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Phenotypic effects induced by knock-down of the *period* clock gene in *Bombyx mori*

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Summary

The lepidopteran *Bombyx mori* is an insect of considerable scientific and economic importance. Recently, the *B. mori* circadian clock gene *period* has been molecularly characterized. We have transformed a *B. mori* strain with a construct encoding a *period* double-strand RNA in order to knock-down *period* gene expression. We observe that this post-transcriptional silencing produces a small but detectable disruption in the egg-hatching rhythm, as well as a reduction in egg-to-adult developmental time, without altering silk production parameters. Thus we show that both circadian and non-circadian phenotypes can be altered by changing *per* expression, and, at a practical level, these results suggest that *per* knock-down may provide a suitable strategy for improving the efficiency of rearing, without affecting silk productivity.

1. Introduction

Circadian rhythms are a basic property of living systems that impose a 24 h temporal organization of behavioural and metabolic activities in many higher and some lower organisms. These rhythms are determined by endogenous oscillators that can be entrained by environmental stimuli. In higher eukaryotes, circadian rhythms are mediated at the molecular level by autoregulatory feedback loops. In the model organism *Drosophila melanogaster*, the *period* (*per*) gene has been demonstrated to play a fundamental role in the control of circadian eclosion and adult locomotor activity rhythms (Hall, 2003). Mutations within *per* can abolish the expression of 24 h behavioural rhythmicity or modify its periodicity, by either increasing or decreasing the circadian cycle in constant darkness (Konopka & Benzer, 1971). Furthermore, circadian clocks have also been implicated in the control of pre-adult developmental time in both *D. melanogaster* (Kyriacou *et al.*, 1990; Paranipe *et al.*, 2005) and the melon fly *Bactrocera cucurbitae* (Miyatake, 1996; Shimizu *et al.*, 1997) in that positive correlations have been observed with both these species between the free-running periods of various clock mutants and various measures of development time. In *Drosophila, per*^{01} null mutants show a faster development than all other *per* genotypes, even under conditions of constant bright light, when PER expression should be minimal and all flies are behaviourally arrhythmic (Kyriacou *et al.*, 1990; Konopka *et al.*, 1989). This suggests that a developmental pleiotropy that is not based on the circadian clock, but which involves the *per* locus, is mediating these pre-adult phenotypes.

B. mori displays circadian rhythms in both egg-hatching behaviour (as quoted in Sehadova *et al.*, 2004) and adult eclosion (Shimizu & Matsui, 1983). These circadian phenotypes are common to many Lepidoptera. In particular, in the giant moth

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Antheraea pernyi, egg-hatching could be controlled by the cycling expression of *Apper* in a restricted number of brain neurons, although no compelling experimental data have been presented (Sauman & Reppert, 1998). While *A. pernyi* encodes a family of three types of sex-linked *per* genes (Gotter *et al*., 1999), *B. mori* is characterized by a single copy of *per*, expressed in both adult heads and peripheral tissues (Iwai *et al*., 2006). Specifically in heads, it is rhythmically expressed under different light : dark (LD) regimes, with a maximum just after lights-off. The *B. mori* putative master clock has been identified in three clusters of brain neurons, where PER protein is expressed with a cycling profile in 12 : 12 LD (Sehadova *et al*., 2004).

In *B. mori* (or *A. pernyi*) no *per* mutant alleles have yet been isolated, so we have used a dsRNAi strategy to produce a gene knock-down (KD). The possibility of using RNAi in *B. mori* was first demonstrated by Quan and co-workers (2002), who showed that the injection of dsRNA into preblastoderm eggs inhibited the expression of a target gene. Subsequently, transgenic silkworms were produced that carried a construct for generating a stable dsRNAi (Isobe *et al*., 2004). In our study, we have generated transgenic silkworms that carry a construct that induces a stable and heritable *per* dsRNAi (*per-IR*).

In our system the *per* KD was able to modestly but significantly alter egg-hatching circadian behaviour in 12 : 12 LD. This is particularly interesting in the light of work by Sauman and co-workers (1996), in which the 'hatching gate' in *A. pernyi* was reported to be disrupted by anti-sense injections of *Apper*. However, this result could not be replicated by the same group, who subsequently retracted their finding (Sauman *et al*., 2000). Our results thus reopen the possibility that *per* may indeed play a functional role in the hatching rhythm of Lepidoptera. In addition, the *per* dsRNAi construct caused a reduction in egg-to-adult developmental time, as predicted by the studies in Diptera mentioned above. Finally, the transgenic *per* KD silkworms did not show any significant modification in female fertility, nor in cocoon silk percentage, important production parameters in sericulture.

2. Methods

(i) *Strain and rearing conditions*

The Nistari polyvoltine Indian strain was obtained by Dr G. Chavancy (Institut National de la Recherche Agronomique, Unité Nationale Séricicole, France). *B. mori* eggs were incubated at 26 ± 1 °C and 85 ± 5% relative humidity (RH). Silkworms were reared in 12 : 12 LD and constant darkness (DD). Larvae were fed with an artificial diet (Cappellozza *et al*., 2005). All the molecular and behavioural experiments were carried out on newly laid eggs, avoiding egg preservation at low temperature (5 °C) after laying.

(ii) per-IR *plasmid construction*

The *per-IR* construct was characterized by two *per* inverted repeats (IR), under the control of the *B. mori Actin* promoter in the *piggyBac*[3 X P3-EGFP] vector (Piccin *et al*., 2001; Horn & Wimmer, 2000).

(a) *First step: Introduction of the* Act *promoter and SV40 terminator in the pBac[3 X P3-EGFP] vector*

A 241 bp *Act* promoter fragment (*Act-prom*) and 282 bp *SV40* terminator (*SV40-Term*) were amplified using the pPIGA3GFP plasmid (Tamura *et al*., 2000) as template. The primers were as follows: (i) for *Act-prom*, *act-5* (5′-AAAAGGCGCGCCCACAGGAAA CAGCTATGACC-3′) and *act-3* (5′-TACTAGTCTGATGCACTGTTCGAGCACACC-3′), both carrying a tail (underlined) containing the restriction sites *Asc*I and *Spe*I, respectively; (ii) for *SV40-Term*, *SV40-5* (5′-CATCAGACTAGTAAGCGGCCGCGACTCT-3′) and *SV40-3* (5′-AAAAAGATCTCGCGTATCGATAAGCTTTAAG-3′), carrying a tail (underlined) containing the restriction sites *Spe*I and *Bgl*II, respectively. The underlined regions in the *act-3* and *SV40-5* primers indicate complementary sequences that permit the joining of the two fragments in a chimeric segment. Therefore, equimolar amounts of the two amplified fragments were mixed together and subjected to PCR with *act-5* and *SV40-3* primers, which allow only the amplification of the 517 pb *Act-SV40* chimeric segment. This tract was then cloned as an *Asc*I–*Bgl*II fragment in the pBac[3 X P3-EGFP] vector, obtaining the pBac[3 X P3-EGFP]-exp vector.

(b) *Second step: Construction of the* per-IR *fragment*

A 825 bp fragment from the *B. mori per* coding sequence was obtained via RT-PCR using the 5′ primer 5′-CGGTACGCAAGAAAAAGCTC-3′ and the 3′ primer 5′-CTGTGGTCCGGATCCTGTATTTC-3′ (positions 991–1011 and 1792–1816, respectively, in GenBank AB179829) and cloned in the pCR2-Topo vector (Invitrogen). This fragment was sequenced to exclude PCR-induced nucleotide substitutions and subcloned as a *Spe*I–*Apa*I fragment in the pBC KS+ vector (Stratagene). In parallel, a 419 bp fragment from the *lacZ* gene, serving as a heterologous spacer to favour hairpin-dsRNA formation, was amplified

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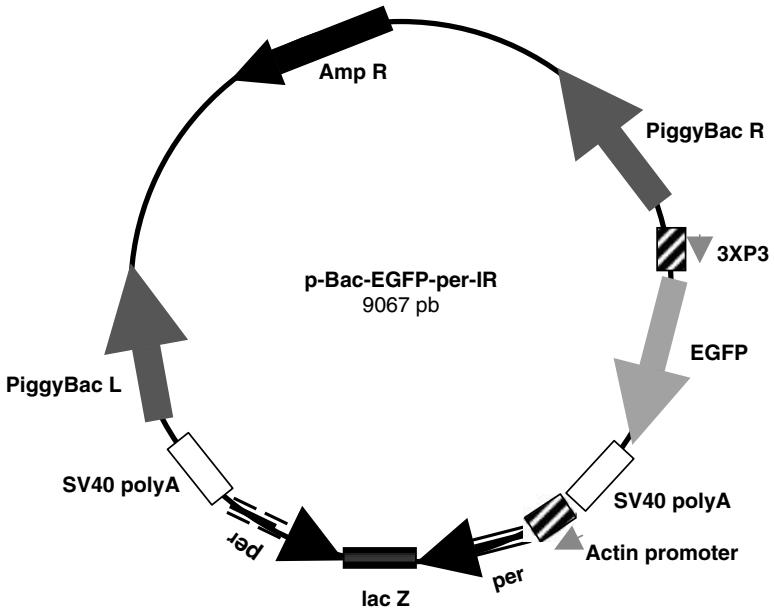

Fig. 1. Schematic representation of the pBac-EGFP-*per-IR* construct. 825 pb *per* inverted repeats separated by the *lacZ* spacer were subcloned between the *Actin* promoter and SV40 poly A terminator in the pBac[3 X P3-EGFP] vector. The marker EGFP is under the control of the 3 X P3 promoter, active at the level of stemmata and in the developing embryonic nervous system (Horn & Wimmer, 2000). *piggyBac* R and L, inverted terminal repeats of *piggyBac*; Amp R, ampicillin-resistance gene.

using the 5′ primer 5′-GCATTATCCGGACCATCC-3′ and the 3′ primer 5′-TCTCTCCAGGTAGC-GAAAGC-3′, both containing an *Ava*II restriction site (underlined). PBC-*per* was then digested using *Kpn*I and *Ava*II restriction enzymes and recircularized in the presence of a 402 bp *Ava*II–*Ava*II *lacZ* fragment and *Ava*II–*Kpn*I *per* fragment, obtained from pCR2-*per* plasmid.

(c) *Third step: Preparation of the pBac-EGFP-*per-IR *construct*

Finally, the *Spe*I–*Spe*I fragment containing the *per-IR* separated by the *lacZ* spacer was subcloned between the *Act* promoter and *SV40* terminator in the pBac[3 X P3-EGFP]-exp vector, obtaining the approximately 9·1 kb pBac-EGFP-*per-IR* (*per-IR*) construct (Fig. 1).

(iii) *Transgenesis and screening of transformed* B. mori

Syncytial preblastoderm wild-type Nistari embryos were injected with a 1 : 1 mixture of pBac-EGFP-*per-IR* construct and helper plasmid, as described in Tamura *et al.* (2000). G1 embryos were screened on the seventh day of development under a fluorescence stereomicroscope, equipped with appropriate filters for GFP detection. From 4353 *Nistari* injected embryos, 463 G0 fertile moths were obtained. After sibling mating, one G1 brood with GFP-positive embryos was isolated, giving a transformation efficiency of 0·2%. The G1 individuals were allowed to develop in a mass culture with non-transformed embryos and the GFP fluorescence was rechecked at the pupal stage. Five GFP-positive single G1 individuals were then selected and backcrossed to wild-type Nistari moths, obtaining five different G2 transgenic broods. Subsequently, in order to obtained stable transgenic lines, GFP-positive individuals of each G2 transgenic brood were selected and separately intercrossed for about 10 generations.

(iv) *DNA extraction and Southern blot analysis*

Genomic DNA (10 μg per sample) was prepared according to Sambrook *et al.* (1989). Wild-type DNAs were digested with *Bam*HI or *Hin*dIII or *Xba*I restriction enzymes, singly or in combination; transgenic DNA samples were digested separately with *Apa*I or *Pst*I or *Sac*I restriction enzymes. Wild-type blots were hybridized with a 160 bp *per* [γ-^{32}P]dCTP probe (1035–1194 in GenBank Accession number AB179829; Takeda *et al.*, 2004). Transgenic blots were probed with a 380 bp *lacZ* [γ-^{32}P]dCTP fragment, corresponding to the portion used as spacer sequence in the transgene. Each Southern blot experiment was repeated twice.

(v) *RT- and real-time PCR*

For RT-PCR, transgenic embryos were collected after 5, 6 and 7 days of development; for quantitative PCR, 7-day-old embryos from transgenic and wild-type

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synchronized cultures in 12 : 12 LD were collected and frozen 3 h after lights-on (Zeitgeber 3 (ZT 3), with ZT 0 and ZT 12 representing lights-on and lights-off, respectively). For each sample, total RNA was extracted from 25 frozen embryos and cDNA was synthesized as in Mazzotta *et al*. (2005). To amplify the *lacZ* spacer in RT-PCR experiments, a nested PCR was carried out. The first PCR was performed by using the 5′ primer 5′-GCTTTCGCTACCTGGAGAGA-3′ and the 3′ primer 5′-GCTGTGGTACACGCTGTGCG-3′, obtaining a 401 pb amplicon. The second was performed employing the 5′ primer 5′-TGTGGTGGATGAAGCCAATA-3′ and the 3′ primer 5′-GATACAGCGCGTCGTGATTA-3′ and 2 μl of the first amplification as template, in order to obtain a 194 bp amplified fragment. Both PCR reactions were carried out using the following programme: 95 °C for 10 min then 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, for 40 cycles, followed by a final 7 min at 72 °C.

The real-time PCR experiments were performed on a Rotor Gene 3000 (Corbett Research), as in Zordan *et al*. (2006). The primers used were the following: 5′-GTTGGGTAACACCGAGGAG-3′ and 5′-CGCTGGAATATGGTGATCGG-3′ for *per* mRNA (positions 2996–3016 and 3212–3233, respectively in GenBank AB179829; Takeda *et al*., 2004) and 5′-TCAATCGGATCGCTATGACA-3′ and 5′-GGAATCCATTTGGGAGCATA-3′ for the housekeeping *rp49* mRNA (positions 7–26 and 142–161 respectively in GenBank AB048205). SYBRGreen (Applied Biosystems) fluorescent molecule was used to generate semiquantitative data. The PCR reaction was carried out using the following programme: 95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s, for 40 cycles, followed by a final 10 min step at 72 °C. The amplification efficiencies of each sample were calculated on the basis of the results of three amplification reactions, with different quantities of the template (30, 60, 120 ng of total reverse-transcribed RNA), according to Pfaffl (2001). Each reaction was done in duplicate. The relative levels of *per* mRNA in both transgenic lines were compared with those of the wild-type at the same developmental stage (chosen as reference). All real-time PCR experiments were performed on three independent RNA extractions.

(vi) *Analysis of egg-hatching circadian behaviour*

For each line, batches of 150–250 eggs, laid in 4 h periods, were checked during development and the numbers of hatched larvae were recorded by three operators at 2 h intervals, as previously described for the lepidopteran *A. pernyi* (Sauman *et al*., 1996; Sauman & Reppert, 1998). For each line the experiments were performed at least twice, in 12 : 12 LD. In order to evaluate the egg-hatching behaviour, each egg was given an hourly score that represented the time it hatched, from the initial observation period which was ZT 19, day 1 (score 1), until ZT 18, day 3 (score 72). Subsequently, for each egg-batch, a 'mean hatching time' score was calculated.

(vii) *Life cycle analysis*

The developmental rate analyses were conducted in 12 : 12 LD and DD.

(a) *Egg collection and egg development analysis*

For each line, 20 mother moths were allowed to lay for 2 h periods. For each environmental condition, 4 h of egg-laying was collected, from ZT 10 to ZT 14 (and at the corresponding CTs, with CT 0 and CT 12 representing the beginning and the end of the subjective day in DD), and subdivided into two 2 h egg-laying sessions. For each 2 h egg-laying session at least three replicates per experimental condition were evaluated. Number of eggs per replicate ranged from 100 to 400. Egg-hatching was examined three times per day (at ZT 0, 4, 12, and at the corresponding CTs) for three consecutive days, and for each transgenic or wild-type egg-laying replicate, the number of hatched individuals per day were recorded.

(b) *Larval cycle*

Fertilized eggs were obtained from silkworms grown in 12 : 12 LD. Ten egg batches for each line were placed in 12 : 12 LD and DD. Hatching larvae were placed in transparent boxes and the number of larvae per box was progressively reduced from 100 for the first instar, to 30 for the fifth instar. The synchronization of the cultures was performed as described in Bosquet *et al*. (1989). Briefly, during moulting periods, relative humidity was progressively decreased to 60–65 % in order to permit food desiccation and to avoid early moulters resuming feeding before late moulters. Feeding was suspended when larvae showed moulting behaviour. Interruptions of feeding in concurrence with moulting periods never lasted more than 24 h, as larvae were well synchronized and feeding was resumed when 80 % of moulting larvae had ecdysed. For each line, three replicates were established. At 4–5 days after spinning, cocoons were cut and pupae were separated from their shells and sexed.

(c) *Eclosion and mating*

New-emerged adults were consecutively recorded for 4–5 days. Three replicates per line for females in 12 : 12 LD and two replicates for females in DD and males in 12 : 12 LD and DD were analysed. The

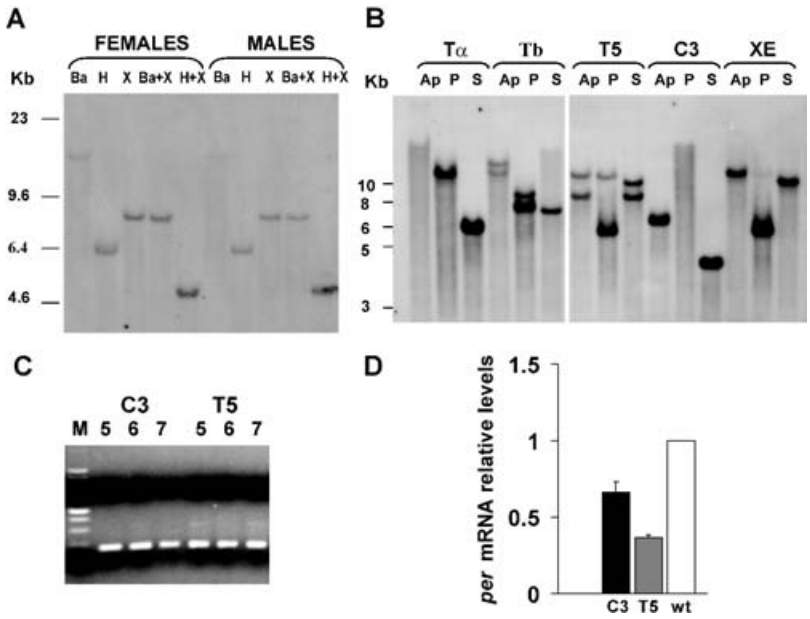

Fig. 2. Molecular characterization of the wild-type and the C3 and T5 transgenic lines. (*A*) Genomic Southern blots of wild-type adult males or females DNAs digested with *BamH*I (Ba), *Hind*III (H) or *Xba*I (X), singly or in combination, and hybridized with 160 bp *per* probe. Letters above lanes identify restriction enzyme(s). (*B*) Genomic Southern blots of transgenic DNAs from adult males digested with *Apa*I (Ap), *Pst*I (P) or *Sac*II (S) and probed with a 380 bp *lacZ* fragment. Ta, Tb, T5, C3 and XE represent five independent transgenic lines. Letters above lanes identify restriction enzyme(s). (*C*) RT-PCR of a *per-IR* transgene fragment on transgenic C3 and T5 embryonic cDNAs, at different developmental stages. Numbers above lanes identify the number of days after fertilization. (*D*) *per* mRNA levels (via real-time PCR) in 7-day-old transgenic C3, T5 and wild-type embryos (mean ± SEM of three independent experiments; one-way ANOVA $F_{2,6} = 57{\cdot}75$, $P < 0{\cdot}001$).

number of individuals per replicate ranged from 30 to 50. Eclosed moths (single couples) were mated for 2 h, males were eliminated and females were allowed to lay eggs.

In order to analyse the data, for each developmental stage, each individual was given a daily score that represented the time it required to complete the specific developmental stage, from day 1 (score 1) to day 3 (score 3) for larval hatching and moults and from day 1 (score 1) to day 5 (score 5) for adult eclosion. Subsequently, for each transgenic or wild-type replicate, a 'mean developmental time' score (the mean time required to complete a specific developmental stage) was calculated.

(viii) *Production parameters*

(a) *Analysis of oviposition*

For each line, 20–30 fertilized females were allowed to lay in 12 : 12 LD. Overall 8 h ovipositions were collected, from ZT 8 to ZT 16. For each genotype, the percentage of eggs laid in each 2 h interval was recorded. In order to analyse the oviposition profile data, each egg was given an hourly score that represented the time it was laid, from ZT 8–10 interval (score 1) to ZT 14–16 interval (score 4) and for each transgenic or wild-type female the 'mean oviposition score' value, an individual index of the temporal pattern of oviposition, was calculated.

(b) *Analysis of cocoon silk ratio*.

For each line, at least 30 female and 30 male cocoons, spun in 12 : 12 LD, were individually weighed. A 'cocoon silk' ratio was calculated as silk shell/cocoon weights.

(ix) *Statistical analysis*

Analysis of variance was performed using one-way ANOVA and the *a posteriori* LSD-least significant difference test with the Statistica 5.0 package (Statsoft Inc.).

3. Results

(i) *Characterization of transgenic* per-IR B. mori

Our data indicate that, unlike *A. pernyi* (Gotter *et al.*, 1999), the wild-type Nistari *B. mori* strain encodes a single *per* gene in both sexes (Fig. 2 *A*). Five transgenic lines were characterized by Southern blot analysis using a *lacZ* fragment as a probe, which allowed the specific identification of the *per-IR* insert (see Section 2 for details). Each sample showed a different

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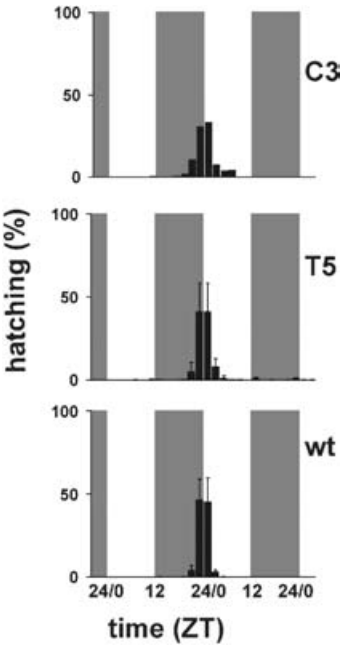

Fig. 3. Percentage of hatching larvae in 12 : 12 LD. Grey bars represent dark periods, open bars light periods of the day. C3 transgenic line (mean of two replicates); T5 transgenic line (mean ± SEM of three replicates); wild-type (mean ± SEM of three replicates). Statistical analysis is provided in the text.

hybridization pattern, variable both in position and number of insertions (Fig. 2*B*). Specifically, three transgenic lines (Ta, C3, XE) carried only one copy of the transgene, while the others (Tb, T5) had two inserts. The C3 and T5 transgenic lines were subsequently selected in order to characterize the effects of *per* post-transcriptional silencing. For both transgenic lines, the *per-IR* insert was actively transcribed 5 days after fertilization, approximately 72 h before hatching, and persisted in all later stages examined, until approximately 24 h before hatching (Fig. 2*C*). In fact, in all tested samples the RT-PCR experiments showed an active transcription of the *lacZ* spacer, characteristic of the *per-IR* transgene and not present in the host *B. mori* genome. Fig. 2*D* shows *per* mRNA expression evaluated via real-time PCR in 7-day-old C3, T5 transgenic and wild-type embryos, sampled at the same time of the day, 3 h after lights-on (ZT 3). The results indicate a partial but nevertheless significant decrease in *per* mRNA levels in both the transgenic lines tested compared with wild-type ($F_{2,6} = 57{\cdot}75$, $P < 0{\cdot}001$).

(ii) *Circadian egg-hatching in transgenic lines*

Fig. 3 shows the temporal profiles of hatching times for C3, T5 transgenic and wild-type individuals in 12 : 12 LD. Wild-type *B. mori* showed modal egg-hatching around the dark–light transition with an 'anticipation' of lights-on. Under LD entrainment conditions both the *per* KD lines showed a similar behaviour to that of the wild-type strain, although it is clear that in both lines, particularly C3, the main 'hatching gate' had spread into the day compared with the control. ANOVA of three replicate experiments for wild-type and T5, and two for C3, revealed no significant differences in the mean temporal interval in which hatching occurs ($F_{2,5} = 0{\cdot}63$, n.s.). However, when we examined the coefficient of variation (CV) for hatching, we obtained a highly significant result, even with such small sample sizes ($F_{2,5} = 13{\cdot}65$, $P < 0{\cdot}01$, CV mean for wild-type = 0·052, T5 = 0·107, C3 = 0·196). *A posteriori* analysis of the mean CVs revealed that the wild-type showed a significantly reduced CV compared with C3 ($P < 0{\cdot}005$) and a marginal effect compared with T5 ($P = 0{\cdot}07$). Thus there appears to be a significant increase in the variances of the 'hatching gate', but not in the means, which would be consistent with a mild disruption of the hatching rhythm.

(iii) *Transgenic* per-IR *life cycle analysis*

We compared the developmental rate of C3 and T5 transgenic lines with that of the wild-type polyvoltine Nistari strain, which at 26 °C shows a life cycle length of approximately 40 days. The rate of each developmental stage was analysed in synchronized cultures of the two transgenic and wild-type lines, in 12 : 12 LD and DD (CT 0, subjective lights-on). Moreover, for each stage, mortality was recorded, but no differences between transgenics and wild-type were found (data not shown). To evaluate the duration of the first developmental stage (from egg to first instar larva), we analysed the developmental time of fertilized eggs laid in two consecutive 2 h periods (ZT 10–12, ZT 12–14, and at the corresponding CTs). From the first day onwards, we noticed an acceleration in development of the transgenic fertilized eggs with respect to those of the control. During *B. mori* embryonic maturation, the egg-shell pigmentation changes because of the progressive maturation of the enclosed embryo (Pang-Chuan & Da-Chuang, 1992). Specifically, at 26 °C, the wild-type Nistari strain shows a uniformly yellow pigmented egg-shell ('yellow' stage) 6 days after fertilization. The following day, the egg-shell shows a dark spot, corresponding to the developing embryonic head ('eye-spot' stage) and, after a further 24 h, it becomes uniformly grey ('blue-egg' stage). For both the transgenic lines the last pigment modification in the egg-shells occurred earlier, in both 12 : 12 LD and DD, so that by 7 days after fertilization, when the wild-type eggs were at the 'eye-spot' stage, most of the transgenic eggs were at the 'blue-egg' stage (Fig. 4*A*). From the eighth day after fertilization, the egg-hatching percentages for C3 and T5 transgenic lines and wild-type strain were evaluated

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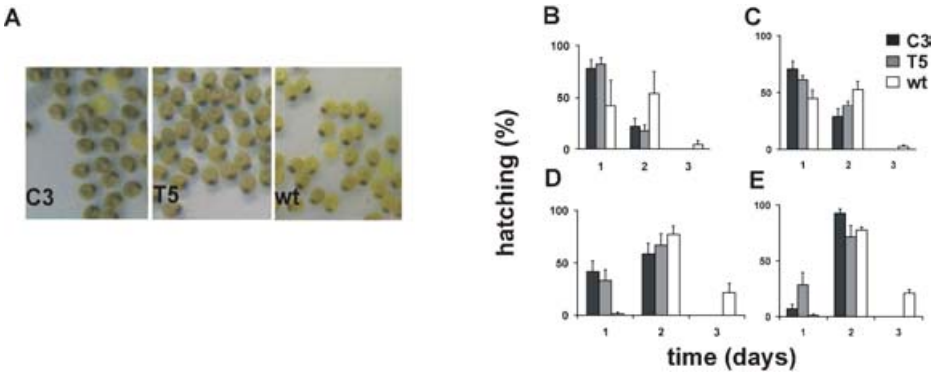

Fig. 4. Egg-hatching in transgenic C3, T5 and wild-type synchronized cultures in 12 : 12 LD and DD. (*A*) Seven-day-old transgenic C3, T5 and wild-type embryos derived from synchronized eggs laid in the ZT 12–14 interval. Percentage of hatched larvae from eggs laid in (*B*) the ZT 10–12 and (*C*) the ZT 12–14 intervals, in 12 : 12 LD, and in (*D*) the CT 10–12 and (*E*) the CT 12–14 intervals, in DD. For each egg-laying interval, the mean ± SEM of at least three replicates is shown.

for three consecutive days (Fig. 4*B–E*). To compare the transgenic and wild-type egg-hatching time, analysis of variance was performed on scores representing the mean time required to hatch, calculated for each transgenic and wild-type replicate ('mean hatching time' score) (Table 1). In both 12 : 12 LD and DD, for each 2 h egg-laying cohort, the transgenic cultures showed a significant acceleration of approximately 24 h in the mean time required to complete the embryonic stage compared with the wild-type. The *a posteriori* comparisons did not reveal any significant differences in the hatching time between the two transgenic lines, for the CT, ZT 10–12 collections, but the T5 line showed a significant acceleration compared with C3 for the subsequent collection in CT 12–14 ($P<0{\cdot}05$; see notes to Table 1).

During the three subsequent moults, no significant differences in the developmental timing between transgenic and wild-type lines were recorded (Table 2). At the last moult, in 12 : 12 LD, most of the transgenic individuals reached the moulting phase 24 h earlier than the wild-type ($F_{2,6}=39{\cdot}59$, $P<0{\cdot}001$; Table 2). In DD, the faster developmental rate of both the transgenic lines was more evident, with the moulting phase approximately 48 h before the controls ($F_{2,6}=271{\cdot}36$, $P\ll0{\cdot}0001$; Table 2). In both 12 : 12 LD and in DD, *a posteriori* comparisons revealed significant differences in the moulting time between the two transgenic lines (see notes to Table 2).

In Fig. 5*A* and 5*B* the percentages of transgenic and wild-type emerging adult females, analysed for five consecutive days in 12 : 12 LD and DD, are reported. The transgenic and wild-type eclosion times obtained in 12 : 12 LD were compared by performing an ANOVA on 'mean eclosion time' scores, calculated for each transgenic or wild-type replicate. The temporal eclosion pattern of both the transgenic lines was approximately 24 h earlier compared with that of the wild-type strain ($F_{2,6}=13{\cdot}82$, $P=0{\cdot}005$; Table 3). In fact the wild-type females showed few emerging individuals on the second day of analysis and the main emerging cohort was on the third day. For both the transgenic lines, few individuals eclosed during the first day of observation, while a massive emergence occurred on the second day, and by the third day both the transgenic lines had essentially completed eclosion. An *a posteriori* comparison did not reveal significant differences in the eclosion profiles between the two transgenic lines (see notes to Table 3). In DD the same experiment was performed twice, giving a similar accelerated temporal eclosion profile for the transgenic lines compared with that of the wild-type.

Table 1. *Egg-hatching time in transgenic C3 and T5 and wild-type* B. mori

Egg-laying interval	Genotype	*N*	Score (mean ± SEM)
ZT 10–12	C3	3	1·22 ± 0·08
	T5	3	1·18 ± 0·06
	Wild-type	3	1·86 ± 0·16[a]
ZT 12–14	C	6	1·29 ± 0·07
	T5	6	1·38 ± 0·03
	Wild-type	6	1·58 ± 0·07[b]
CT 10–12	C3	3	1·50 ± 0·12
	T5	3	1·68 ± 0·09
	Wild-type	3	2·20 ± 0·09[c]
CT 12–14	C3	6	1·93 ± 0·37
	T5	6	1·71 ± 0·10
	Wild-type	6	2·20 ± 0·04[d]

Score represents the mean time required to hatch (comparison between C3, T5 and wild-type for eggs laid in: [a] ZT 10–12 interval: $F_{2,6}=12{\cdot}47$, $P<0{\cdot}01$; *post-hoc* LSD tests were not significant between C3 and T5; [b] ZT 12–14 interval: $F_{2,15}=6{\cdot}30$, $P<0{\cdot}01$; *post-hoc* LSD tests were not significant between C3 and T5; [c] CT 10–12 interval: $F_{2,6}=11{\cdot}74$, $P<0{\cdot}01$; *post-hoc* LSD tests were not significant between C3 and T5; [d] CT 12–14 interval: $F_{2,15}=13{\cdot}40$, $P<0{\cdot}001$; *post-hoc* LSD test: C3 vs T5, $P=0{\cdot}03$).
N, number of replicates.

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Table 2. *Timing of larval development in transgenic C3 and T5 and wild-type* B. mori

Genotype	Moult	Moulting larvae (% ± SEM) 12 : 12 LD		Score (mean ± SEM) 12 : 12 LD	Moulting larvae (% ± SEM) DD			Score (mean ± SEM) DD
		First day	Second day		First day	Second day	Third day	
C3	I	28 ± 14	72 ± 14	1·62 ± 0·14	96 ± 0·2	4 ± 0·2	0 ± 0	1·09 ± 0·03
	II	12 ± 6	88 ± 6	1·87 ± 0·06	79 ± 8	21 ± 8	0 ± 0	1·21 ± 0·08
	III	79 ± 3	21 ± 3	1·21 ± 0·02	100 ± 0	0 ± 0	0 ± 0	1 ± 0
	IV	48 ± 3	52 ± 3	1·52 ± 0·03[a]	43 ± 6	57 ± 6	0 ± 0	1·57 ± 0·03[b]
T5	I	17 ± 11	83 ± 11	1·83 ± 0·11	78 ± 5	22 ± 5	0 ± 0	1·22 ± 0·04
	II	18 ± 5	81 ± 5	1·82 ± 0·05	76 ± 7	24 ± 7	0 ± 0	1·23 ± 0·07
	III	97 ± 1	3 ± 1	1·12 ± 0·04	100 ± 0	0 ± 0	0 ± 0	1 ± 0
	IV	67 ± 4	33 ± 4	1·33 ± 0·03[a]	21 ± 7	79 ± 7	0 ± 0	1·76 ± 0·07[b]
Wild-type	I	22 ± 6	78 ± 6	1·78 ± 0·05	76 ± 3	24 ± 3	0 ± 0	1·24 ± 0·03
	II	12 ± 3	87 ± 3	1·88 ± 0·03	77 ± 10	23 ± 10	0 ± 0	1·23 ± 0·10
	III	80 ± 4	20 ± 4	1·19 ± 0·04	83 ± 13	11 ± 7	0 ± 0	1·29 ± 0·16
	IV	22 ± 4	78 ± 4	1·79 ± 0·03	1 ± 0·7	1 ± 0·7	98 ± 0·07	2·97 ± 0·008

Score represents the mean time required to complete a specific developmental stage (comparison for the IV moult between C3, T5 and wild-type: [a] in 12 : 12 LD $F_{2,6} = 39{\cdot}59$, $P < 0{\cdot}001$, *post-hoc* LSD test: C3 vs T5 $P < 0{\cdot}001$ and [b] in DD $F_{2,6} = 271{\cdot}36$, $P \ll 0{\cdot}0001$, *post-hoc* LSD test: C3 vs T5 $P < 0{\cdot}01$).

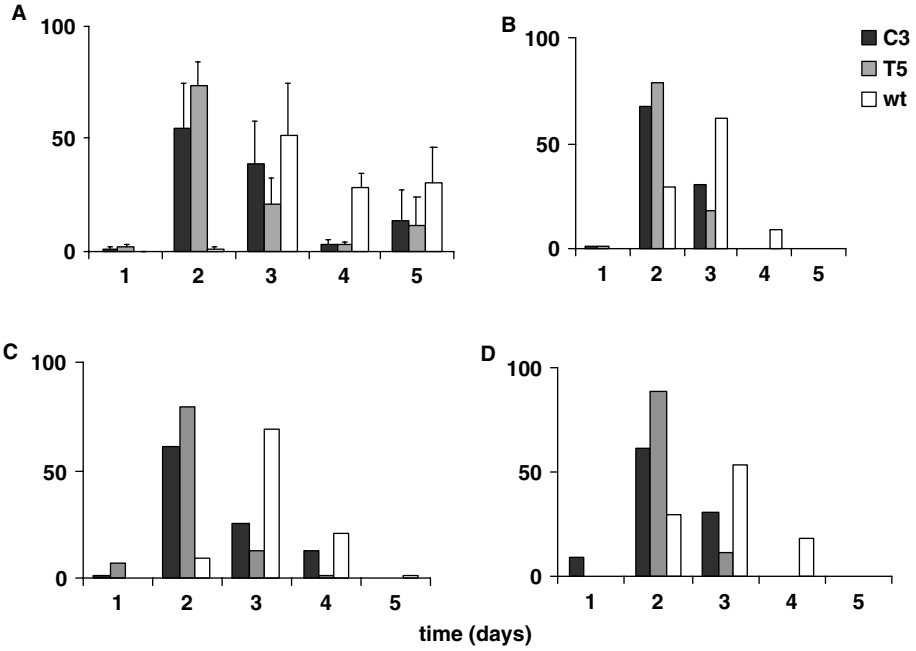

Fig. 5. Adult eclosion in transgenic C3, T5 and wild-type synchronized cultures for 5 consecutive days in 12 : 12 LD and DD. Percentage of eclosion in (*A*) females in 12 : 12 LD (mean ± SEM of three replicates); (*B*) females in DD (mean of two replicates); (*C*) males in 12 : 12 LD (mean of two replicates); (*D*) males in DD (mean of two replicates).

Moreover, a comparable 24 h acceleration was detected on analysing the emerging activity in males, in both 12 : 12 LD and DD (Fig. 5*C*, *D*).

(iv) *Estimation of transgenic* per-IR *productivity*

To estimate the productivity parameters for the *per* KD lines, we compared the fertility of C3 and T5 transgenic females with those of wild-type, in 12 : 12 LD. Therefore we recorded the number of eggs laid per female in an 8 h interval, from ZT 8 to ZT 16; no significant differences between genotypes were found ($F_{2,69} = 0{\cdot}59$, $P = 0{\cdot}56$; Table 4). Fig. 6 shows egg-laying temporal profile for transgenic and wild-type females evaluated for four consecutive 2 h periods (ZT 8–10, ZT 10–12, ZT 12–14, ZT 14–16 intervals). The statistical analysis shows that the 'mean oviposition time' of both types of transgenic females was not

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Table 3. *Eclosion timing in transgenic C3 and T5 and wild-type* B. mori *in 12 : 12 LD*

Genotype	Score (mean ± SEM)
C3	2·50 ± 0·21
T5	2·26 ± 0·15
Wild-type	4·05 ± 0·37[a]

Score represents the mean time required to eclose in 12 : 12 LD ([a] comparison between C3, T5 and wild-type $F_{2,6} = 13{\cdot}82$, $P = 0{\cdot}005$; *post-hoc* LSD test: C3 vs T5 $P = 0{\cdot}58$, n.s.).
Number of replicates = 3.

Table 4. *Female fertility and oviposition time in 12 : 12 LD*

Genotype	No. of females	No. of eggs (mean ± SEM)	Score (mean ± SEM)
C3	23	286 ± 20	3·09 ± 0·035
T5	29	291 ± 15	3·11 ± 0·04
Wild-type	20	264 ± 18	3·22 ± 0·07

Comparison between number of laid eggs by C3, T5 and wild-type females: $F_{2,69} = 0{\cdot}59$, $P = 0{\cdot}56$, n.s. For each genotype, score represents an index of the temporal pattern of oviposition. It was calculated as the mean of the values obtained for each analysed female in the ZT 8–16 interval (see Section 2 for details), in 12 : 12 LD (comparison between C3, T5 and wild-type females score values: $F_{2,69} = 1{\cdot}80$, $P = 0{\cdot}17$, n.s.).

significantly different from that of controls, over this 8 h interval ($F_{2,\ 69} = 1{\cdot}80$, $P = 0{\cdot}17$, n.s.; Table 4).

Table 5 reports the silk percentage for transgenic and wild-type males and females, calculated as the cocoon shell/whole cocoon weight ratio. Both C3 and T5 transgenic males and females showed a significant decrease in the whole cocoon weight with respect to the wild-type by ANOVA (males: $F_{2,87} = 9{\cdot}2$, $P < 0{\cdot}001$; females: $F_{2,87} = 4{\cdot}56$, $P < 0{\cdot}01$) but no significant modifications in the cocoon shell weight (males: $F_{2,87} = 0{\cdot}11$, $P = 0{\cdot}89$, n.s.; females: $F_{2,87} = 3{\cdot}0$, $P = 0{\cdot}054$, n.s.). These modifications suggest a slight but significant increment in the silk percentage for males of both the transgenic lines with respect to the wild-type ($F_{2,87} = 4{\cdot}03$, $P < 0{\cdot}05$). In the transgenic females no significant effect was observed ($F_{2,87} = 1{\cdot}51$, $P = 0{\cdot}23$, n.s.).

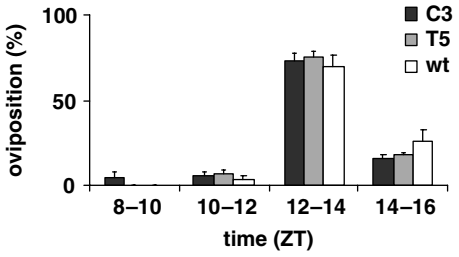

Fig. 6. Oviposition of transgenic C3, T5 and wild-type females during an 8 h interval in 12 : 12 LD. Percentage of oviposition (mean ± SEM) of transgenic 23 C3, 29 T5 and 30 wild-type females during four consecutive periods (ZT 8–10, ZT 10–12, ZT 12–14, ZT 14–16 intervals).

4. Discussion

The 7-day-old *per-IR* transgenic embryos showed a significant decrease of *per* mRNA (in the 40–75% range) with respect to the wild-type, suggesting that the *B. mori* RNAi molecular machinery is active in the induction of the post-transcriptional silencing from at least approximately 24–48 h before hatching. This level of reduction is clearly not a complete knock-down and this might be due to positional effects of the insert(s) in the host *B. mori* genome, which might influence the *Actin* promoter activity in the transgene. Partial and variable mRNA KD levels were also obtained in *Drosophila melanogaster*, when an *Actin*-driven early RNA interference was induced following a similar strategy (Zordan *et al.*, 2006). The *per* mRNA reduction obtained in *B. mori* KD transgenic lines is nevertheless at least at about the level that would be expected in a heterozygous *per* null mutation in this species. In *D. melanogaster*, per^{+}/per^{01} individuals, heterozygous for the recessive per^{01} null allele, show a periodicity slightly longer than normal (24·6 ± 0·2 vs 24·0 ± 0·1; Citri *et al.*, 1987; Dushay *et al.*, 1990), revealing an apparent dosage effect. In addition, partially dominant behavioural effects due to heterozygosity for the classic mutants per^{s} and per^{L1} can also be detected (Konopka & Benzer, 1971; Dushay *et al.*, 1990). In *Bombyx mori*, the *per* KD levels obtained in both the *per-IR* transgenic lines were able to detectably modify the circadian gate of egg-hatching behaviour, under entrainment conditions. There was no difference in the mean hatching time of the strains, but the variation around that time was significantly elevated in the transgenic lines, particularly C3, compared with the wild-type. These results indicate a modest disruption of the hatching rhythm when *per* mRNA is downregulated under these conditions, and, to our knowledge, the first direct demonstration of an association between *per* expression and a circadian phenotype in Lepidoptera. A much more dramatic effect on the hatching rhythm was observed with *per* anti-sense injection in *A. pernyi* (Sauman *et al.*, 1996), but these results were later withdrawn (Sauman *et al.*, 2000). Our data are supportive of the original results of Sauman and co-workers (1996), suggesting that their RNAi experiments in the giant silkmoth may have worked at least once. However, one inconsistency in our own work with *B. mori* is that the transgenic strain T5, which had the largest KD of about 75% of *per*

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Table 5. *Transgenic productivity: silk percentage*

Genotype		Whole cocoon weight (mean ± SEM)	Cocoon shell weight (mean ± SEM)	Silk % (mean ± SEM)
C3	M	0·77 ± 0·01	0·075 ± 0·001	9·6 ± 0·21
	F	1·08 ± 0·01	0·093 ± 0·001	8·6 ± 0·16
T5	M	0·77 ± 0·01	0·073 ± 0·001	9·5 ± 0·17
	F	1·05 ± 0·02	0·094 ± 0·002	8·9 ± 0·16
Wild-type	M	0·84 ± 0·01	0·074 ± 0·001	8·9 ± 0·14
	F	1·12 ± 0·01	0·100 ± 0·001	9·0 ± 0·12

mRNA, did in fact show the least disruption to the hatching gate, whereas C3, which had an approximately 40% KD, showed a more impressive spreading of the hatching gate. However, real-time PCR was performed on whole embryos before hatching, so it is quite likely that position effects between the two transgenic lines on the *Actin* driver may have generated a patchy distribution of *per* KD in the relevant cells for eclosion. Immunohistochemical examination of the PER protein levels will help to map the brain *per* KD target neurons in these transgenic embryos.

In addition, *per* mRNA KD modified *B. mori* developmental timing, in both 12 : 12 LD and DD. This appears to be a real effect of a faster growth, rather than a carry-over from the more variable eclosion gating of the transgenics, because the mean and modal values for eclosion were not significantly different. Also the eggs were collected in 2 h windows for this experiment, thereby minimizing the 'spread' of subsequent eclosions. In fact, we observed significant accelerations in embryonic development, the last larval moulting phase, and during pupal-adult transition, reminiscent of the effect of the *per*-null mutation in *Drosophila* (Kyriacou *et al*., 1990). Specifically, the last larval moult seemed to be the most sensitive developmental phase, with the strongest temporal anticipation under constant conditions. In contrast, the duration of earlier larval stages was unaffected. These data generally resemble those obtained on *D. melanogaster*, where different *per* mutations are able to modify the *Drosophila* developmental timing, under different environmental conditions (Kyriacou *et al*., 1990).

We cannot completely exclude the possibility that the phenotypic effects exhibited by our *per* KD *B. mori* lines transgenic lines can derive, at least in part, from alteration in the expression of 'off-target' genes. For a non-model organism such as *B. mori*, web-based tools are not available for planning dsRNA constructs that limit off-target effects, as for *Drosophila*, *Caenorhabditis* and mouse (Arziman *et al*., 2005). The production and analysis of further *B. mori* lines, transgenic for constructs in which the *IR-per* involves a different *per* sequence, would be useful in this respect. Nevertheless, it would be an extreme coincidence if the results that we have obtained with *B. mori*, which so nicely parallel those for *D. melanogaster*, were due to such non *per*-specific targeting of the dsRNAi construct.

In *Drosophila*, *per* is expressed in all developmental stages and the translocation of PER protein from cytoplasm to nucleus is fundamental for the ticking of the endogenous clock (Hall, 2003). In *B. mori*, the *per* gene is actively transcribed in larva, adult, testes and ovaries (Iwai *et al*., 2006). Here, we have demonstrated *per* mRNA expression during embryogenesis. PER cellular localization has been investigated in the *B. mori* adult brain and PER-like signals have been detected in the cytoplasm of different neuronal populations of dorsolateral protocerebrum, which represent the conserved site of the circadian clock for many insects (Sehadova *et al*., 2004; Zavodska *et al*., 2003). In addition, PER staining was also observed in some neurons of the *pars intercerebralis* and in nerve fibre arborizations in the corpora cardiaca and corpora allata, organs involved in moulting and metamorphosis (Sehadova *et al*., 2004). In *A. pernyi*, the dorsolateral PER-expressing neurons of each hemisphere are very close to, and probably communicate with, two neurons expressing the prothoracicotropic hormone (PTTH), a fundamental neuropeptide regulating post-embryonic development in Lepidoptera (Sauman & Reppert, 1996). After release from axonal projections in the corpora allata, PTTH induces the production and secretion of ecdysone in the prothoracic gland. Ecdysone, together with juvenile hormone (JH) of the corpora allata, regulates moulting phases and metamorphosis (Bollenbacher & Granger, 1985; Mizoguchi & Kataoka, 2005). PTTH-expressing neurons with position and projection patterns similar to those of *A. pernyi* have been identified in *B. mori* fifth instar larval brains (Kawakami *et al*., 1990; Dai *et al*., 1995). These observations in *B. mori* suggest possible pathways of connections between PER- and PTTH-expressing neurons that may be important in the control of PTTH release during development. In

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addition, the PER-positive arborizations to the corpora allata may indicate a PER regulatory activity on JH levels. The effect of a general *per* KD on developmental timing might therefore be explained by a modification in the humoral equilibrium during development. Moreover, the diverse sensitivity of the different developmental stages to *per* mRNA depletion might be explained by hypothesizing a variable efficiency of *per* RNAi due to variations in the levels of *per-IR* insert transcription or in the RNAi activation during development. Alternatively, we might assume that the different developmental stages are characterized by different sensitivities to humoral modifications caused by *per* mRNA depletion.

Taken together, these results suggest that the clock gene *period* is involved in the control of similar developmental processes in different species. Recent studies also demonstrate a similar dual function of *mPer* in mammals, the genetic disruption of *mPer* (1 and 2) in mice causing a total loss of circadian rhythmicity, premature ageing, and an enhancement of a neoplastic phenotype (Fu *et al*., 2002; Chi Lee, 2006). At a practical level, the reduction in developmental time induced in *B. mori* by *per* KD does not compromise female egg productivity or the egg-laying temporal profile. In addition, it does not alter the cocoon silk percentage compared with that of the polyvoltine wild-type Nistari strain. Both female fertility and cocoon silk percentage are considered to represent productivity parameters in sericulture, so maintaining their integrity in these transgenic lines is rather encouraging. Perhaps transfer of the *per* dsRNAi construct into a monovoltine line (which has different hormonal regulation and longer developmental profile in comparison with a polyvoltinerian), may enhance the effect of *per* dsRNAi. Consequently *per* mRNA depletion via *per* KD transgenesis in *B. mori* may provide a viable commercial strategy to improve the efficiency of rearing, without affecting silk productivity in either mono- or polyvoltine strains. Finally, another application of *per* dsRNAi may be in the selective transfer of the above-described *per* construct onto the W sex chromosome. As the silkworm female sex karyotype is ZW, while that of the male is ZZ, the faster development of female larvae may result in an easier and earlier separation of the two sexes, which could reduce the laborious operation of pupal sexing in order to obtain crosses for silkworm egg production.

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