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# Hypothermia modulates circadian clock gene expression in lizard peripheral tissues

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Submitted 31 May 2006; accepted in final form 22 June 2006

**Vallone, Daniela, Elena Frigato, Cristiano Vernesi, Augusto Foà, Nicholas S. Foulkes, and Cristiano Bertolucci.** Hypothermia modulates circadian clock gene expression in lizard peripheral tissues.*Am J Physiol Regul Integr Comp Physiol* 292: R160–R166, 2007. First published June 29, 2006; doi:10.1152/ajpregu.00370.2006.—The molecular mechanisms whereby the circadian clock responds to temperature changes are poorly understood. The ruin lizard *Podarcis sicula* has historically proven to be a valuable vertebrate model for exploring the influence of temperature on circadian physiology. It is an ectotherm that naturally experiences an impressive range of temperatures during the course of the year. However, no tools have been available to dissect the molecular basis of the clock in this organism. Here, we report the cloning of three lizard clock gene homologs (*Period2*, *Cryptochrome1,* and *Clock*) that have a close phylogenetic relationship with avian clock genes. These genes are expressed in many tissues and show a rhythmic expression profile at 29°C in light-dark and constant darkness lighting conditions, with phases comparable to their mammalian and avian counterparts. Interestingly, we show that at low temperatures (6°C), cycling clock gene expression is attenuated in peripheral clocks with a characteristic increase in basal expression levels. We speculate that this represents a conserved vertebrate clock gene response to low temperatures. Furthermore, these results bring new insight into the issue of whether circadian clock function is compatible with hypothermia.

ectotherms; cryptochrome; period; vertebrate

THE CIRCADIAN CLOCK IS A HIGHLY conserved feature of plants and animals. By generating circadian rhythms in many aspects of physiology, it enables organisms to anticipate and thereby make appropriate adaptations to day-night changes in their environment (6). The circadian clock is synchronized ("entrained") with the daily environmental cycle primarily by signals ("zeitgebers") such as light and temperature (8, 32).

It had long been thought that the circadian clock was the function of a limited number of specialized pacemaker structures. These central pacemakers, such as the suprachiasmatic nucleus (SCN) of the hypothalamus, the retina, and in lower vertebrates, the pineal gland were considered to be responsible for generating all circadian rhythms within the organism (21, 36, 40). However, evidence has accumulated over the past few years, using molecular tools to monitor clock function, that suggests the existence of autonomous peripheral clocks in most cells and tissues (29, 42, 43). It appears that the vertebrate circadian timing system is composed of a set of independent pacemakers in addition to central pacemakers such as that located in the SCN (15). The function and regulation of these peripheral pacemakers have now become an important additional subject of investigation.

The molecular core components involved in central and peripheral oscillators are largely conserved from arthropods to mammals. Many of the clock component molecules characterized to date are transcription factors that function within transcriptional autoregulatory feedback loops, composed of positive and negative elements (25). For instance, in mammals, basic helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) domain transcription factors CLOCK and BMAL1 act as positive regulators, and three PERIOD proteins (PER1, PER2, and PER3) and two CRYPTOCHROME proteins (CRY1 and CRY2) operate as negative regulators (2). This molecular framework appears to be applicable to nonmammalian vertebrates. For instance, in the chicken pineal gland, mRNA levels of clock gene homologs (*cPer2*, *cClock*, *cBmal1*, and *cBmal2*) exhibit daily fluctuations and both cCLOCK:cBMAL1 and cCLOCK:cBMAL2 heterodimeric complexes up-regulate *cPer2* transcription, which is subject to down-regulation by cPER2 protein, suggesting that these chicken clock genes also constitute an autoregulatory feedback loop (30). Similarly, in most zebrafish tissues, rhythmic expression of clock gene homologs has been documented and the basic features of the autoregulatory feedback loop seem to be conserved (41).

Although light has received a considerable amount of attention as an environmental signal that entrains the circadian clock, in ectotherms, temperature also represents a potent zeitgeber (12, 37). Furthermore, the period length of the circadian cycle is adjusted to remain relatively constant over a range of temperatures in free running conditions (temperature compensation; 28, 31). In a recent study, we have documented how shallow temperature cycles and temperature steps influence clock gene expression and entrain the circadian clock in the zebrafish. Furthermore, the amplitude of cycling clock gene transcription is strongly influenced by temperature in this species, a mechanism possibly contributing to temperature compensation (22). Historically, the ruin lizard *Podarcis sicula* has been established as an ideal model to investigate the effects

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of temperature on the circadian clock in vertebrates for the following reasons: *1*) It is an ectotherm that naturally experiences an impressive range of temperatures during the course of the year (39); *2*) its circadian clock has been extensively studied at the behavioral, hormonal, and neural levels (13, 40); and *3*) low-amplitude temperature cycles have been shown to entrain behavioral circadian rhythms (12). However, a lack of molecular tools for studying the clock in this animal has excluded its use for investigating the molecular basis of the temperature response. In a recent study, we reported the sequence of a partial length cDNA for the *P. sicula* homolog of *Per2* (26). In the SCN, the site of the primary circadian pacemaker in the ruin lizard, *lPer2* is rhythmically expressed and its expression pattern is similar to that in the SCN of the house sparrow, quail, and mouse. Furthermore, we showed that rhythmic expression of *lPer2* in the SCN is strongly attenuated by exposure of the lizard to low temperatures (26).

Here, we report the cDNA coding sequences for *lCry1*, *lPer2,* and *lClock* in *P. sicula.* We document their tissue specific expression pattern, as well as performing an accurate phylogenetic analysis relative to other vertebrate homologs. We subsequently study the temporal expression pattern of these genes in the heart and eye of lizards maintained under light-dark (LD) cycles and constant darkness (DD) either at 29°C or 6°C. We show that exposure to low temperatures attenuates rhythmic expression of clock genes considerably, as well as raising their basal expression levels. Our findings provide valuable new insight into how the molecular mechanism of the clock responds to low temperatures.

## MATERIALS AND METHODS

*Animal collection.* Ruin lizards (*Podarcis sicula*) were collected from the area of Ferrara (Italy, longitude: 12°21′44′′ E, latitude 45°03′72′′ N). After capture, lizards were transported to the lab where they were exposed for a period of up to 1 wk to natural daylight (thus natural photoperiodic and light intensity conditions). Temperature conditions in the lab varied depending on the time of day and season, thus providing thermoperiodic conditions reflecting the natural environment. During this period, food (*Tenebrio molitor* larvae) and water were supplied ad libitum.

*Experimental design.* Lizards ($n = 128$) were transferred from the initial "natural daylight" conditions to environmental chambers for 2 wk at 29°C under a 12:12-h light-dark cycle, with light provided by full-spectrum cool fluorescent tubes (Osram, Germany) [lights on from zeitgeber time (zt) 0 to 12 with an intensity of 900 lux]. Subsequently, lizards were subdivided into two groups. These were maintained in an LD cycle at 29°C ($n = 64$) or at 6°C ($n = 64$). All lizards were transferred to 6°C at the same phase (zt6). After 1 wk, lizards ($n = 32$ for each set of experimental conditions) were killed from zt0 every 3 h for 24 h ($n = 4$ per time point). The remaining 64 lizards were kept in DD for 3 days and then killed from circadian time (ct) 0 every 3 h for 24 h ($n = 4$ per time point). In 12:12-h light-dark cycles, activity onset occurred predominantly around lights-on (zt0). During the first days after release from a 12:12-h light-dark cycle into DD conditions, locomotor activity rhythms persisted with a circadian free running period, and activity onset continued to occur around the phase of projected lights-on (ct0: subjective dawn) (4). Thus zts and cts mark the same phases and can be used to identify equivalent time points of sampling between the LD and DD tests. Visual inspection of the lizards at 6°C confirmed their state of immobility. Both in LD and DD at 29°C, food were supplied ad libitum, while no food was supplied during the exposure to 6°C (since immobilized lizards did not feed).

Lizards were killed rapidly by decapitation to minimize acute changes in gene expression. Dissected organs (brain, eye, lung, heart, liver, and skeletal muscle) were immediately frozen in dry ice. Certain lizards presented regenerated tails at capture, presumably the consequence of attacks by predators. From these lizards, the tails were also harvested. Enucleated eye samples included retina, cornea, sclera, lens, lens muscles, and iris. We did not dissect the retina from the eye to avoid any damage to the outer segments of the cones, a possible site of circadian oscillators. Dissections in dark conditions were performed under dim red light (<1 lux).

This research adhered to the American Physiological Society's Guiding Principles and to the guidelines established by the Italian Ministero della Salute and Max-Planck-Society for the care and use of laboratory animals.

*Cloning and sequencing.* Total RNA was isolated from ruin lizard organs and tissues using Trizol reagent (Invitrogen, Milano, Italy) according to the manufacturer's instructions. To initially obtain partial cDNA sequences, single-stranded cDNA was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen, Milano, Italy). Clock genes were amplified by PCR using Taq DNA Polymerase (Invitrogen, Milano, Italy) with primers designed by Primer3 software (35) on the basis of sequence of the *Gallus gallus* homologs (*Clock*, GenBank accession no. AF246959; *Cry1*, accession no. NM 204245). The primers used for clock gene amplification were (forward): 5′-GGTAATGCTCGGAAGATGGA-3′; (reverse): 5′-CCTTGAATGCTTCCCCCTCCG-3′ for *lClock* and (forward): 5′-GTCCGTCAGGGGCTCCGGCTCCC-3′; reverse: 5′-CACCACATTTTTCATCATGGTCATC-3′ for *lCry1*. For *lPer2*, PCR was performed with primers (forward: 5′-AATGCAGATATGTTTGCTGTAGC-3′; reverse: 5′-TGAAACTGGACCAGCTAGTATCC-3′) specific for the *P. sicula lPer2* gene (accession no. AY465113). PCR reactions were subjected to 35 of the following temperature cycles: 94°C for 30′′, T°C for 30′′, and 72°C for 45′′, where T°C = 49°C for *lPer2* and *lClock*, and 59°C for *lCry1*. Negative control reactions containing water or RNA instead of cDNA were included in the PCR reactions. Bands of the predicted sizes (309 bp for *lClock*, 550 bp for *lCry1,* and 682 bp for *lPer2*) were cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The ruin lizard clock gene cDNA fragments were sequenced and compared with the GenBank database by using the BLAST algorithm (3). Additional cDNA sequences were subsequently obtained using a 5′-3′ SMART RACE cDNA amplification kit (BD Bioscience-Clontech, Palo Alto, CA), and then coding sequences were deposited in GenBank (accession no. AY465113 for *lPer2*, DQ376040 for *lCry1,* and DQ376041 for *lClock*). Expression of the *β-actin* gene was assayed to serve as a control. Amplification of the partial nucleotide sequence of ruin lizard *β-actin* was performed using primers (forward: 5′-TGTGATGTTGATATCCGAAAGG-3′; reverse: 5′-TGATCCACATCTGCTGGAAA-3′), which were designed on the basis of the *G. gallus β-actin* sequence (accession no. NM205518). PCR reactions were subjected to 30 of the following temperature cycles: 94°C for 30′′, 49°C for 30′′, and 72°C for 45′′. The PCR product was then cloned and sequenced as described above. The *lβ-actin* sequence was deposited in GenBank (accession no. DQ015917). All sequencing reactions were performed with the ABI Prism "Big Dye" Terminator Cycle sequencing kit and were analyzed by the central sequencing service of the Max Planck Institute using ABI 3700 sequencing machines. Sequencing reactions were subjected to the following temperature profile 96°C/20 s, 50°C/10 s, 60°C/4 min for 30 cycles.

Nucleotide coding sequences were converted into amino acid sequences by ExPASy Proteomics Server (http://www.expasy.org/tools/dna.html), and protein domains were predicted using SMART (http://smart.embl-heidelberg.de).

*RNA expression.* mRNA expression levels for each of the clock genes in a range of different organs and tissues were measured using RT-PCR (SuperScript III One-Step RT-PCR System; Invitrogen, Milano, Italy) with 1 μg of total RNA extracts. To avoid temporal fluctuations in clock gene expression, RNA were extracted from

tissues collected at the same time point (zt6). PCR reactions were subjected to 30 of the following temperature cycles: 94°C for 30″, T°C for 30″ and 72°C for 1′ where T°C = 49°C for *lClock* and *lβ-actin*, 48°C for *lPer2* and 58°C for *lCry1*. Amplification reactions were analyzed by electrophoresis on 1.5% agarose gels and amplification of the *lβ-actin* transcript was used as a control.

RNase protection assays (RPA) for gene expression in the eye and heart under various lighting conditions and at 29°C or 6°C were performed using a standard protocol (42). For *lPer2*, an antisense probe of 395 nt covering the nucleotide sequence AY465113 between nt 1088 and nt 1476 was prepared. For *lCry1*, an antisense probe of 534 nt covering the nucleotide sequence DQ376040 between nt 567 and nt 1101 was prepared. A 280 bp *lβ-actin* fragment was generated by RT-PCR using zebrafish sequence primers for the *β-actin* gene (accession No. AF057040): forward: 5′ TATCCACGAGACCACCTTCAACTCCAT 3′, reverse: 5′ AACGATGGATGGGCCAGACTCATCGTA 3′. This fragment was subcloned into pGEMTeasy and then linearized with NcoI before synthesizing an antisense probe with SP6 RNA polymerase.

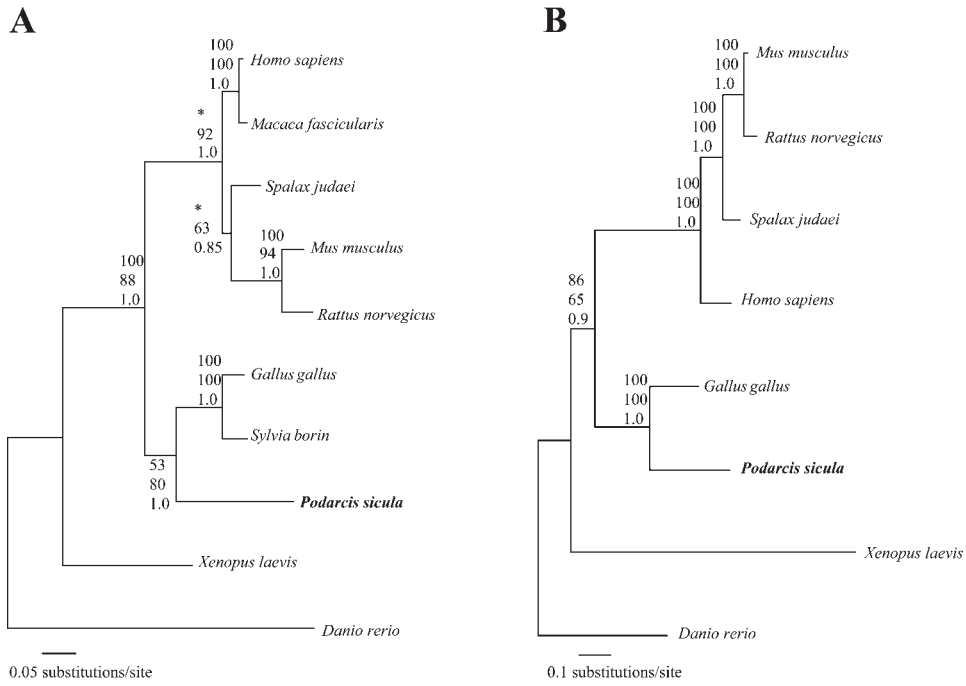
Each RNA sample used in the RPAs represented a pool prepared by mixing equal amounts of RNA extracts obtained from lizards treated under identical conditions (temperature, lighting regimes, and time of harvesting). All of the RPAs were performed at least 3 times. Autoradiographs were typically exposed between 1 and 3 days. Autoradiographs were quantified following scanning on an imaging densitometer (Bio-Rad, Hercules, CA) using Metamorph software (Molecular Devices, Sunnyvale, CA). All the statistical analysis was performed using GraphPad Prism software (version 4.0). One-way ANOVA followed by Bonferroni's test was used to compare single time points with the others in the same set of RPA samples and thus to assess the presence or absence of rhythmicity. Two-way ANOVA was used to measure the effect of temperature on the basal level of gene expression in lizards raised at 29°C vs. 6°C. The *P* value was fixed to $<0.05$.

*Phylogenetic analysis.* Sequences from the *lClock*, *lPer2,* and *lCry1* loci have been aligned with the homologs from eight different species (*Homo sapiens, Rattus norvegicus, Mus musculus, Spalax judaei, Gallus gallus, Podarcis sicula, Xenopus laevis,* and *Danio rerio,*) using Clustal X (38). An additional four (*Spalax carmeli, Spalax galili, Coturnix coturnix,* and *Tyto alba*) and two species (*Macaca fascicularis* and *Sylvia borin*) were added for the *lPer2* and *lCry1* analysis, respectively. Phylogenetic inferences were conducted with maximum parsimony (MP; Ref. 9), maximum likelihood, ML (10) and Bayesian inference (BI; Ref. 20) methods. The best-fit model of nucleotide substitution was selected using the Akaike Information Content (1) approach as implemented in Modeltest 3.6 (33). The general-time-reversible model (23), with a gamma-shaped distribution for among-site rate variation (alpha value equal to 1.21, 1.58, and 3.36 for *lClock*, *lPer2,* and *lCry1*, respectively), was chosen for each locus and employed in the ML and BI analyses. In the MP and ML analyses, we adopted the Tree Bisection Reconnection branch-swapping algorithm with 150 and 100 random addition-sequence replicates, respectively. Robustness of the phylogenetic trees generated by MP and ML was tested by using the nonparametric bootstrap (11) with 1,000 pseudoreplicates. The above analyses were performed with the software package PAUP* (version 4.0). The BI analyses were carried out with the program MrBayes (version 3.1) (19). The Monte Carlo Markov Chain length was 2,000,000 generations with a sampling frequency of 100 generations. The log-likelihood values for sampled trees were stabilized after almost 100,000 generations; we used the last 15,000 out of the 20,000 total trees to estimate Bayesian posterior probabilities (Bpp). From these trees, a 50% majority-rule consensus tree was constructed with PAUP*. Sequence from *D. rerio* was used as outgroup to root the trees.

## RESULTS

*Lizard clock genes.* Historically, the ruin lizard has proven a valuable model ectotherm to study the response of the vertebrate circadian timing system to changes in ambient temperature. However, to date, there are very few tools available for molecular analysis of the clock in this species. The cDNA sequences of vertebrate clock genes cloned from various species have revealed regions of high sequence conservation. We targeted these sequences with an RT-PCR-based approach to clone three different clock genes from the ruin lizard representative of the positive and negative limbs of the core transcription-translation feedback loop. We obtained cDNA sequences for *lCry1*, *lPer2,* and *lClock* homologues. The full-length *lCry1* cDNA-coding sequence consists of a 1,860-bp open reading frame (ORF) encoding a 619-amino acid sequence that contains pterin and FAD (flavin-adenine dinucleotide) binding domains. The deduced protein shows a high degree of similarity (>83%) with other vertebrate cryptochromes. In particular, the pterin and FAD domains are extremely conserved and showed >85% similarity with respect to the mammalian and avian counterparts. The *lPer2* cDNA contains a 2,157-bp ORF encoding a 719 amino acid protein, while *lClock* cDNA contains a 1,599-bp ORF encoding a 533-amino acid protein. Like all other lPER2 and lCLOCK orthologs, they contain PAS domains (255 and 270 amino acids long, respectively), including PAS-A, PAS-B, and PAC motifs. Furthermore, lPER2 has a nuclear export signal (NES; spanning between amino acids 436 and 445) and a casein kinase I binding site (CKI; between amino acids 657 and 670). The N-terminal region of lCLOCK contains a bHLH domain (amino acids 40 to 90). Comparison of the predicted amino acid sequence of the lCLOCK protein with other vertebrate orthologs indicated a high degree of sequence similarity (>76%). lPER2 has the highest sequence similarity with a house sparrow (86%) homolog, whereas the similarity with mammalian orthologs is 58–59%. As expected, the highest percentages of similarity are present in conserved domains and motifs (lPER2: PAS > 79%, NES > 60%, CKI > 95%; lCLOCK: bHLH > 95%, PAS > 86%). Ortholog clock gene protein sequence alignments are shown in *Supplemental Fig. 1*.

*Phylogenetic analysis.* To determine the phylogenetic relationship between the ruin lizard clock genes and other vertebrate orthologs, we analyzed each gene separately with three methods, namely MP, ML, and BI. The alignment of *lClock* resulted in a sequence of 1,578 bp in length, (A, 32%; C, 20.6%; G, 21.2%; and T, 26.2%) with 696 polymorphic sites (495 transitions, 349 transversions, and 28 indels) and 407 parsimony-informative sites. The *lPer2* alignment gave rise to a sequence of 2,501 bp (A, 27.6%; C, 25.8%; G, 25.2%; and T, 21.4%) with 1,798 polymorphic sites (1,024 transitions, 815 transversions, and 544 indels) and 1,016 parsimony-informative sites. Finally, the alignment of *lCry1* produced a sequence of 1,797 bp (A, 26.7%; C, 22.7%; G, 25.4%; and T, 25.2%), with a total of 830 polymorphic sites (545 transitions, 365 transversions, and 132 indels) and 570 parsimony-informative sites. The three distinct methods used for phylogenetic inference gave substantially identical results, so that only the ML trees are presented in Fig. 1. A general pattern clearly emerged from the three distinct trees: *Podarcis sicula* is always the sister group of the bird species being compared for each locus. This result is highly supported by bootstrap and Bayesian posterior probability values, higher than 80% and 0.95, respec-



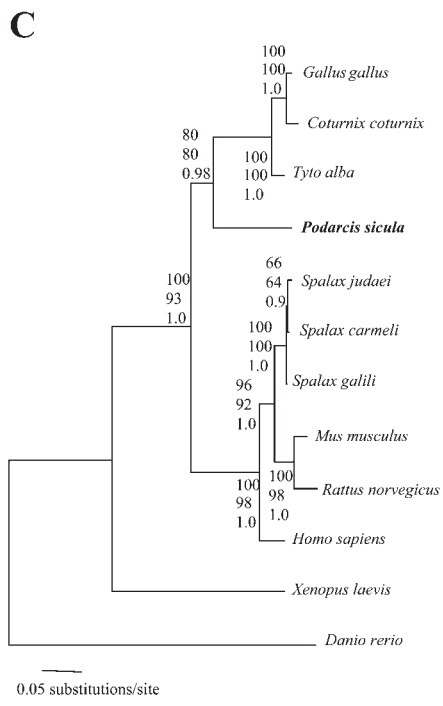




Fig. 1. Maximum likelihood (ML) tree of the *lCry1* (*A*), *lPer2* (*B*), and *lClock* (*C*) sequences. On each node are reported (from the top to the bottom) maximum parsimony (MP) and ML bootstrap values (based on 1,000 pseudo-replicates) and Bayesian posterior probabilities values, respectively. * in *A* denotes that in this position, there is a discrepancy between MP and the other two methods. In fact, MP posits *Spalax judaei* as a sister group of the *Homo sapiens/Macaca fascicularis* cluster.

tively, the only exception being the MP bootstrap values for *lCry1*.

The expected relationships among the Tetrapoda taxa have been confirmed by the inferences based on the three genes analyzed here. Considering that bootstrap and Bayesian posterior probability values are usually higher than 70% and 0.95 at the major nodes, we can conclude that the phylogenetic trees presented here are strongly supported (16, 19).

*Clock oscillations in peripheral tissues: effects of light and temperature.* We next tested the expression profile of these lizard clock genes by RT-PCR in a panel of seven organs and tissues (brain, eye, lung, heart, liver, skeletal muscle, and regenerated tail collected at zt6). *lClock*, *lCry1,* and *lPer2* were expressed in all organs and tissues tested with tissue-specific differences in absolute levels (Fig. 2).

Ruin lizards are ectotherms, and it has been clearly demonstrated that at low temperatures (6°C), the animals cease locomotor activity and become dormant (26). A previous study of *lPer2* expression in the SCN (26) would tend to indicate that the clock "stops" at lower temperatures. Does the expression pattern of the genes reported in this paper also support this notion? We analyzed clock gene expression in the eye and the

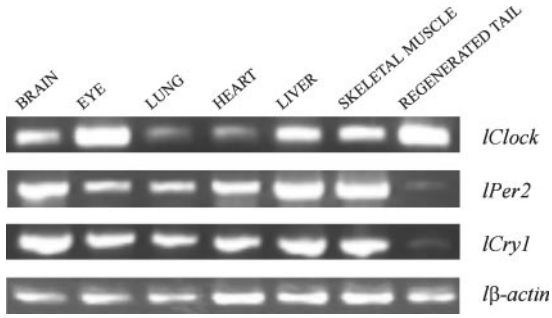



Fig. 2. RT-PCR analysis of lizard clock gene expression in a range of tissues and organs collected at zeitgeber time (zt)6. RT-PCR reactions were performed as described in MATERIALS AND METHODS. Ethidium bromide-stained gel pictures are representative of typical experimental results obtained in five independent experiments. Lizard clock genes were expressed in all organs and tissues tested.

heart, as previous studies have confirmed the presence of circadian oscillators in these organs in a wide range of vertebrates (5, 42, 43). We examined the expression of *lPer2* and *lCry1* at 29°C and 6°C under LD and also in DD conditions 3 days after transfer from a LD cycle, using RPAs. In the eye (Fig. 3), our results in LD at 29°C reveal a circadian rhythm of expression for *lPer2* (Fig. 3*A*) with a peak between zt3 and zt6 ($P < 0.001$, one-way ANOVA) and *lCry1* with a broader peak between zt6 and zt12 ($P < 0.001$) (Fig. 3*B*). The circadian rhythms of expression of *lPer2* and *lCry1* also persist in constant darkness DD (Fig. 3, *C* and *D*, respectively), with peaks shifting to ct12 ($P < 0.05$).

In contrast, at 6°C, a severe attenuation of cycling expression was observed in LD and DD for both genes in the lizard eye ($P > 0.05$). Remarkably, an elevated basal level of expression was observed for *lPer2* and *lCry1* at 6°C compared with 29°C, most notably in LD cycle conditions. Two-way ANOVA reveals that this temperature-dependent difference in basal level of expression is statistically significant for both genes under LD and DD conditions ($P < 0.0001$ and $P < 0.001$, respectively).

Comparable results were obtained for the expression of *lPer2* and *lCry1* in the heart (Fig. 4). At 29°C, *lPer2* expression is rhythmic ($P < 0.001$) in LD with a peak between zt3 and zt6 and rhythmic expression persisted in DD (Fig. 4, *A* and *C*) with a peak at zt9 ($P < 0.01$). Elevated expression ($P < 0.001$) but with no significant oscillation ($P > 0.05$) was detected at 6°C in both LD and DD for *lPer2* in the heart (Fig. 4, *A* and *C*). The expression of *lCry1* at 29°C oscillated ($P < 0.001$) in LD and DD conditions (Fig. 4, *B* and *D*) with a peak at zt3–zt6 and zt12, respectively ($P < 0.001$). Again, elevated expression ($P < 0.0001$) but no significant oscillation ($P > 0.05$) was detected at 6°C in LD or DD for *lCry1* in the heart.

These results are consistent with the notion that at 6°C, circadian clock function is severely attenuated both in central (26) and peripheral circadian oscillators.

## DISCUSSION

The aim of our investigation was to study the circadian clock of reptiles at the molecular level. For this purpose, we cloned the cDNA coding sequences of three clock genes, orthologs of vertebrate *Cry1, Per2,* and *Clock*, in the ruin lizard *Podarcis sicula*. Comparison of the predicted lizard clock protein sequences with other vertebrate orthologs revealed high levels of identity, particularly in functional domains, indicating conservative selective pressure. Our phylogenetic analysis of lizard *lClock*, *lCry1,* and l*Per2* genes was performed for the three genes separately. We adopted this approach to highlight the evolutionary relationships between each *P. sicula* clock gene and its orthologs in other vertebrate species. Not only did the three distinct methods of phylogenetic inference give identical results, but the statistical support was always very high, with bootstrap and Bayesian posterior probabilities values at the major nodes higher than 75% and 0.95, respectively. These trees clearly showed *P. sicula* to be a sister species of the bird

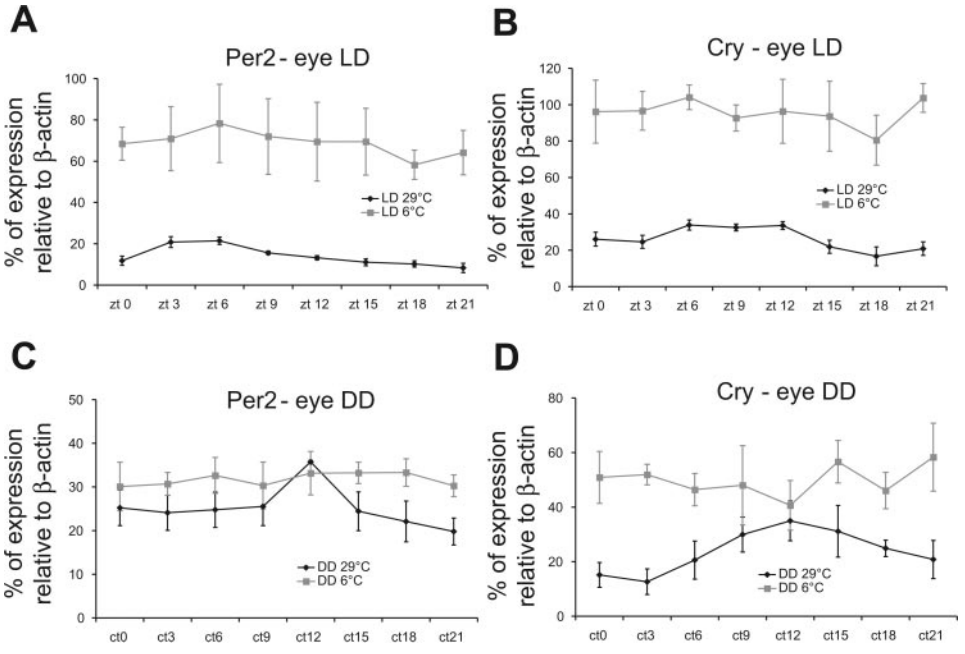



Fig. 3. The effects of ambient temperature and lighting regime upon l*Per2* and l*Cry1* expression in the lizard eye. RPA results for *lPer2* (*A*) and *lCry1* (*B*) mRNA expression in light-dark (LD) cycle conditions and *lPer2* (*C*) and *lCry1* (*D*) expression in constant darkness (DD) conditions. Quantified autoradiograph results were normalized for *lb-actin* expression and then expressed as percentage (%) of β-*actin* expression levels. On the *x*-axes are indicated the harvesting times (zeitgeber time and circadian time; zt and ct). Results obtained at 29°C and 6°C are plotted on the same axes (black line and diamonds, gray line and squares, respectively) to emphasize the increase in basal clock gene expression at lower temperatures. Consequently, the robust cycling of gene expression at 29°C is visually less evident.

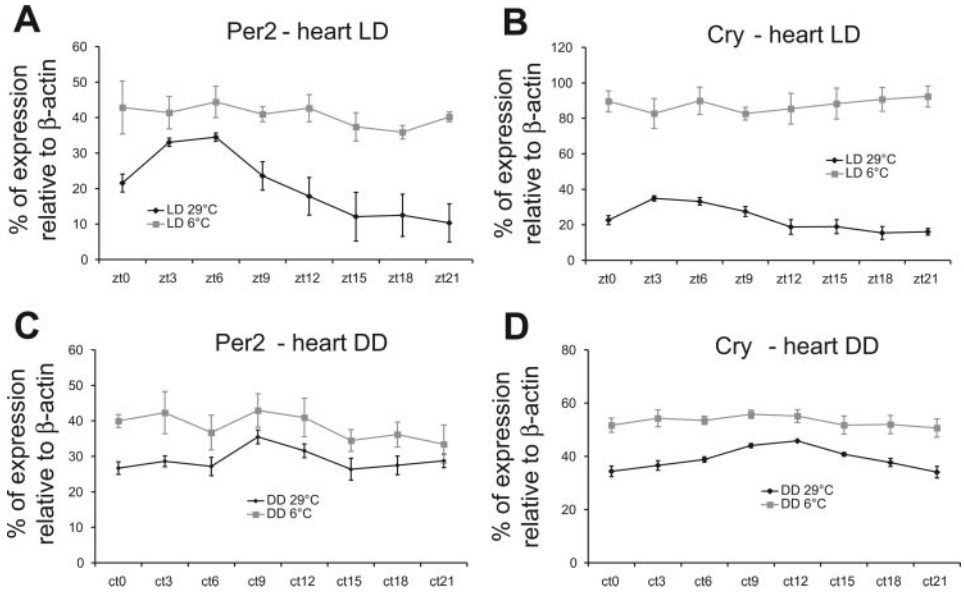



Fig. 4. The effects of ambient temperature and lighting regime upon *lPer2* and *lCry1* expression in the lizard heart. Comparable analysis to that shown in Fig. 3. RPA results for *lPer2* (*A*) and *lCry1* (*B*) mRNA expression in LD cycle conditions and *lPer2* (*C*) and *lCry1* (*D*) expression in DD conditions.

species being compared. Thus a strict evolutionary relationship between reptilian and avian clock gene sequences is evident that retraces classic phylogeny.

Consistent with observations in other vertebrates (5, 42, 43), each of the three lizard clock genes is expressed in all tissues and organs tested. This result confirmed a previous investigation showing the expression of *lPer2* in different tissues of ruin lizard (7). Interestingly, we also showed that clock genes are expressed in regenerated tails. Tail loss in lizards is followed by tail regeneration. The regrowing tail is composed of a cartilaginous tube, replacing the original vertebral column and containing a rudimentary spinal cord, muscle, and skin tissues. Given the expression of clock components in the regenerated tail, it is tempting to speculate on a possible role for the circadian clock in regulating the process of regeneration. A relationship between regrowth of the tail and the circadian timing system has been previously indicated in lizards in the case of the Gecko *Hemidactylus flaviviridis.* The pineal gland appears to mediate the photoperiodic stimulation of the regeneration process in this species (34). Notably, in mammals, liver regeneration is also influenced by the circadian timing system, since it restricts entry of hepatocytes into mitosis to occur only at certain times of the day (27).

Our main goals were *1*) to verify the existence of circadian oscillators in reptile peripheral organs and tissues, as previously demonstrated in zebrafish, birds, and mammals (5, 42, 43) and *2*) to study the effects of temperature, a strong environmental timing cue for ectotherms, on circadian clock gene expression in the peripheral oscillators. Our results demonstrated that the clock genes, *lCry1* and *lPer2*, are rhythmically expressed both in LD and DD in the eye and heart of lizards. A preliminary, low-resolution analysis has also revealed comparable rhythmic clock gene expression in other tissues (data not shown). These data suggest the widespread presence of peripheral circadian oscillators in this species, as appears to be the case in other vertebrates. The temporal expression pattern of both genes in the eye and the heart showed a circadian rhythm with phases comparable to their mammalian and avian counterparts (5, 43).

The ruin lizard *P. sicula*, our animal model, is a heliotherm, a precise thermoregulator and an actively foraging diurnal lizard (14, 39). In northern and central Italy, ruin lizards are active in the field all year round, with the exception of the winter period between December and February, during which lizards mainly retreat into underground burrows. An average ambient temperature of 6°C, typical for a northern-central Italian winter season, induces hypothermia in lizards (14, 39). Historically, the ruin lizard was one of the first animal models used to study the effects of temperature on the circadian timing system. In two pioneering studies, Hoffmann (17, 18) demonstrated the temperature compensation and entrainment by low-amplitude temperature cycles of circadian behavioral rhythms in ruin lizards.

Here, we show that hypothermia conditions have a profound effect on clock gene expression both in the eye and heart. Specifically, both in LD and in DD conditions, exposure of lizards to 6°C for 1 wk resulted in an increased basal level of clock gene expression with a coincident loss of rhythmicity. These results appear to indicate that circadian oscillator function in lizard tissues and organs is severely attenuated when the animals are exposed to low temperatures. Moreover, our previous investigations in *P. sicula* have shown that *lPer2* expression in the SCN, which is rhythmic at 29°C, becomes constantly elevated at low temperatures (26). The absence of a transcript oscillation does not automatically exclude the possibility that protein levels or even posttranslational protein modifications might continue to oscillate. Alternatively, desynchronization of single cell oscillators could be another possible explanation for the absence of overt transcript oscillations in the whole organ at the low temperatures.

A similar effect of temperature on clock gene expression in peripheral oscillators has been found in another ectotherm, the zebrafish (22). In this species, an ambient temperature decrease resulted in an increase in basal expression of the *Per4* gene and a shallower oscillation. Furthermore, the amplitude of rhythmic transcription directed by E-box enhancer elements is significantly reduced at lower temperatures. These results were obtained in larvae, as well as cell lines, indicating that this represents a cell

autonomous response. Taken together, the zebrafish and lizard results suggest that we have revealed a general vertebrate clock gene expression response to temperature. Interestingly, in *Neurospora*, circadian clock function is also restricted to a permissive range of temperatures, a property that has been linked with translational control of the FREQUENCY protein (24).

Now in possession of tools to study the molecular basis of the lizard circadian clock, one can contemplate exploring more aspects of the influence of temperature on lizard circadian physiology. For example, it could be interesting to investigate how 24 h temperature cycles are able to induce bimodal activity rhythms more effectively during the summer than in the winter time (12). Bimodality appears to represent a key seasonal survival strategy for lizards to avoid overheating around midday during the summer.

## GRANTS

N. S. Foulkes and D. Vallone were supported by funds from the Max Planck Society (Germany) and the Centre National de la Recherche Scientifique (France). C. Bertolucci, A. Foá and E. Frigato received funding from the University of Ferrara, Italy.

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