

## Molecular cloning and characterization of the clock gene *period2* in the testis of lizard *Podarcis sicula* and its expression during seasonal reproductive cycle

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### Abstract

Clock genes are known to oscillate with circadian rhythmicity in the central clock structure, the suprachiasmatic nucleus of the hypothalamus, and also in peripheral tissues. Reproduction is a peripheral activity that is strongly influenced by a circadian clock in many organisms. Most mammals that exhibit a seasonal cycle are able to decode the daily changes in light across the year and to translate these in hormonal signals that regulate reproductive cycles. Expression of many clock genes has been revealed in mouse testis, although transcription of these genes seems to be constitutive in 24 h, suggesting that these genes may play in the testis a different role with regard to the central clockwork function.

The seasonal breeding lizard *Podarcis sicula* represents an attractive model for studying some developmental and differentiation phenomena, such as gonadal maturation, since in the adult male the testis shows a spring full activity and a complete summer regression. Experimental data seem to suggest that in lizard the environmental factors, as photoperiod and temperature, affect the endogenous elements, although the interaction mechanisms are unknown.

It is known that temperature signals have a direct influence on clock processes such as transcription, translation, protein phosphorylation and degradation. In addition, most data show that the expression of circadian clock genes, such as *period2*, is affected by length of photoperiod. In this way, the core clockwork may also decode seasonal information.

Here we report the cloning, sequencing and bioinformatic analysis of *period2* gene, isolated from the testis of lizard *P. sicula*, and its expression both in the testis and in other tissues during the different phases of the seasonal cycle. RT-PCR assays enlighten the presence of transcript in testis, brain, heart, liver and kidney in all the phases analysed. Moreover, real time quantitative PCR assays detect a peak of *per2* testicular expression during gonadal regression.

Our preliminary results cannot clearly demonstrate the involvement of *per2* gene in seasonal reproductive cycle of male lizard *P. sicula*, but its presence in the testis may suggest a role of this gene during spermatogenesis. Besides, our work can provide numerous starting points to clarify the role of *per2* during seasonal reproductive cycle.

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**Keywords:** Comparative genomics; Warm-blooded species; Reptilians; Circadian rhythms; Quantitative expression

**Abbreviations:** *per2*, *period2*; SCN, suprachiasmatic nucleus; *ck1e*, casein kinase 1e; *cry1*, cryptochrome1; *cry2*, cryptochrome2; *per1*, *period1*; *per3*, *period3*; *per*, *period*; PAS, Per (*period* circadian protein)–Arnt (Ah receptor nuclear translocator protein)–Sim (single-minded protein); bHLH, basic-helix–loop–helix; LP, long photoperiod; SP, short photoperiod.

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## 1. Introduction

The circadian clock is a biological rhythm that persists under constant environmental conditions (light, dark, and temperature) with a period length of ~24 h (Reppert and Weaver, 2001). The circadian timing system consists of an input pathway, connecting the clock to the environment, the clock itself and output pathways. The site of the *master biological clock* in vertebrates has been localized to discrete neural anatomical structures within the nervous system. These structures are the suprachiasmatic nucleus (SCN) of hypothalamic region, the lateral eyes, and the pineal complex (Menaker and Tosini, 1996). Although these structures are present in all vertebrates, their contribution to the circadian system may vary considerably among classes and even within the same class. The SCN, for example, is the central circadian pacemaker in mammals (Weaver, 1998), while the pineal gland is a central component in the regulation of circadian rhythmicity of reptiles and other non-mammalian vertebrates (Underwood, 1990).

Molecular bases of circadian clock seem to be conserved during Metazoa evolution, although the role of single components differs between insects and vertebrates (Dunlap, 1999).

To date, eight core circadian genes have been identified. They are *casein kinase 1 $\epsilon$*  (*ck1 $\epsilon$* ); *cryptochrome1* (*cry1*) and *cryptochrome2* (*cry2*); *period1* (*per1*), *period2* (*per2*), and *period3* (*per3*); *clock* and *bm11*. The three *per* genes encode PER–ARNT–SIM (PAS) domain proteins that function in the nucleus but do not directly bind to DNA. The *clock* and *bm11* genes encode basic-helix–loop–helix (bHLH)–PAS transcription factors. The levels of mRNAs and proteins of these genes, except those of *clock* and *ck1 $\epsilon$* , oscillate robustly during 24-h circadian periods (Reppert and Weaver, 2001; Young and Kay, 2001).

Recently, correlations between expression of circadian genes and photoperiod were observed. The length of day, in fact, seems to influence circadian duration and intensity of expression of some circadian genes. In Syrian hamster, in experimental conditions [long photoperiod (LP) and short photoperiod (SP)], *per1*, *per2* and *per3* are expressed longer under LP than under SP in SCN, while in pars tuberalis the amplitude of *per1* peak is 3-fold higher under LP than under SP (Tournier et al., 2003). These experimental evidences seem to indicate that circadian expression pattern of clock genes is sensitive to seasonal photoperiod variations; in this way, the core clockwork may decode some seasonal information.

Many vertebrates use the annual change in day length to time such important behavioural and physiological events such as migration, moulting and reproduction. The adaptive significance of such photoperiodic responses is obvious: the animals can anticipate and prepare for adverse conditions and can confine reproduction to the time of year most conducive to the survival of the individual and its offspring.

Reproduction is a peripheral activity that is strongly influenced by a circadian clock in most organisms (Goldmann, 1999; Turek et al., 1984). Insights concerning the circadian control of reproduction come from studies with insects (Gvakharia et al., 2000; Giebultowicz and Joy, 1992). Recent data indicate that *Drosophila* mutants in circadian genes showed a decreased sperm

release and a reduced reproductive fitness (Beaver et al., 2002). In mammals, experiments concerning the influence of circadian rhythms on reproduction are not as complete as with insects. However, in hamster, short photoperiod causes decreased sperm production and testicular regression (Honrado et al., 1991).

Among vertebrates, the lizard *Podarcis sicula*, living in the neighborhood of Naples, shows a seasonal rhythm in reproductive function (Angelini and Botte, 1992). In particular, male genital apparatus is well developed in spring–half summer (April–half July) (*full activity*), when an intense spermatogenetic and endocrine activity was revealed (Angelini and Botte, 1992; Andò et al., 1992), while at the end of July, when reproduction is over, the testis shrinks (*summer regression*) and only Sertoli cells and spermatogonia are left in its regressed seminiferous tubules. In autumn, a recrudescence of spermatogonial mitosis causes production of sperm, which, however, is not released into the tubule lumen (*autumnal recrudescence*). Afterwards, a winter stasis follows, characterized by the block of spermatogenetic activity. Only in March, when the photoperiod lengthens and the temperature increases, does the spermatogenic activity undergo a renewal (Angelini and Botte, 1992).

The lizard testis is an attractive model for studying circadian clock expression since, as mentioned above, genital apparatus is well developed in spring when day length increases progressively, while at the end of July, as day length gradually decreases, the testis shrinks and only Sertoli cells and spermatogonia are left in its regressed seminiferous tubules (Angelini and Botte, 1992).

In order to identify the factors involved in seasonal maturation of testis in lizard *P. sicula*, we have investigated about some genes, which may be regulated by environmental factors and, for this reason, possibly involved in this seasonal phenomenon. Our attention has been addressed to *period2*, a crucial gene involved in molecular mechanism controlling circadian clock in most organisms (Shearman et al., 1997), whose expression is affected by length of photoperiod (Tournier et al., 2003; Nusslein-Hildesheim et al., 2000).

## 2. Materials and methods

### 2.1. Animals

Adult specimens of male lizards (*P. sicula*) were captured in the neighborhood of Naples (Italy) during the three principal phases of reproductive cycle, which correspond to May (phase of *full gonadal activity*), July (*testicular regression phase*) and December–January (*winter stasis*). The animals were maintained in terraria under natural photothermal conditions and fed with meal worms and fresh vegetables ad libitum. The lizards were killed under anaesthesia; testes, heart, liver, kidney and brain were aseptically excised and quickly frozen in liquid nitrogen for RNA extraction. The sacrifice was achieved always in the morning, between 10:00 a.m. and 12:00 a.m., to avoid possible gene expression variation due to a different circadian time. The experiments were performed under the approval of institutional committees (Department of Health); all efforts

were made to avoid animal suffering and to minimize the number of animals used.

## 2.2. RNA extraction and RNA reverse transcription

Total RNA was extracted from tissues following Chomczynski and Sacchi (1987). The yield and quality of RNA were assessed by the 260/280 nm optical density ratio and by electrophoresis under non-denaturing conditions on 1.8% agarose gel.

1.5 µg of total RNAs were retro-transcribed to obtain cDNA, using Superscript II First Strand Synthesis System for RT-PCR kit (Invitrogen), following manufacturer's instructions. 1 µl of cDNA preparation was used to perform RT-PCR assays.

## 2.3. Sequence analysis

Degenerated primers to amplify *period2* cDNA were projected using a protein alignment, obtained by CLUSTALW algorithm, using PER2 amino acidic sequence from different mammalian and non-mammalian species. The sequence of the oligonucleotides was deduced from a particularly conserved amino acidic region of PAS domain. In Table 1, amino acidic sequences, from which primer sequences were derived, primer sequences, and RT-PCR products name, size and amplification conditions were reported. To clone and sequence lizard *per2* coding region, a primer walking strategy was performed, carrying out RT-PCR assays with different types of primer: a non-degenerated primer, designed on the previously obtained nucleotidic sequence and a degenerated one deduced from the alignment of an amino acidic sequence. Purified fragments were cloned in pCRII vector, using TOPO-TA cloning kit (Invitrogen). After cloning, fragments were sequenced through an ABI PRISM 3100 sequencing system (Perkin Elmer), using M13 reverse and T7 universal primers. Sequencing results were analysed by the use of BLASTX algorithm.

## 2.4. Preparation of PolyA(+) RNA

Poly A+ mRNA from testis of lizards captured and sacrificed in May was isolated using Poly A Pure mRNA Isolation System (Ambion), which is based on the elution of polyA mRNA from oligo (dT) cellulose columns, according to the manufacturer's protocol. ~2 µg of polyA(+) mRNA were isolated from 12 µg of total RNA.

## 2.5. 5' RACE and 3' RACE

cDNA to perform 5' and 3' RACE was obtained using Marathon™ cDNA Amplification Kit (Clontech), from 1 µg of poly A+ RNA extracted from testis, following the supplier's protocol. cDNA was diluted 250-fold in Tricine-EDTA buffer, provided with the kit. Both 5' and 3' RACE were performed by nested PCR, using a Hybaid PCR Express cycler (Celbio).

5' RACE: reaction mix for the first amplification round was composed of 2.5 µl cDNA 1:250 dilution, 0.2 mM of each dNTP, 0.2 µM of both Adaptor primer 1 and Gene Specific Primer 1 (RACE-PER-R: 5'-ACTCCTGGGTGGTAGACT-CAACTCC-3'), 1× cDNA PCR reaction buffer, 1× Advantage 2 Polymerase Mix and H<sub>2</sub>O up to 25 µl. Amplification was performed by denaturing cDNA at 94 °C for 30 s, then 30 cycles of 30 s at 94 °C and 4 min at 68 °C were carried out. The amplification product of the first round was diluted 50-fold; 2.5 µl of this dilution was used for the second round, carried out in a reaction mix composed of 0.2 mM of each dNTP, 0.2 µM of both Adaptor primer 2 (provided with kit) and Gene Specific Primer 2 (RACE-PER-R2: 5'GGTGCCAGAACTCCACAA-ATTTGGC-3'), 1× cDNA PCR reaction buffer, 1× Advantage 2 Polymerase Mix and water up to 25 µl. The reaction was performed with a "touch down" amplification program: 30 s at 94 °C for 1 cycle, 5 s at 94 °C and 4 min at 72 °C for 5 cycles, 5 s at 94 °C and 4 min at 70 °C for 5 cycles, then, 5 s at 94 °C and 4 min at 68 °C for 25 cycles.

Table 1  
Primer sequence and PCR amplification conditions of *per2* transcript

Primer name	Amino acidic sequence	Nucleotidic sequence (5'→3')	Fragment name and size	PCR settings			
				Denaturation	Annealing	Extension	
L-per1°F	DMFAVA	GAYATGTTYGCAGTSGCAGT	PER1°F/R,	Time (s)	30	45	45
L-per1°R	FQDVDER	CGTTCRTCSACRTCYTGRAA	470 bp	Temperature (°C)	95	48	72
				35 cycles			
L-per2°F	–	AGTTGTTTGTTCAGGACGT	PER2°F/R,	Time (s)	30	45	45
L-per2°R	NEDVFAA	GCWGCRAASACRTCCTCRIT	320 bp	Temperature (°C)	95	48	72
				35 cycles			
L-per3°F	–	AACTGGTCCCTTGAACGAAG	PER3°/4°F/R,	Time (s)	30	45	45
L-per4°R	DKKPQPE	TCWGGYTGWGGYTTYTTRTC	819 bp	Temperature (°C)	95	50	72
				35 cycles			
L-per5°F	–	ACAGCAGCACCATAGTTCAT	PER5°/6°F/R,	Time (s)	30	45	45
L-per7°R2	MMTYQLP	GGVARYTGRTADGTATCAT	991 bp	Temperature (°C)	95	50	72 (1° round)
				Temperature (°C)	95	50	72 (2° round)
				30 cycles			
L-per6°bisR	PPLFQSR	CGRSWYTGRAAVARWGGWGG		Time (s)	30	45	45
L-per7°F2	–	AGCCTCACCTCCATGTGCC	PER7°F/R,	Temperature (°C)	95	48	72
L-per7°R	LMANTDD	RTCRCTDGTTRTTDGCATVA	538 bp	Temperature (°C)	95	48	72
				40 cycles			

3' RACE: reaction mix for first round contained 2.5  $\mu$ l cDNA dilution, 0.2 mM for each dNTP, 0.2  $\mu$ M of both Adaptor primer 1 and Gene Specific Primer 1 (RACE-PER-F: 5' GGCAGTGGAACAGGAAGTAGTGGAA-3'), 1 $\times$  cDNA PCR reaction buffer, 1 $\times$  Advantage 2 Polymerase Mix and H<sub>2</sub>O up to 25  $\mu$ l. cDNA was denatured at 94 °C for 30 s, then 30 cycles of 30 s at 94 °C and 4 min at 68 °C were carried out. The product of the first round was diluted 50-fold; 2.5  $\mu$ l of this dilution were used for the second round, carried out in a reaction mix with 0.2 mM of each dNTP, 0.2  $\mu$ M of both Adaptor primer 2 and Gene Specific Primer 2 (RACE-PER-F2: 5'CATCTGGCGGCTG-ATGGCAAACACA-3'), 1 $\times$  cDNA PCR reaction buffer, 1 $\times$  Advantage 2 Polymerase Mix and water up to 25  $\mu$ l. The reaction was performed with the same "touch down" amplification program used to perform the second round of 5' RACE.

Both 5' and 3' RACE products were extracted from 1.5% agarose gel and purified with Qiaquick gel extraction kit (Qiagen). Purified fragments were cloned in pCRII vector, using TOPO-TA cloning kit (Invitrogen). After cloning, fragments were sequenced through an ABI PRISM 3100 sequencing system, using M13 reverse and T7 universal primers. Sequencing output data were analysed using BLASTX algorithm.

## 2.6. Expression analysis of *per2* gene

RT-PCR assays were performed with cDNA extracted from the testes, brain, liver, kidney and heart of lizards captured in May, July and January. The reaction mixtures, containing 1  $\mu$ l of cDNA, 0.2 mM of each dNTP, 0.3  $\mu$ M of each primer (Percontr-F 5'-ATGTTTGCCGTAGCTGTTTC-3', Percontr-R 5'-CTTGGGGTGTGTGTAGTTGT-3'), 1 $\times$  Perkin Elmer buffer, 0.5 U of Perkin Elmer Amplytaq and H<sub>2</sub>O up to 10  $\mu$ l, were denatured at 95 °C for 1 cycle, then the mixtures were subjected to the following PCR conditions: 40 cycles of 95 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s, plus a final extension at 72 °C for 5 min. Amplifications were carried out using a Robocycler® Gradient 96 (Stratagene). The samples were loaded on 1.5% agarose gel.

## 2.7. Real time quantitative PCR

Total RNAs from lizard testis and brain, extracted in different phases of reproductive cycle (May, July and January), were retro-transcribed as previously indicated. Real time PCR was carried out with a DNA Engine Opticon 2 System (MJ Research, Boston), using SYBR Green to monitor dsDNA synthesis. The differences in *per2* expression were normalized with respect to  $\beta$ -actin expression. Primers were designed using oligo4 software. Each reaction was performed in a 20  $\mu$ l reaction volume containing 4  $\mu$ l of a 1:32 dilution of testis and brain cDNA preparation, 10  $\mu$ l of 2 $\times$  DyNAmo HS SYBR Green qPCR Kit (Finnzyme), and 0.25  $\mu$ M of each primer (*per2* amplification, 245 bp product: PER913F: 5'-GCTTCATCC-AGTGACAGCAA-3' and PER1157R: 5'-GCCAAACTCCTC-TGACAAGC-3';  $\beta$ -actin amplification, 264 bp product: ACTQF: 5'-CTCATGCCATCCTGCGACTT-3' and ACTQR: 5'-GGCAATAAACTTCCCAACAC-3'). For each period of

reproductive cycle (May, July and January), experiments were performed twice and in triplicates. The amplification protocol was as follows: 1 cycle of 15 min at 95 °C, 35 cycles of 95 °C for 20 s, 58 °C (annealing for *per2* gene primers) and 63 °C (annealing for  $\beta$ -actin gene primers) for 20 s, 72 °C for 20 s, plus an extension at 72 °C for 1 min. May sample was chosen as phase zero. The relative level of expression was calculated with the formula  $2^{-\Delta\Delta ct}$ .

## 2.8. Northern blot analysis

For Northern blot analysis, 30  $\mu$ g of total RNA, isolated from testes and brain of lizards sacrificed during the phase of full gonadal activity, was subjected to electrophoresis on 1.2% agarose-formaldehyde gel. Ribosomal component of RNA was used as molecular weight marker. Samples were transferred overnight onto nylon membrane (Hybond N+, Amersham) using 20 $\times$  SSC. After blotting, RNA was fixed on the filter by exposition on UV. Prehybridization was performed at 42 °C for 4 h in 50% deionized formamide. Hybridization was performed in the same solution, with a probe of *per2* cDNA, 744 bp long, designed on a PAS domain region (obtained by PCR, using the following primers: PERcontr-F 5'-ATGTTTGCCGTAGCT-GTTTC-3', PERsonda-R 5'-TTCGTTCAAGGGACCAGTTC-3') or with a probe of  $\beta$ -actin cDNA (obtained by PCR, using following primers: Act-1F 5'-ATGGAAAARATYTGCCAY-CA-3', Act-2R 5'-GCDGTRATTTCTTYTTCAT-3'). Probes were labelled with  $\alpha$ -<sup>32</sup>P-dCTP using Prime-a-Gene Labeling System (Promega). The probe specific activity was  $1 \times 10^9$  cpm/ $\mu$ g. Hybridization was achieved at 42 °C overnight. Filter was washed at 65 °C twice with 2 $\times$  SSC, 0.2% SDS and once with 0.1 $\times$  SSC, 0.2% SDS. Dried filter was exposed to X-ray film (Hyperfilm, Amersham) for 6 days (*per2* probe) or overnight ( $\beta$ -actin probe).

## 3. Results

### 3.1. Identification of *P. sicula per2* transcript

In order to identify *P. sicula per2* ortholog, RT-PCR experiments were performed using degenerated primers. These oligonucleotides were projected using an alignment of PER2 aminoacidic sequences, obtained by CLUSTALW program, by which protein PER2 sequences, available in Genbank databases, from *Gallus gallus*, *Coturnix japonica*, *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens* were compared. Amino acidic sequences used to design the couple of primers, corresponding to PAS domain region, show high conservation between various species. RT-PCR experiments were carried out using cDNA obtained from total RNA extracted from the testis of lizards captured in May, at a phase of full gonadal activity. The amplification fragment obtained was cloned and sequenced and the 470 bp sequence was analysed through BLASTX. The putative amino acidic sequence coding this portion of transcript showed homology with PER2 protein from various species. In fact, with respect to *G. gallus* and *C. japonica* protein, the identity was 83%, (positivity 89%);



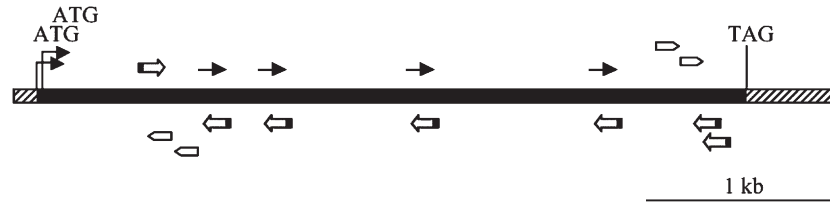


Fig. 1. Schematic diagram illustrating *per2* transcript isolated from lizard testis. Black box indicates the putative coding region while dashed boxes specify, respectively, 5'-UTR and 3'-UTR. Primers used to sequence the transcript are indicated (black arrows: non-degenerated primers; grey arrows: degenerated primers; and white arrows: RACEs primers). Two putative translation start sites and a putative stop codon are shown.

comparison with the protein of species phylogenetically more distant from lizard, such as *M. musculus*, showed an identity and a positivity, respectively, of 74% and 85%.

### 3.2. Cloning, sequencing and bioinformatic analysis of *P. sicula per2* full length transcript

To obtain the complete coding region sequence of *per2* from lizard testis, a primer walking strategy was used, taking advantage of the 470 bp sequence previously obtained. RT-PCR assays were performed using, in each experiment, a primer pair set in which an oligonucleotide was designed on the nucleotide sequence previously obtained and a degenerated oligonucleotide by using PER2 amino acidic sequence alignment from various species. This reiterated approach allowed the cloning and sequencing of the majority of *per2* gene coding region (Fig. 1).

### 3.3. Rapid amplification of cDNA ends (5'-RACE and 3'-RACE)

The sequence of the whole *per2* transcript has been obtained by carrying out 5'-RACE and 3'-RACE experiments, made using cDNA obtained from polyA(+) mRNA from *P. sicula* testis (Fig. 1).

5'-RACE experiment has provided five fragments, which were cloned, sequenced and analysed with BLASTX. Only the 770 bp fragment has shown homology with the 5' end of bird PER2 protein. The identity and the positivity with *G. gallus* PER2 were, respectively, 62% and 72%. Moreover, in this region, we have identified two AUG, which may be used in translation initiation, although no Kozak consensus sequence was found. However, this consensus sequence, generally, is not present in the mRNA of *per2* gene of other organisms.

3' RACE experiment has provided 5 fragments, four of them showing homology with the 3' end of *per2* cDNA. The smaller

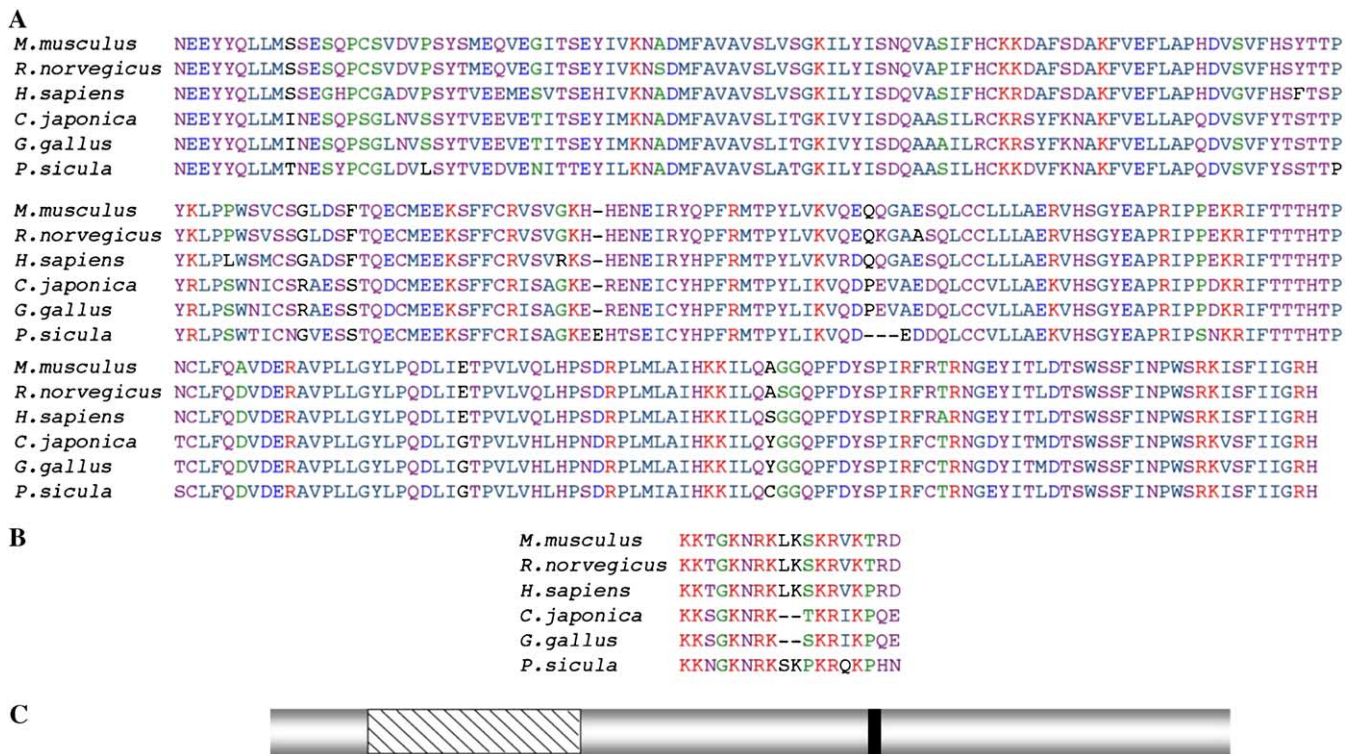


Fig. 2. Multiple alignment of the amino acidic sequence of PER2 PAS domain (A) and nuclear localization signal (B). Amino acids showing the same colour have similar chemical–physical property. [Hydrophobic and/or aromatic: GALIVMFYWP; small: GASTCPADN; polar: NQSTCYDEKR; positive (basic): KRH; and negative (acid): DE]. (C) Schematic representation of putative lizard PER2 protein. Dashed box indicates the presumed PAS domain; black box shows the putative nuclear localization signal.

one, 249 bp long, does not show a stop codon. The other fragments of 559, 658 and 671 bp, respectively, presented the same sequence and the same putative stop codon UAG; they showed, however, a different length in the putative untranslated region. The identity and the positivity of 671 bp long fragment with *C. japonica* PER2 were, respectively, 75% and 86%.

### 3.4. Sequence analysis

The putative complete sequence of *P. sicula per2* cDNA was 4285 bp long (GenBank Accession no. AJ874691). The presumed protein was composed of 1268 amino acids (by using the first ATG). The identity and the positivity with *C. japonica* PER2 was, respectively, 62% and 70%, while the identity and the positivity with *G. gallus* PER2 was 61% and 69%. These values are lower when the *P. sicula* protein is compared with mammalian PER2. The CLUSTALW alignment showed the presence of a putative PAS domain, [Fig. 2A (280 amino acids long, localized from amino acid 131 to 410)] and of a putative nuclear localization signal, [Fig. 2A (18 amino acids long, restricted from amino acid 790 to 807)]. Both regions are extremely conserved; in fact, PAS domain showed an identity of 85% with respect to *C. japonica* PER2, whereas the identity with mouse PER2 PAS domain was of 79%. The overall organization of putative *P. sicula* PER2 protein is depicted in Fig. 2B.

### 3.5. Expression analysis of *P. sicula per2*

In order to investigate if *per2* gene was expressed in other tissues besides the testis and to determine if the expression was restricted to a particular period of *P. sicula* reproductive cycle, RT-PCR assays were performed using cDNA obtained from total RNA, extracted from the kidney, liver, heart and brain of animals captured in May (full gonadal activity), in July (gonadal regression period), and in January (winter stasis). The experiments were achieved using a couple of primers amplifying a 440 bp fragment of PAS domain coding region. All tissues extracted from all periods and analysed showed PER2 expression (Fig. 3).

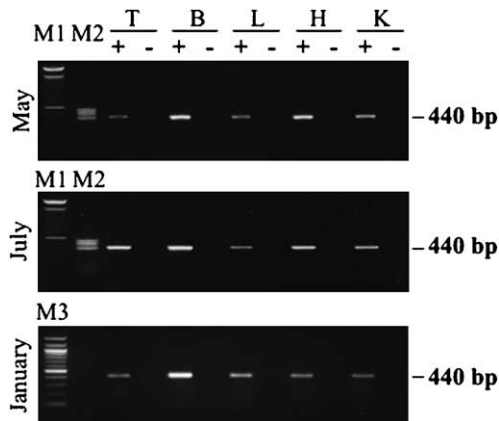


Fig. 3. Expression analysis of *per2* gene in *P. sicula* in different tissues during full gonadal activity (May), gonadal regression period (July) and winter stasis (January). T: testis; B: brain; L: liver; H: heart; and K: kidney. M1, M2 and M3: molecular weight markers. +: retrotranscribed RNA and -: no RT negative control.

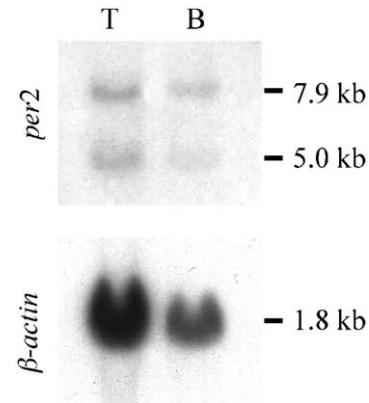


Fig. 4. Northern blot analysis of *per2* gene. Each lane contains 20  $\mu$ g of total RNA, extracted, respectively, from testis (T) and brain (B) during full gonadal activity. The blot was probed with a specific *per2* probe, then with  $\beta$ -actin probe (loading control). Exposure times were 6 days for *per2* probe and overnight for  $\beta$ -actin probe.

### 3.6. Northern blot analysis

To identify the whole length of *per2* gene transcript in testis and to compare it with the brain transcript, Northern blot analysis was performed using total RNA extracted from the

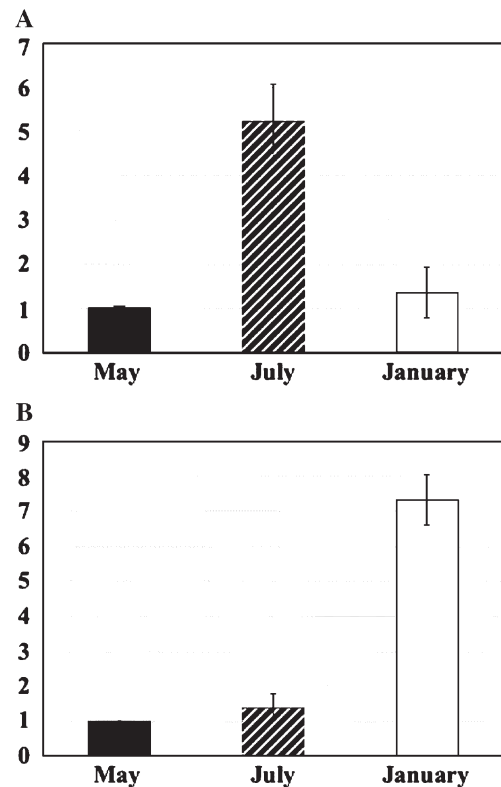


Fig. 5. Expression analysis of *per2* gene by real time quantitative PCR. The expression was assayed starting from RNA extracted from testis (panel A) or brain (panel B) of lizards sacrificed, respectively, during full gonadal activity (May), gonadal regression period (July) and winter stasis (January). *per2* expression levels were normalized with respect to the internal control  $\beta$ -actin. Full gonadal activity phase was chosen as reference expression. Each experiment was performed twice and in triplicate.

testis and brain of animals captured during the phase of full gonadal activity. In both tissues, the presence of two major transcripts was revealed, with approximate sizes of 7.9 and 5 kb (Fig. 4). Brain fainter expression is probably due to the reduced RNA amount loaded.

### 3.7. Real time quantitative PCR

To estimate the testicular expression of *P. sicula per2* in specific stages of reproductive cycle, real time quantitative PCR assays were performed using RNA from testis of lizards captured in May, July and January. In Fig. 5A, a quantitative variation in testicular expression of this gene is shown. In particular, expression level in May and in January is similar, while in July the expression is five folds higher.

To verify if a similar expression variation was evident also in the brain, which represents the principal location of circadian clockwork, we performed the same experiment using RNA extracted from this tissue. Surprisingly, in the brain, the greatest expression has been revealed during winter stasis, while in the phase of full gonadal activity and in the period of gonadal regression it seems to be 7 folds lower (Fig. 5B).

## 4. Discussion

It is clear that reproduction is strongly influenced by a circadian clock (Goldmann, 1999; Turek et al., 1984) and expression of clock genes, such as *mper1*, *mper2*, *bmal1*, *mcry1* and *clock*, was revealed in mouse testis. Transcription of these genes, however, seems to be constitutive in 24 h. The fact that there is no cycling of circadian genes in the mouse testis raises the question as to their function. Alvarez et al. (2003) speculate that these genes participate in spermatogenesis. Interestingly, they found CLOCK and mPER1 expression restricted to separate and specific developmental stages of spermatogenesis, with CLOCK protein localized in developing acrosome of round spermatids while mPER1 protein is expressed into the nucleus of type B spermatogonia and condensing spermatids (Alvarez et al., 2003). Moreover, recent data indicate that *Drosophila* mutants in circadian genes showed decreased sperm release and reduced reproductive fitness (Beaver et al., 2002).

To understand the role of PER2 in seasonal cycle of *P. sicula*, we characterized the lizard *per2* ortholog and analysed its expression both in the testis and in other tissues during the different phases of reproductive cycle.

The comparison of the transcript sequence and the putative protein of lizard testis *per2* with the transcript and protein of other vertebrates enlightens its conservation among vertebrates even if we ignore that the molecular partner of lizard PER2 is a member of CRYPTOCHROME family, as found in the SCN of other vertebrates. The presence of PAS domain and nuclear localization signal indicate that putative PER2 protein can form a dimer and its function can be carried out in the nucleus.

Accordingly, *per2* gene expression was revealed also in the other tissues of the lizard. RT-PCR assays have enlightened the presence of transcript in the testis, brain, heart, liver and kidney.

Moreover, its expression was found during the principal phases of reproductive cycle: full gonadal activity, gonadal regression and winter stasis. Real time quantitative PCR assays, achieved to identify any eventual expression variation in testis and brain during the different phases of reproductive cycle, have detected a peak of *per2* testicular expression during gonadal regression (5 folds higher), while it was comparable during full gonadal activity and winter stasis. Instead, in the brain, a peak of expression (7 folds higher) was enlightened during winter stasis, while during full gonadal activity and testicular regression, *per2* expression was comparable. These data indicate that *per2* expression is changeable among the different phases of reproductive cycle both in the testis and in the brain.

Daily environmental temperature changes as light or photoperiod are characteristic Zeitgeber signals of the circadian clock of many organisms that can directly affect the clock mechanisms by accelerating or slowing component processes involved in transcription, translation as well as post-translation modifications of protein (Rensing and Ruoff, 2002; Shultz and Kay, 2003).

Most data have shown that *per2* expression in suprachiasmatic nuclei is affected by the length of photoperiod (Tournier et al., 2003; Nuesslein-Hildesheim et al., 2000). In addition, alteration of *per2* expression pattern in the brain of lizard at low temperature was recently published (Magnone et al., 2005), indicating that the activity of the molecular feedback loop in the central clock structure is modified by temperature.

Starting in this issue, we have hypothesized that *per2* gene may decode environmental signal, playing a role during reproductive cycle of male lizard *P. sicula*; in this way, a variation of circadian signal may be converted into a circannual signal. However, since the observed variations are not synchronous between two tissues, we suggest that *Per2* gene might play a testicular role different from the brain function.

We aimed at demonstrating that *per2* was involved in seasonal reproductive cycle of male lizard *P. sicula*. Our preliminary results cannot clearly demonstrate its involvement in this phenomenon, but we have showed that expression of *per2* gene is changeable both in the testis and in the brain during the different phases of reproductive cycle. Moreover, its presence in the testis may suggest a role of this gene during spermatogenesis. Our work can provide numerous starting points to clarify the role of *per2* during seasonal reproductive cycle.

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