. Coding and deduced amino acid sequence of *C. capitata* period gene. Nucleotides are numbered on the left and amino acids on the right. Introns positions are indicated by filled triangles and numbered above. The end of translation is marked by an asterisk (*). A second stop codon, in frame with the first, is shown in boldface type and underlined. Two overlapping polyadenylation signals are double underlined. Regions conserved in PER proteins of other insect species, C1 through C6, as defined by Colot and colleagues (1988), are indicated on the top of the coding sequence. The PAS (PAS A and PAS B), CLD, Thr-Gly (TG), CCID and NLS domains are indicated (according to Huang *et al*., 1993; Vosshall *et al*., 1994; Saez & Young, 1996; Chang & Reppert, 2003).

5′-AA**GCGGCCGC**CCACACCCGCAGTTGCTG-CTGACC-3′ (58934–58957 in GenBank AE003425; the *Not*I site is in boldface) and the 3′ primer 5′-CA**GAGCTC**CATAATCGCAAAAGAAAA-3′ (61385–61403 in GenBank AE003425; the *Sac*I site is in boldface). This fragment was subcloned in the

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pCRII-TOPO vector (Invitrogen) and automatically sequenced to check for errors. Subsequently, it was extracted and fused downstream to the approximately 10·4 kb chimeric fragment in the pBluescript vector.

Fourth step. The resulting approximately 13·4 kb fragment, containing the 7·3 kb 5′ *D. melanogaster per* region, the 3·1 kb *C. capitata per* cDNA and the 3 kb *D. melanogaster* 3′UTR, was finally subcloned as *Kpn* I–*Sac*II segment in the pW8 vector, obtaining the p*MC1* construct for P-element transformation.

Transformation of *Drosophila* was carried out according to Spradling (1986). Six independent transgenic lines were obtained (21, 34, 119, 156, 192 and 197), chromosomally mapped, balanced and then made homozygous. *In situ* hybridization on third instar salivary gland chromosomes was used to map the position and the number of inserts in each line using a 2 kb fragment of *white* cDNA as a probe and the DIG-DNA Labelling and Detection Kit (Roche).

(vi) *Northern analysis*

Adult *Ceratitis* flies 3–4 days old were reared for at least 3 days in 12 : 12 LD at 23 °C and heads were collected by freezing at 3 h intervals on day 4. Adult flies reared in 12 : 12 LD for 3 days were also placed in constant darkness (DD), and harvested over the first and the second day of DD. Approximately 30 μg of total RNA extracted from 15 heads per time point were subjected to electrophoresis through a 1% agarose-formaldehyde gel, blotted onto nylon membrane and hybridized with DNA probes labelled with [α-^{32}P]dCTP by random priming (New England Biolabs). The probes used were the same 5′ 1·8 kb fragment of *C. capitata period* gene described in Section (iv), and an approximately 0·9 kb fragment corresponding to the 5′ end of *C. capitata actin* cDNA (*act*; positions from 626 to 1555 according to GenBank M76614). For quantification of the signals, each film was analysed with Quantity One 4.2.0 Software (Bio-Rad). Relative abundance of *per* mRNA was defined as a *per*/*act* ratio and normalized to the highest time point value for each experiment.

(vii) *Western blotting*

Samples of 15 fly heads were taken at 3 h intervals in 12 : 12 LD at 23 °C for at least 3 days and collected on day 4. Adult flies reared in 12 : 12 LD for 3 days were also placed in DD, and heads harvested at 3 h intervals over two circadian cycles in the first and second day of DD. For each sample, approximately 100 μg of total proteins was extracted and subjected to western blotting as in Edery *et al*. (1994). The anti-PER primary antibody used was an affinity-purified rabbit polyclonal antibody against a synthetic 17 amino acid peptide KKHRESRGRTGEKSKK corresponding to residues 808–823 of the *D. melanogaster* PER protein (364 anti-*Dm*PER, 1 : 50; Neosystem, France). This region is highly conserved in *C. capitata* PER (16 of 17 amino acids), and corresponds to residues 670–685 (Fig. 1). A mouse anti-HSP70 (1 : 10 000, Sigma) was employed to immunodetect HPS70, used as a loading control. An anti-rabbit IgG-HRP (1 : 3000; Bio-Rad) and an anti-mouse IgG-HRP (1 : 5000; Sigma) were used as secondary antibodies. Positive immunoreactivity was visualized using a chemiluminescence system. For quantization of the immunodetected signals, each film was analysed with the Quantity One 4.2.0 Software (Bio-Rad). Relative abundance of PER was defined as a ratio with HSP70 (PER/HSP) and normalized to the highest time point value for each experiment.

(viii) *Behavioural analysis*

Adult male flies 4–5 days old of wild-type (*Oregon-R*), *per^{01}* and *pMC1 Drosophila* transgenic lines in *per^{01}* and *per^{+}* backgrounds, were analysed with respect to their circadian locomotor activity as described previously (Piccin *et al*., 2000). Locomotor activity of *Ceratitis capitata* adult flies was studied by using the TriKinetics activity monitoring system (Waltham, MA). Flies were entrained in a 12 : 12 LD cycle for 3 days and subsequently free-run in DD for 7 days at 18, 23 and 28 °C. Periodicity was calculated by spectral analysis performed with the CLEAN algorithm of Roberts *et al*. (1987), enhanced by a Monte Carlo simulation to generate 95% and 99% confidence limits for the estimation of the significance of the peaks, as described in Zordan *et al*. (2005). In addition, all the behavioural data were analysed by autocorrelation, and only flies that showed significant periods with both statistical analyses were judged as 'rhythmic', as described in Peixoto *et al*. (1998). Mean phase and onset values were calculated for morning and evening activity peaks on the third day of 12 : 12 LD, and given in hours from the last lights-on and lights-off transition (values are negative when occurring before the transition), respectively. Onset was defined as the mean time between the beginning of locomotor activity and the maximum of the peak as previously described in Majercak *et al*. (2004). In order to compare profiles between individuals and between genotypes, the data for locomotor activity in DD for each fly were standardized according to the procedure of Tauber *et al*. (2003).

(ix) *Statistical analyses*

Analysis of variance was performed using a one-way ANOVA for northern and western blot data and a two-way ANOVA for behavioural activity data, using the STATISTICA 5.0 package (Statsoft).

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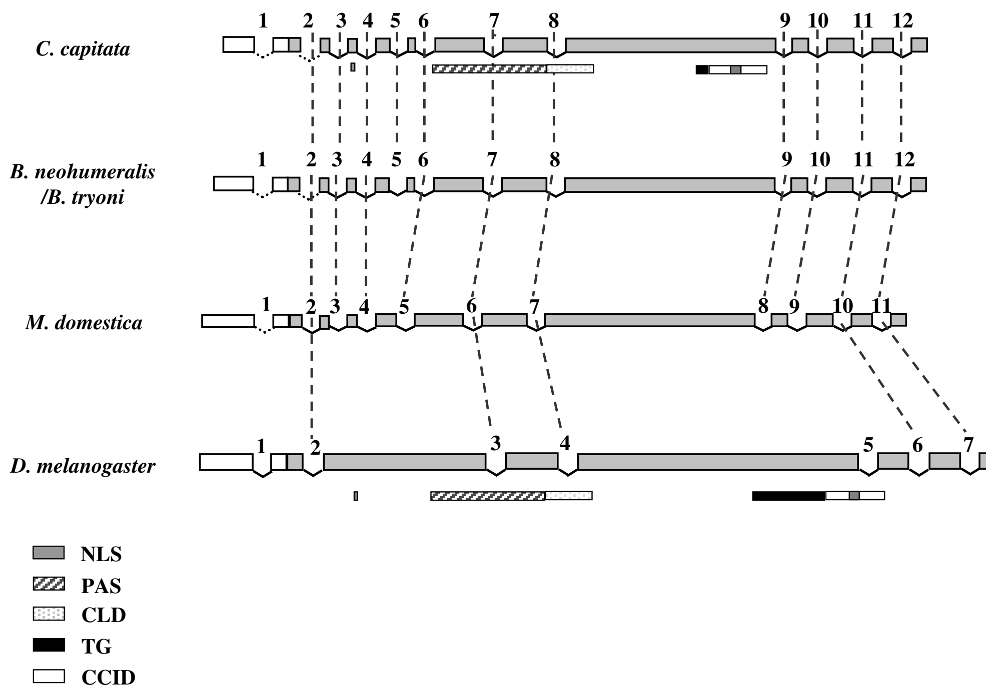

Fig. 2. *period* intron/exon comparison. A schematic representation of *C. capitata*, *B. neohumeralis*/*B. tryoni*, *M. domestica* and *D. melanogaster* period intron/exon structures is given. Open boxes represent the 5′UTR. Introns are numbered. *Ceratitis* and *Bactrocera* introns 1 and 2 and *Musca* intron 1 are represented by a dotted line to indicate that their length and complete sequence have not been determined. Introns conserved between the species are connected by dashed lines. Conserved functional domains are indicated and labelled.

3. Results

(i) *Cloning of the* C. capitata per *homologue*

C. capitata per (*Ccper*) spans approximately 15 kb from the starting codon to the putative polyadenylation signal. This dramatic increase in size compared with its *Drosophila* orthologues (Citri *et al.*, 1987; Colot *et al.*, 1988; Thackeray & Kyriacou, 1990) has also been documented in *B. neohumeralis*, *B. tryoni* and *Musca domestica per* (Piccin *et al.*, 2000; An *et al.*, 2002), and is due to changes in intron–exon structure. RT-PCR and 5′-RACE performed on head RNA extracts provided a coding sequence of approximately 3·1 kb which includes 352 bp of 5′ untranslated sequence (Fig. 1). A second stop codon, in frame with the first, has been identified from nucleotide 3578. Two overlapping polyadenylation signals are predicted from nucleotides 3753 to 3763 (Fig. 1). The *Ceratitis per* transcript contains an open reading frame (ORF) encoding an inferred protein of 1035 residues with a putative molecular weight of 116 kDa (Fig. 1).

(a) *Intron–exon structure*

C. capitata per has 12 introns, the first localized in the 5′UTR and the second revealing the presence of a 68 bp sequence that shares 82% identity with the *mariner* transposase pseudogene from *Rivellia quadrifasciata* (Accession number: U91383; Robertson *et al.*, 1998). These data support the ability of the *mariner* transposon family to undergo horizontal transfer even between taxonomically rather distant organisms (Lampe *et al.*, 2003). A comparison of intron number and size between the relevant species is illustrated in Fig. 2 and Table 1. It shows five additional introns in *C. capitata* compared with *Drosophila*, and one additional intron compared with *Musca* (intron 5), as well as a general increase of intron size with respect to both *Drosophila* and *Musca* (Table 1). Except for intron 5, all other introns within the coding region are located in well-conserved intron/exon boundaries in *C. capitata* and *M. domestica*.

(b) *Coding sequence*

The overall pattern of conserved (c) and non conserved (nc) regions, introduced by Colot *et al.* (1988), is preserved in *Ceratitis* (Fig. 1). The nuclear localization signal (NLS; Vosshall *et al.*, 1994; Saez & Young, 1996), amino acids (aa) 72 to 76; the two PAS regions (PAS A aa 194 to 243 and PAS B aa 344 to 396; Huang *et al.*, 1993); the cytoplasmic localization domain (CLD) that spans aa 399 to 458; the CKL:CYC inhibition domain (CCID) with the

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Table 1. *Size (bp) of period introns in* C. capitata, B. neohumeralis/B. tryoni, M. domestica *and* D. melanogaster

Intron	*C. capitata*	*B. neohumeralis/B. tryoni*	*M. domestica*	*D. melanogaster*
1	Not sequenced	Not sequenced	Not sequenced	2000
2	>7000	Not sequenced	5000	61
3	184	155	72	Absent
4	111	107	64	Absent
5	620	1100	Absent	Absent
6	641	1400	63	Absent
7	74	86	56	64
8	73	56	63	62
9	92	66	68	70
10	71	800	71	Absent
11	2498	1100	61	64
12	199	166	60	58

internal NLS that span aa 647 to 858 and aa 692 to 719 respectively (Chang & Reppert, 2003), are all conserved in *C. capitata*. The sites to which the *Drosophila* per^{L}, per^{S} and per^{01} mutations have been mapped (Baylies *et al*., 1987; Yu *et al*., 1987) are perfectly conserved in *Cc*PER. The *Cc* PER threonine–glycine (TG) repeat (aa 640 to 646), as in *Bactrocera* and *Musca*, has not undergone the dramatic expansion in size observed in the *Drosophila* genus (Costa *et al*., 1991; Peixoto *et al*., 1992, 1993). In general, levels of identity of *Cc*PER compared with *Drosophila* PER were very high (>70%) for each domain that we examined, and dropped only for the CCID (48%) and DBT interacting domain (aa 1 to 311; 62%).

We also calculated the Relative Synonymous Codon Usage (RSCU) index for *C. capitata period* coding sequence (GCUA, v1.0; McInerney, 1998). This analysis was performed for the full-length cDNA, for the PAS, the non-PAS-conserved and the non-conserved coding sequences separately. A general low codon bias was observed for the full-length cDNA, except for CGU (Arg) codon, which showed a RSCU index of 2·36 (out of 6 codons coding for Arg) in the full-length cDNA analysis, and reached 4·24 in the PAS coding sequence when analysed separately. These numbers appear to be consistent with the fact that *per* is not a highly expressed gene (Grantham *et al*., 1981).

(ii) *Molecular phylogeny of the PER proteins*

Sequences from *C. capitata*, four species of *Drosophila* (*D. melanogaster*, *D. virilis*, *D. yakuba* and *D. pseudoobscura*), two species of *Bactrocera* (*B. tryoni* and *B. neohumeralis*), *Lucilia cuprina*, *Musca domestica*, *Chymomiza costata* and the lepidopteran *Antheraea pernyi* were aligned using Clustal W (Thompson *et al*., 1994), in order to reveal any significant differences between the species tree and the PER protein tree. We were interested in the evolution of the conserved regions (Colot *et al*., 1988), and therefore compared the PAS domain, implicated in protein dimerization, with the evolution of non-PAS sequences.

Fig. 3A and B represents phylogenetic trees obtained with the amino acids from the PAS region (which also includes the *Ceratitis* PER CLD domain: aa 184 to 442; Fig. 1), and the *Ceratitis* PER conserved non-PAS sequence (aa 1–78, 179–183, 443–637, 649–721, 762–932, 947–1035; Fig. 1). The PAS tree places *D. melanogaster* and *D. yakuba* closer to *Ceratitis* and *Bactrocera*, and farther from the other *Drosophila* species. Although the bootstrap value associated with the ancestral node common to these species is low, a similar result was obtained by Piccin *et al*. (2000) with the *Musca* sequence, whose unorthodox position relative to *D. melanogaster*/*yakuba* and *D. pseudoobscura*/*D. virilis* is maintained in this phylogeny. On the other hand the non-PAS tree follows the species tree, as also shown in Piccin *et al*. (2000).

(iii) C. capitata per *mRNA levels oscillate both in LD and in DD*

The temporal expression of *Ccper* mRNA was determined by northern blot in 12:12 LD at 23 °C. In these conditions, no alternatively spliced forms were revealed in any of the sampled ZTs. The approximately 6 kb unique *per* mRNA form showed a significant daily oscillation in abundance, with a peak at ZT9 and a mean fivefold amplitude ($F_{8,18}=7{\cdot}29$, $P<0{\cdot}0003$; Fig. 4A, B). A significant daily rhythm in *per* mRNA expression was maintained also in DD for the first 48 h after switching from 12:12 LD, but with maximum levels at CT15 and CT39, revealing a 6 h phase difference after shifting to DD ($F_{16,33}=4{\cdot}08$, $P<0{\cdot}0003$; Fig. 4C, D). However, an increase in the baseline values leads to a dampening in the amplitude of the oscillation during the second day of DD,

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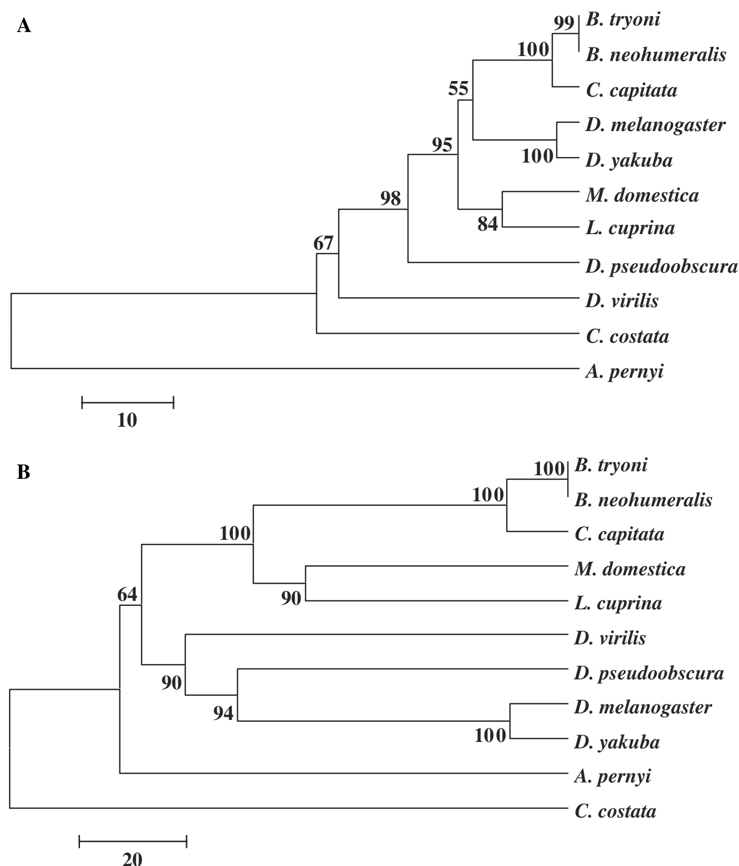

Fig. 3. Phylogeny of different fragments of PER protein. Tree A represents the molecular phylogeny of the PAS region (residues 238–496 of the *D. melanogaster* sequence) and tree B that of the non-PAS-conserved sequences (residues 1–79, 233–237, 497–691, 767–842, 926–1114, 1130–1224 of the *D. melanogaster* sequence). Both trees were obtained with the 'Number of Differences' distance matrix and the UPGMA method. Bootstrap values derived from 500 resamplings of the data set are shown at the nodes. GenBank sequences used in this analysis were: *D. melanogaster*, M30114; *D. virilis*, X13877; *D. yakuba*, X61127; *D. pseudoobscura*, X13878; *M. domestica*, AAD39163; *L. cuprina*, Y19108; *A. pernyi*, U12769; *C. costata*, AB014477; *B. tyroni*, AF480840; *B. neohumeralis*, AF480839.

probably reflecting a gradual loss of synchrony between individual heads.

(iv) C. capitata *PER protein does not oscillate in LD or DD*

Western blot of *Ceratitis* head extracts using anti-*Dm*PER antibody 364 identifies an approximately 120 kDa band that can be ascribed to *Ceratitis* PER (Fig. 5A, lane 1). This band is in fact observed in *D. melanogaster* flies transgenic for *C. capitata* PER (lane 2), but is absent in both *D. melanogaster* arrhythmic mutant per^{01} (lane 3) and Oregon-R (lanes 4 and 5). Analysis of *C. capitata* PER protein levels was performed in 12:12 LD as well as during 2 days of DD at 23 °C. No evidence for rhythmic oscillation of protein levels was observed, either in LD ($F_{7,16}=1{\cdot}74$; $P=0{\cdot}17$; Fig. 5B, C) or in DD ($F_{16,33}=0{\cdot}99$; $P=0{\cdot}48$; Fig. 5D, E). In all cases data were taken from three replicate experiments.

(v) *Behavioural rhythms in* C. capitata *and transgenic* D. melanogaster

The locomotor activity of males from *C. capitata* ISPRA strain and six *D. melanogaster* transgenic lines bearing the *C. capitata per* gene was monitored at 18, 23 and 28 °C for 3 days in 12:12 LD followed by 7 days in DD. The histograms in 12:12 LD for *C. capitata* were compared with those for *D. melanogaster* Oregon-R, which shows similar activity patterns to per^{+} transformants (Sawyer *et al.*, 1997). Unlike Oregon-R, which shows a bimodal pattern at the higher temperatures, no bimodality of locomotor activity was found in *C. capitata*, but a single broad peak during the daytime was present at every temperature (Fig. 6A, B). Mean locomotor phase and onset values were recorded on the third day of 12:12 LD at 18, 23 and 28 °C. No significant differences in the phase values were observed ($F_{2,69}=0{\cdot}70$, $P=0{\cdot}50$) but a significant change in onset was observed, which at 18 °C fell 2 h after lights-on, and at 29 °C occurred

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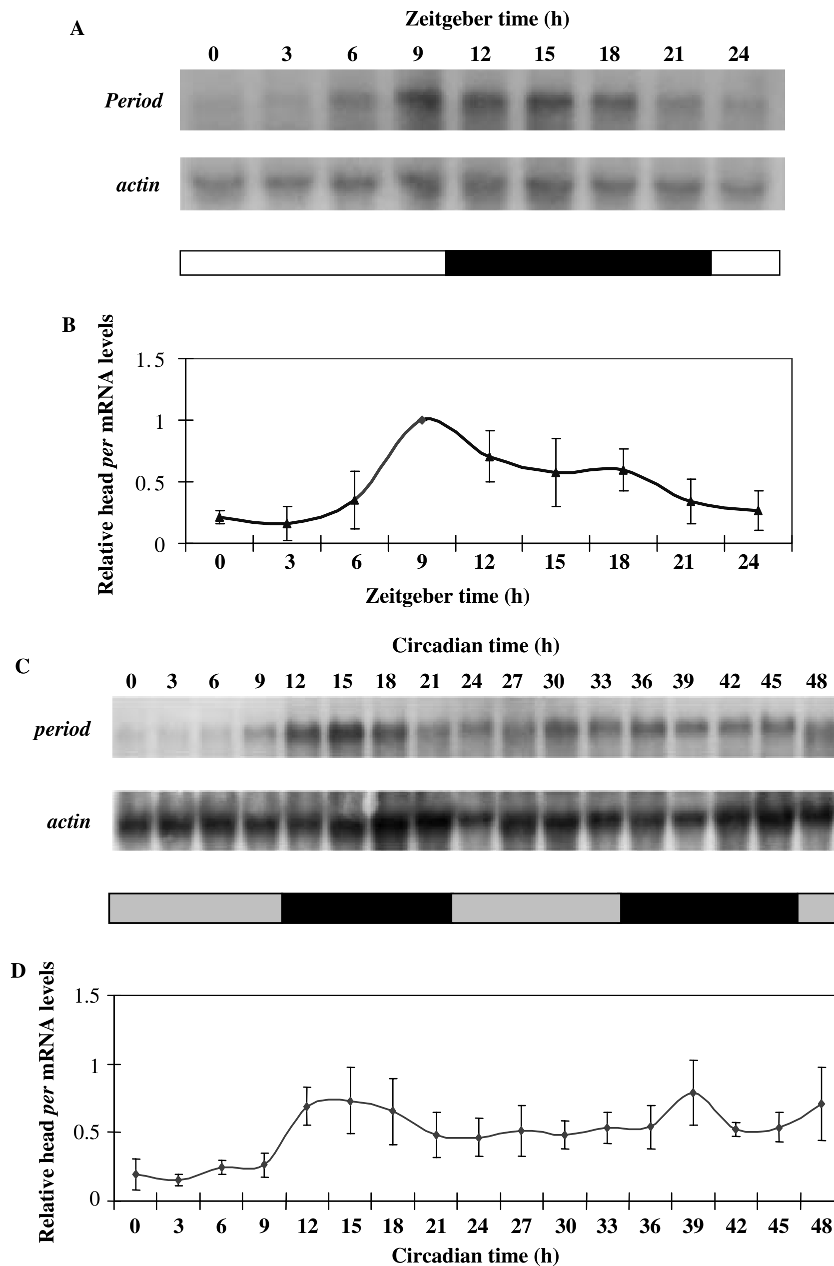

Fig. 4. Cycling of *period* mRNA levels in *Ceratitis capitata*. Adult heads were collected every 3 h intervals in 12 : 12 LD or over the first and second day of DD, at 23 °C. A unique band of approximately 6 kb was identified with *per* DNA probe. (*A*) Representative northern blot hybridized with *per* (top) and *actin* (bottom) DNA probes. ZT0 was lights-on and ZT12 was lights-off, as indicated by black and white bars. (*B*) Quantification of *per* mRNA levels. Relative abundance of *per* mRNA was defined as a ratio with *act* (*per*/*act*), normalized to the highest value. Points represent mean levels ± standard deviation of three independent experiments. *per* mRNA shows significant cycling ($F_{8,18} = 7{\cdot}29$, $P = 0{\cdot}0003$). (*C*) Representative northern blot hybridized with *per* (top) and *actin* (bottom) DNA probes. Circadian Time refers to subjective time under constant conditions; grey bars indicate the subjective day and black bars the subjective night. (*D*) Quantification of *per* mRNA levels. Points represent mean levels ± standard deviation of three independent experiments. *per* mRNA shows significant cycling ($F_{16,33} = 4{\cdot}08$, $P = 0{\cdot}0003$).

1·5 h before lights-on ($F_{2,69} = 26{\cdot}90$, $P < 0{\cdot}0001$; Table 2).

All the transgenic lines that were behaviourally tested carried one autosomal copy of the *pMC*1 construct in a per^{01} background. The locomotor activity patterns in 12 : 12 LD were analysed for those transgenic *pMC1* flies that showed a circadian activity in the subsequent free-running condition. In many cases (40–80 % of the rhythmic *pMC1* transgenics, depending on the line tested) the flies showed a bimodal locomotor activity profile, very similar to *D. melanogaster* control flies, with anticipatory activity corresponding to the lights-on and -off transitions (morning and evening peaks) at every temperature

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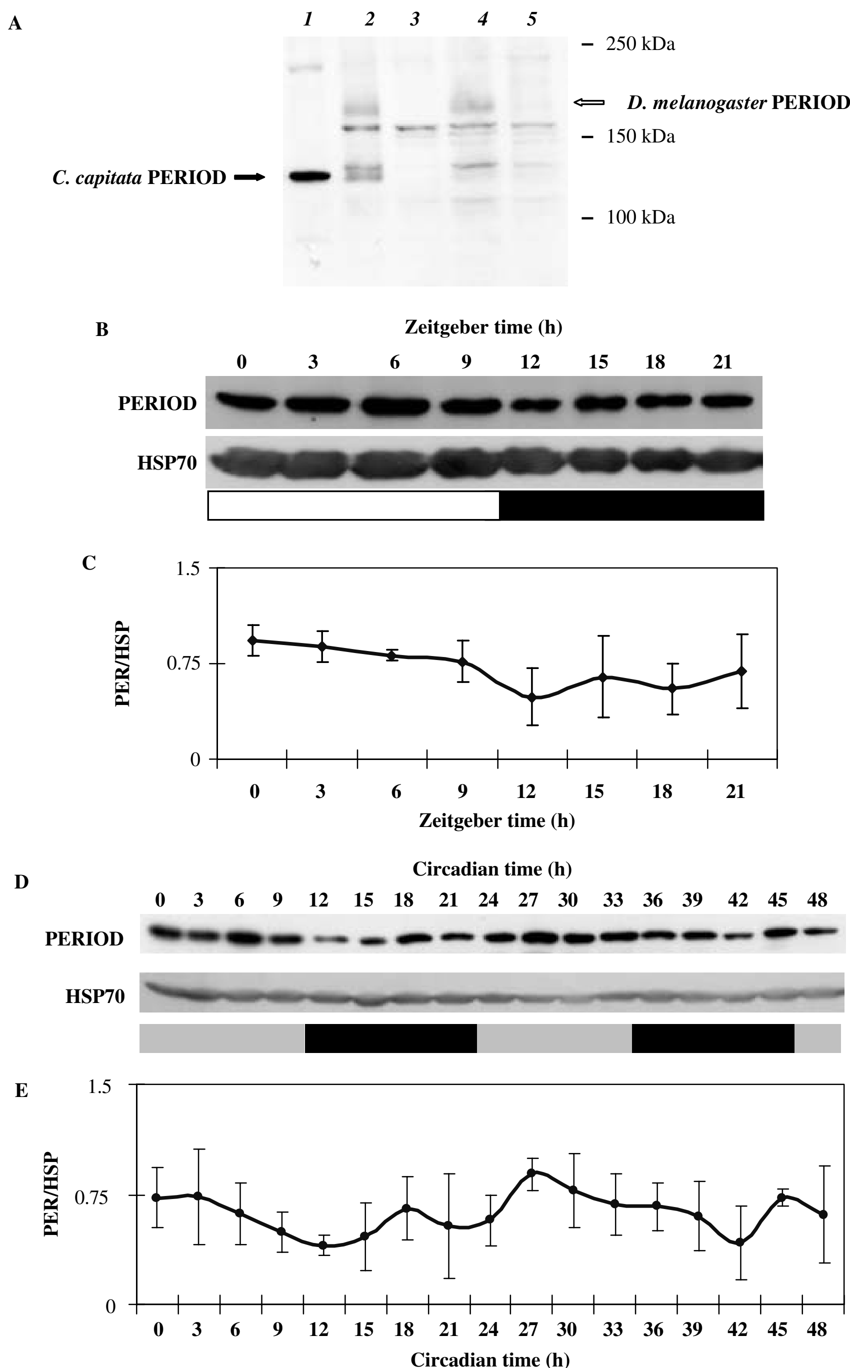

Fig. 5. PER protein in *Ceratitis capitata*. (*A*) Western blot (with purified 364 anti-*Dm*PER antibody) on adult head extracts from *C. capitata* collected at ZT21 (lane 1), 192*MC1 D. melanogaster* transgenic line in per^{+} background, collected at ZT18 (lane 2), *D. melanogaster* arrhythmic mutant per^{01} (lane 3) and *D. melanogaster* Oregon-R flies collected at ZT18 (lane 4; expected peak of expression) and ZT9 (lane 5; expected trough of expression). Empty arrow indicates the approximately180 kDa signal corresponding to *Drosophila* PER and the filled arrow shows a band of estimated 120 kDa, detected solely in *Ceratitis* and in the *D. m.* 192*MC1* transgenic line. (*B*) Representative western blot of *Ceratitis* adult head extracts probed with 364 anti-*Dm*PER antibody (top) and an anti-HSP70 (bottom). Flies heads were collected at 3 h intervals in 12:12 LD cycle at 23 °C. ZT0 was lights-on and ZT12 was lights-off, as indicated by black and white bars. (*C*) Quantification of PER levels. Relative abundance of PER was defined as a ratio with HSP70 (PER/HSP), normalized to the highest value and mean levels ± standard deviation of three independent experiments are shown. No significant PER cycling was observed ($F_{7,16}=1{\cdot}74$, $P=0{\cdot}17$). (*D*) Representative western blot of *Ceratitis* adult head extracts probed with 364 anti-*Dm*PER antibody (top) and an anti HSP70 (bottom). Flies' heads were collected every 3 h or over the first and second day of DD, at 23 °C. Circadian Time refers to subjective time under constant conditions; grey bars indicate the subjective day and black bars the subjective night. (*E*) Quantification of PER levels. Relative abundance of PER was defined as a ratio with HSP70 (PER/HSP), normalized to the highest value. Mean levels ± standard deviation of three independent experiments are shown. No significant PER cycling was observed ($F_{16,33}=0{\cdot}99$, $P=0{\cdot}48$).

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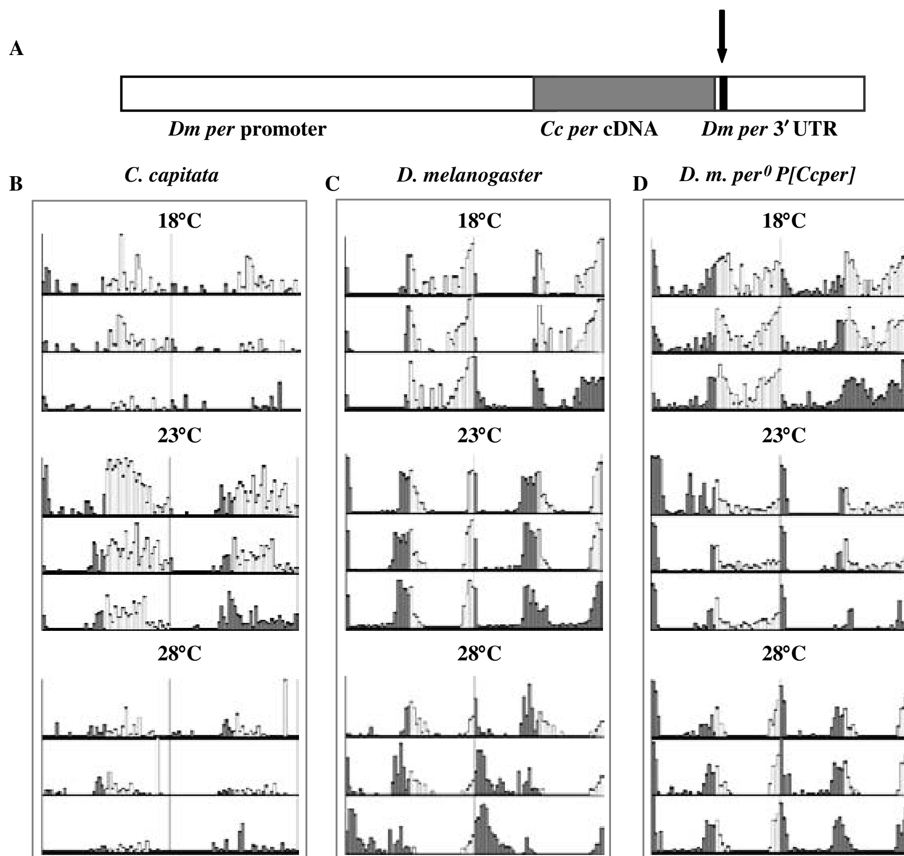

Fig. 6. Locomotor activity pattern of *C. capitata*, *D. melanogaster* Oregon-R control flies and *pMC1* transgenic line in 12 : 12 LD at 18, 23 and 28 °C. (*A*) Schematic representation of the *MC1* transgene. White boxes represent the *D. melanogaster* regulatory regions, the grey box corresponds to the *C. capitata per* cDNA. The arrow indicates the 89 bp *D. melanogaster per* 3′UTR thermally regulated intron. (*B*)–(*D*) Single-fly double-plotted activity histograms over 3 days in LD and the first day in DD for *C. capitata* (*B*), *D. melanogaster* Oregon-R control flies (*C*) and homozygous 156*MC1* transgenic line in *per*^{01} background (*D*). Each bar corresponds to 30 min of activity events; black and white bars correspond to the activity during dark and light periods.

tested (Fig. 6C). On the other hand, in the remaining cases it was not possible to determine the morning and/or evening peak, because any possible underlying rhythmicity appeared to be masked by light-stimulated locomotor activity. Mean phase and onset for morning and evening peaks at 18, 23 and 28 °C were calculated on the third day of 12 : 12 LD for *pMC1* transgenic lines and compared with those of *D. melanogaster* Oregon-R control flies (Table 2). Focusing attention on 156*MC1* transgenic flies, which showed bimodal activity at every temperature tested, significant temperature-dependent variations were observed only for the evening activity peak (Evening Onset: $F_{2,27}=15{\cdot}58$; $P<0{\cdot}0001$; Evening Phase: $F_{2,27}=16{\cdot}53$; $P<0{\cdot}0001$), not for morning activity (Morning Onset: $F_{2,22}=0{\cdot}50$, $P=0{\cdot}61$; Morning Phase: $F_{2,22}=0{\cdot}63$, $P=0{\cdot}54$).

C. capitata adults were also monitored in DD. The percentage of rhythmic flies in DD varied with temperature, with lower values observed in the coldest condition (Table 3). Among the rhythmic flies, the mean period values were around 22–23 h, with no significant differences between temperatures ($F_{2,46}=0{\cdot}62$, $P=0{\cdot}54$). Fig. 7 shows an analysis of locomotor activity in DD, in which each individual fly's period is normalized to a 24 h period. This analysis confirms the unimodal nature of *Ceratitis* locomotor activity (Fig. 7A) that was also observed in LD. Transgenic lines were also analysed for their ability to rescue circadian locomotor activity rhythms in DD at different temperatures (Table 3). In the *per*^{01} background, the *Ccper* transgene showed low rescue, from 0 to 68 %, depending on the line and the temperature considered. Periods were highly variable between 18 and 34 h, although the means were within the circadian range and no significant differences in period were observed between the three temperatures for any of the lines. Fig. 7C shows histograms in DD for the transgenic line 156*MC1*, which gave the best rescue. Only significantly rhythmic animals are included in the analysis. At 18 °C we observed a subjective morning peak, as in *C. capitata* (but not Oregon-R; Fig. 7A–C), as well as an early evening peak, as in Oregon-R (but not *Ceratitis*).

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Table 2. *Morning and evening peaks (onset and phase values) of homozygous* pMC1 *transgenic lines,* D. melanogaster *Oregon-R control flies and* C. capitata *on the third day of 12 : 12 LD at 18, 23 and 28 °C*

	Morning peak			Evening peak		
	Onset ± SEM Phase ± SEM (*N*)			Onset ± SEM Phase ± SEM (*N*)		
Line	18 °C	23 °C	28 °C	18 °C	23 °C	28 °C
*w, per^01^*; 21*MC1*	−2·38 −0·5 (2)	−1·68 ± 0·54 −0·45 ± 0·52 (10)	−2·00 ± 0·3 −1·25 ± 0·43 (4)	−2·75 −1·25 (2)	−2·85 ± 0·61 −1·85 ± 0·55 (10)	−2·13 ± 0·56 0·00 ± 1·00 (4)
*w, per^01^*; 34*MC1*	−2·63 ± 0·58 −1·13 ± 0·57 (6)	−2·14 ± 0·28 −0·85 ± 0·24 (27)	−3·83 −2·50 (2)	−1·75 ± 0·32 −1·2 ± 0·11 (6)	−2·73 ± 0·36 −1·2 ± 0·36 (27)	−2·25 −0·5 (2)
*w, per^01^*; 119*MC1*	−1·83 ± 1·05 −0·67 ± 0·44 (3)	−2·15 ± 1·06 −1·1 ± 0·83 (5)	ND	−3·58 ± 0·94 −2·17 ± 0·88 (3)	−3·36 ± 0·5 −2·33 ± 0·4 (9)	ND
*w, per^01^*; 156*MC1*	−1·38 ± 0·64 −0·3 ± 0·61 (10)	−2·14 ± 0·35 −1·21 ± 0·22 (7)	−2·03 ± 0·66 −0·81 ± 0·69 (8)	−2·67 ± 0·19 −0·83 ± 0·3 (15)	−5·00 ± 0·91 −3·92 ± 0·95 (6)	−0·92 ± 0·52* −0·56 ± 0·44* (9)
*w, per^01^*; 192*MC1*	−2·69 ± 0·84 −1·50 ± 0·89 (4)	1·75 3·00 (1)	ND	−3·5 ± 0·8 −1·38 ± 1·05 (4)	−3·5 −2·00 (1)	ND
*w, per^01^*; 197*MC1*	−1·63 ± 0·56 −0·13 ± 0·56 (4)	−0·25 ± 0·63 −0·33 ± 0·6 (3)	ND	−3·90 ± 0·46 −2·36 ± 0·39 (7)	−3·08 ± 0·6 −2·00 ± 0·5 (3)	ND
OR-R	−1·28 ± 0·22 0·6 ± 0·12 (29)	−2·50 ± 0·3 −0·58 ± 0·23 (24)	−3·03 ± 0·34** −1·32 ± 0·31** (17)	−3·29 ± 0·18 −0·84 ± 0·08 (29)	−2·06 ± 0·16 −0·38 ± 0·08 (24)	−0·88 ± 0·22** 1·12 ± 0·19** (17)
C. capitata	1·9 ± 0·31 6·22 ± 0·54 (27)	0·21 ± 0·43 5·52 ± 0·72 (17)	−1·34 ± 0·30*** 6·41 ± 0·35 (28)			

Values are given in hours from the last lights-on (morning peak) and lights-off (evening peak) transition, with negative values indicating that they occur before the transition.
(*N*) indicates the number of flies tested.
* Significant delay of the evening peak between 18 °C and 28 °C in 156*MC1* transgenic line (Evening Onset: $F_{2, 27} = 15{\cdot}58$, $P < 0{\cdot}0001$; Evening Phase: $F_{2, 27} = 16{\cdot}53$, $P < 0{\cdot}0001$).
** Significant variations of the morning and evening peaks between 18 °C and 28 °C in *D. melanogaster* Oregon-R control flies (Morning Onset: $F_{2, 67} = 10{\cdot}77$, $P < 0{\cdot}0001$; Morning Phase: $F_{2, 67} = 21{\cdot}80$, $P < 0{\cdot}00001$; Evening Onset: $F_{2, 67} = 39{\cdot}10$, $P < 0{\cdot}00001$; Evening Phase: $F_{2, 67} = 78{\cdot}82$, $P < 0{\cdot}00001$).
*** Significant difference in the onset values between 18 °C and 28 °C in *C. capitata* ($F_{2, 69} = 26{\cdot}90$, $P < 0{\cdot}0001$).

At 23 and 28 °C, the profiles for the transformants seemed to be intermediate between the two parental species, giving rather 'flatter' (average) profiles.

4. Discussion

The cloning of the *C. capitata period* orthologue and the comparative analysis with *D. melanogaster*, *M. domestica*, *B. tryoni* and *B. neohumeralis* shows that the *Ceratitis* gene is very closely related to *Bactrocera per*, with regard to number, position and length of introns. Such a tight correlation between the two species has already been observed in the organization of the *Adh1* and *white* loci (Gomulski *et al*., 1997, 2001). All these genes share a dramatic increase in size, which is due to an increase in both number and size of introns. Several studies have indicated that there may be a positive correlation between intron length and genome size, which is stronger when homologous genes are considered (Moriyama *et al*., 1998; Deutsch & Long, 1999). *C. capitata* and *B. tryoni* have similarly sized genomes of approximately 5×10^8 bp, and therefore longer introns would be expected in these species compared with *D. melanogaster*, whose genome size is $1{\cdot}7 \times 10^8$ bp (Adams *et al*., 2000). Moreover, such long introns tend to accumulate insertions, such as repetitive sequences and transposable elements (Stephan

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Table 3. *Free-running periods of homozygous* pMC1 *transgenic lines, in* per^{01} *and* per+ *background, and* C. capitata *males in DD at different temperatures*

Line	% Rescue (*N*)			Period (in hours) ± SEM		
	18 °C	23 °C	28 °C	18 °C	23 °C	28 °C
w, per^{01}; 21*MC1*	12 (25)	28 (39)	16 (32)	25·57 ± 0·69	22·88 ± 1·13	21·99 ± 0·97
w, per^{01}; 34*MC1*	28 (40)	47 (74)	9 (44)	24·81 ± 0·79	25·17 ± 0·69	22·81 ± 0·83
w, per^{01}; 119*MC1*	20 (20)	27 (48)	0 (11)	26·81 ± 0·83	24·21 ± 1·22	–
w, per^{01}; 156*MC1*	68 (22)	24 (29)	38 (26)	24·03 ± 0·37	24·55 ± 1·13	22·97 ± 0·68
w, per^{01}; 192*MC1*	31 (16)	9 (11)	10 (10)	22·12 ± 0·9	22·34	23
w, per^{01}; 197*MC1*	21 (47)	13 (30)	0 (10)	25·71 ± 1·28	22·86 ± 1· 16	–
w, per^{01}	3 (31)	3 (29)	0 (27)	18·01	24·01	–
w, per^{+}; 21*MC1*	–	90 (20)	–	–	24·41 ± 0·19	–
w, per^{+}; 34*MC1*	–	97 (33)	–	–	24·41 ± 0·14	–
w, per^{+}; 119*MC1*	–	94 (29)	–	–	24·33 ± 0·14	–
w, per^{+}; 156*MC1*	–	95 (19)	–	–	23·87 ± 0·1	–
w, per^{+}; 192*MC1*	–	100 (15)	–	–	24·02 ± 0·1	–
w, per^{+}; 197*MC1*	–	95 (20)	–	–	24·32 ± 0·14	–
OR-R	100 (24)	100 (21)	100 (30)	23·73 ± 0·01	23·71 ± 0·02	24·84 ± 0·04
C. capitata	41 (29)	50 (10)	86 (28)	22·66 ± 0·18	23·23 ± 0·67	22·27 ± 0·36

OR-R and *w*; *per^{01}* represent the *D. melanogaster* Oregon-R control flies and the arrhythmic mutant *per^{01}* strain respectively. No significant differences in the period length between 18 and 28 °C have been found (ANOVA test *w, per^{01}*; 21*MC1* $F_{2,16}=1{\cdot}32$, $P=0{\cdot}29$; *w, per^{01}*; 34*MC1* $F_{2,47}=0{\cdot}71$, $P=0{\cdot}49$; *w, per^{01}*; 156*MC1* $F_{2,28}=1{\cdot}04$, $P=0{\cdot}36$; *C. capitata* $F_{2,38}=0{\cdot}26$, $P=0{\cdot}76$).
(*N*) indicates the number of flies tested.

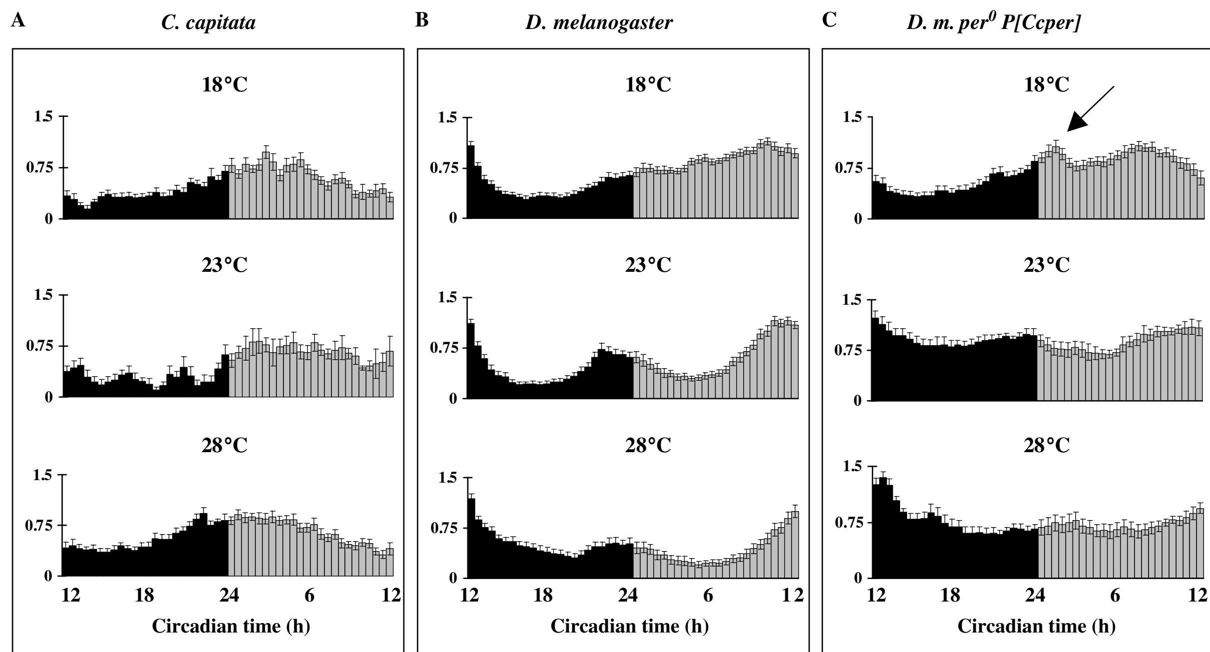

Fig. 7. Free-running locomotor activity profiles of *C. capitata*, *D. melanogaster* Oregon-R control flies and homozygous 156MC1 transgenic line at 18, 23 and 28 °C. Activity events were collected in 30 min bins. Black and grey bars correspond to the activity during subjective night and subjective day respectively. (*A*) Mean activity histograms for *C. capitata* in DD at 18 °C (29 flies), 23 °C (5 flies) and 28 °C (28 flies). (*B*) Mean activity histograms for *D. melanogaster* Oregon-R control flies in DD at 18 °C (27 flies), 23 °C (23 flies) and 28 °C (30 flies). (*C*) Mean activity histograms of transgenic line 156*MC1* at 18 °C (15 flies), 23 °C (9 flies) and 28 °C (10 flies).

et al., 1994). It is noteworthy that degenerate forms of *mariner* transposable elements have been observed in long introns of both *white* and *period* genes, both in *Ceratitis* (Gomulski *et al.*, 2001; this study) and in *Bactrocera* (Bennett & Frommer, 1997; An *et al.*, 2002).

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Sequence comparison of PER proteins reveals high levels of conservation, mostly in the N-terminal region, indicating that important PER functions are performed by this part of the protein. This region contains the PAS domain, a protein–protein interaction module which is also known to have important sensory and signalling functions (Gu *et al*., 2000). In the *Drosophila* clock the PAS domain promotes the dimerization of PER with its molecular partners TIM, CLOCK and BMAL. The phylogeny of the PAS regions shows that *Ceratitis* PAS clusters more closely to *D. melanogaster* and *D. yakuba* PAS regions than suggested by the phylogenetic distance of *Ceratitis* from these *Drosophila* species, as already observed with the *Musca* sequence (Piccin *et al*., 2000). This is probably due to the fact that most of the amino acids in the PAS domain are under selective constraint and that this region of the PER protein cannot evolve independently, but instead is constrained to coevolve in concert with the dimerization domains of its conspecific molecular partners.

At the transcriptional level, we observed a clear daily rhythm for *C. capitata per* mRNA, both in LD and in DD. However, in LD the mRNA peak occurs during the day, resembling those of mammalian *Per1* mRNA (ZT7; Hastings *et al*., 1999) and the marine snail *Bulla gouldiana* (ZT5; Constance *et al*., 2002), rather than those of other insects, which peak at the light–dark transition or early in the night phase (e.g. *Drosophila* (ZT13; Hardin *et al*., 1990), *L. cuprina* (ZT12; Warman *et al*., 2000) or *Chymomyza costata* (ZT15; Kostal & Shimada, 2001)). The heterogeneity between organisms in the *per* mRNA peak in LD supports the hypothesis of species-specific regulatory mechanisms in clock gene expression (Constance *et al*., 2002). Furthermore, the unexpected 6 h shift of the *Ccper* mRNA peak observed immediately after transfer from LD to DD conditions suggests the possibility of the presence in *C. capitata* of two different regulatory mechanisms in modulating *per* expression, which would act alternatively when a strong Zeitgeber such as light is present or in constant darkness. Nonetheless, as the environmental conditions change from LD to DD, rather intriguingly, the 6 h shift in the mRNA peak does not seem to have direct effects on the behavioural circadian output (i.e. the locomotor activity). One needs to bear in mind here that mRNA profiles from the head are averaging the dynamics of the *Ccper* transcript over several tissue types, and we expect that the tissue that contributes most is probably the eyes. The neuronal populations that express *Ccper* and generate the circadian behaviour (see Grima *et al*., 2004; Stoleru *et al*., 2004) may well show rather different mRNA profiles (e.g. Peng *et al*., 2003).

On the other hand, CcPER protein is maintained at constant levels both in LD and in DD. This is in contrast to the data from other insect species such as *Drosophila* (Hall, 2003), *L. cuprina* (Warman *et al*., 2000) or *Thermobia domestica* (Zavodska *et al*., 2003), where studies carried on whole head extracts revealed a circadian oscillation in PER levels, with a peak during the dark period. However, western blots of head extracts inevitably include the eyes, which, at least in *Drosophila*, carry the major portion of PER antigenicity. Thus, as explained in the case of mRNA analysis, it could be that in the central pacemaker cells of *Ceratitis*, PER protein cryptically cycles in the canonical way expected for the Diptera. Immunocytochemical analysis with appropriate reagents would therefore represent the next step in this comparative analysis.

To our knowledge, this is the first study on the circadian locomotor activity of *Ceratitis capitata*. Our results show a diurnal activity with a unimodal profile in LD cycles (*Drosophila*, under the same conditions, shows a bimodal profile of activity) and with little evidence for the modulation of behaviour by temperature (Majercak *et al*., 1999, 2004; Collins *et al*., 2004). This might suggest that the temperature-sensitive 3′ splicing that occurs in *D. melanogaster* and which moves evening locomotor activity earlier into the day phase under colder conditions is not present in *Ceratitis* (Majercak *et al*., 1999, 2004; Collins *et al*., 2004). Other *Drosophila* species such as *D. pseudoobscura* and *D. virilis* do not have the same 3′ thermosensitive *per* splicing mechanism (B. Collins & C. P. Kyriacou, unpublished observations), so as *Ceratitis* generally inhabits warmer regions than the cosmopolitan *D. melanogaster*, it is perhaps not altogether surprising that *Ceratitis* does not have such a mechanism. This hypothesis seems to be sustained also by the ability of the chimeric *period* transgene to drive the temperature-dependent modulations of the evening component of LD activity. As the chimeric transgene carries the *D. melanogaster* thermally regulated 3′ intron (Majercak *et al*., 1999, 2004; Collins *et al*., 2004), we might imagine that in transformant flies the splicing of this intron is controlled efficiently at level of the dorsal lateral neurons (LN_ds), which drive the evening peak in 12 : 12 LD conditions (Grima *et al*., 2004; Stoleru *et al*., 2004). Moreover, we also observed a temperature-sensitive modulation of morning activity in wild-type flies. While this phenomenon has not been commented on previously, we have detected in the data of both Majercak *et al*., 1999 (see their figure 1A) and Collins *et al*. 2004 (see their figure 4) similar thermally regulated morning behaviour in *D. melanogaster*. However, this is not carried over into the *pMC1* transformants. It would therefore be of considerable interest to examine whether the 3′ *per* splicing is relevant to this morning peak modulation in *D. melanogaster*.

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The transformant lines carrying *Ccper*, like those carrying *A. pernyi* and *D. pseudoobscura per* (Petersen *et al.*, 1988; Levine *et al.*, 1995; Peixoto *et al.*, 1998), did not give robust rescue of rhythms in arrhythmic mutant *per^01^* in DD. This could be due to the fact that the *Ceratitis per* cDNA was used, so regulatory regions that might be present in intronic sequences were lacking. Alternatively, the poor level of rescue might imply an alteration of the interactions of CcPER with the *Drosophila* nuclear translocation and/or phosphorylation machinery(ies). Nevertheless, in the transgenic line in which rhythmicity was re-established, a bimodal locomotor activity was observed at 18 °C, with a clear subjective morning peak which was reminiscent of *Ceratitis* diurnal activity, but not *D. melanogaster* behaviour. While this result taken in isolation might be considered as not very significant or relevant, the fact that *D. pseudoobscura per* transfers the *D. pseudoobscura* species-specific behavioural profile to *D. melanogaster* hosts (Petersen *et al.*, 1988; Tauber *et al.*, 2003) suggests that a similar phenomenon is occurring with *Ceratitis per*. This effect is not seen as dramatically at higher temperatures, where the transformants appear to show a generally intermediate phenotype between those of the parental species. This suggests that either the transgene conveys basic rhythmicity to the host which is not strong enough to provide a fully wild-type behavioural profile, or that the transgene conveys species-specific aspects of locomotor patterns but is not robust enough to fully 'convert' the host to a *Ceratitis* pattern.

The study of such transgenes in the heterospecific host offers opportunities to examine the evolution of gene regulation, because unlike *de novo* mutants, which are usually identified and isolated because of their dramatic effects on the phenotype, their phenotypes are usually much more subtle. This is because the gene or protein under study has coevolved with its partner molecules under natural selection, and changed its interactions with these partners along the different lineages. Thus, unlike an amorph, which renders its normal molecular interactions null and void, an interspecific transgene would probably maintain many of these interactions but at different levels, thereby providing a paradigm by which to test the evolution of these interactions.

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