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Circadian expression of the clock gene *Per2* is altered in the ruin lizard (*Podarcis sicula*) when temperature changes

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Abstract

When exposed to the cold, the body temperature of the ruin lizard (*Podarcis sicula*), an ectothermic vertebrate, comes into equilibrium with that low environmental temperature. During this time the behavioral output of the circadian clock, locomotor activity, disappears. We tested the activity of the circadian clockwork at low temperature (6°C) by following the expression of one of its essential components, the *Period2* (*Per2*) gene. Here we show that lizard *Per2* (*lPer2*) expression, which is rhythmic and paralleling the behavioural rhythm of locomotor activity at higher temperature (29°C), becomes constantly high at low temperature. When lizards are re-exposed to high temperature, rhythmic *lPer2* expression is re-established after two days of adaptation and coincides with onset of locomotor activity. The alteration of the *lPer2* expression pattern at low temperature indicates that the activity of the molecular feedback loop is modified under these conditions.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: circadian clock, reptile, ectotherm, entrainment, SCN

1. Introduction

The mechanism that keeps track of time and therefore allows the organism to anticipate upcoming daily changes, is termed circadian clock. The circa 24 hour rhythm generated by this clock is driven by a set of 'clock' genes that produce interlocking autoregulatory feedback loops [1]. Circadian rhythms display a stable period length under constant conditions and under a broad range of temperatures, a property called "temperature compensation" [2]. Only in a few ectotherms and mammals have some circadian output rhythms been shown to dampen or disappear during hypothermia and hibernation [3-5]. However, in all of these cases it is difficult to decide whether the dampening of the rhythm is due to an arrest of activity in the central clockwork or to a general slow-down in metabolic rate linked to hypothermia.

To answer the question whether low temperature affects the circadian clockwork, we studied the expression of an essential 'clock' gene, *Period2 (Per2)*, in the central pacemaker of the ruin lizard (*Podarcis sicula*) during hypothermia. The ruin lizard lacks the internal ability to control body temperature [6] and can be forced into hypothermia at any time by lowering the external temperature below 10 °C. We have previously shown that the master circadian pacemaker in ruin lizards is located in the suprachiasmatic nuclei (SCN) of the hypothalamus [7]. Because of their small body size and ectothermy, the lizard SCN can be considered a model for studying the direct effects of temperature on the circadian clock. Moreover, in ruin lizards one of the main outputs of the circadian clock, the rhythm of locomotor activity, disappears at low temperature. Therefore, the

molecular mechanism of the circadian clock in the SCN can be explored separately from behavioral activity.

We have cloned a partial cDNA of the lizard *Per2* gene (*lPer2*) and performed in situ hybridization experiments under various temperature conditions. Our results indicate that at low temperature, when there is no locomotor activity feedback on the clock, expression of lizard *Per2* is high with no significant circadian amplitude in the SCN.

2. Materials and methods

lPer2 cDNA cloning

RT-PCR was performed with primers specific for the quail *Per2* gene [8]; forward primer: 5'-AATGCAGATATGTTTGCTGTTGC-3', reverse primer: 5'-TGAAACTGGACCAGCTAGTGTCC-3'. The resulting PCR product of 682 nucleotides in length was cloned into the pCRII-TOPO vector using the TOPO TA cloning system (Invitrogen). The partial *Podarcis sicula Per2* cDNA was sequenced and deposited in GenBank (Accession number AY465113).

Animals in LD and DD at 29 °C and at 6 °C

Lizards (N=72) were housed in an environmental chamber at 29 °C under a 12:12 LD cycle and then subdivided into two groups. One group (N=36) was maintained at 29 °C in LD, the other (N=36) was moved to 6 °C in LD at ZT12 and kept at low temperature for 4 days. Animals (N=16 for each condition) were sacrificed by decapitation at 4 different time points (N=4 per time point). The remaining forty lizards were kept in DD at 29 °C (N=20) and at 6 °C (N=20) for two days and then decapitated at 4 circadian times (N=5 per time point).

Animals in DD during and after the transitions from 6 °C to 29 °C

Each lizard (N=48) was put into an individual tilt-cage (30X15X11 cm) for locomotor recording [7]. Tilt-cages were placed inside environmental chambers and were connected to a computer-based data acquisition system (DataQuest III, Minimitter, Sunriver, OR)

for monitoring locomotor activity. Lizards were allowed to entrain to a LD 12:12 cycle for 14 days and then put in DD for two days. On the third day in DD, the temperature was lowered to 6 °C in the middle of the subjective day, that is at circadian time (CT) 6. On the fourth day at 6 °C, lizards were re-exposed to 29 °C in the middle of the projected subjective night (CT18). The lizards were decapitated and their brains were collected at different time points (N=3 per time point) at the time of transition from 6 to 29 °C (transition day, CT 12, 17, 19, 22), and during the first, second and third day at 29 °C (CT6, 12, 18, 24). We tested four different behavioral protocols of "cooling-warming" (cooling at CT6/warming at CT6; cooling at CT6/warming at CT18; cooling at CT18/warming at CT6; cooling at CT18/warming at CT6) and we observed that when ruin lizards are exposed to high temperature at mid-day (CT6) after a period at low temperature, there is a phase advance in the onset of locomotor activity by about 4 hours, independently on the CT when they were cooled. In contrast, exposing lizards to 29 °C at mid night (CT18) evokes a phase delay of about 10 hours. We chose the behavioral protocol "cooling at CT6/warming at CT18" for our experiments for two reasons: first phase delays are larger than advances; second, sampling is easier in the phase delay paradigm because onset of activity can be more easily predicted from the previous high temperature period. "Cooling at CT6-warming at CT18" allowed a more precise sampling of the lizard brains. To minimize imprecision due to the individual variability we did not sacrifice the lizards at the predicted CT0 based on the actogram, but instead allowed onset of activity in order to determine CT0 for each individual lizard. Collected brains were processed for in situ hybridization and *lPer2* expression in the SCN was quantified as described below.

In situ hybridization and statistical analysis

Brains were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin and subsequently cut in coronal sections at 7 μm thickness. Sense and antisense riboprobes labelled with ^{35}S-rUTP were made from the *lPer2* fragment (Accession number AY465113) and hybridized to the sections as previously described [9]. After exposure of the sections to x-ray film, densitometric analysis was performed (Densitometer GS-700, program Molecular Analyst, Bio-Rad Inc.) to evaluate the intensity of the radioactive signal in the SCN. The differences in expression levels among time points (LD 12:12 - 29 °C; LD 12:12 -6 °C; DD - 29 °C; DD - 6 °C) were analyzed using a one-way ANOVA with a subsequent Bonferroni post-hoc test (GraphPad Prism4, GraphPad Software Inc., San Diego, CA). For comparison of temperatures (LD 12:12 at 29 °C vs 6 °C and DD at 29 °C vs 6 °C), a two-way ANOVA with a subsequent Bonferroni post-hoc test was applied. The maximal value of *Per2* expression at CT0/24 at 6°C was set to 100%.

3. Results

To understand the molecular clockwork in lizards we performed RT-PCR to clone a part of the lizard *Per2* gene (*lPer2*). The cDNA fragment of *lPer2* we obtained is part of the coding sequence for the PAS (Per-Arnt-Sim) domain found in the Per2 protein of other vertebrates. Amino acid sequence alignments of *lPer2* with those from other vertebrates gave the following percentages of identity: *lPer2* versus sparrow and quail *Per2* (AY007259 and AB029890) = 86 %, *lPer2* versus mouse *Per2* (AF036893) = 79 %, *lPer2* versus human *Per2* (AB002345) = 78 %. Most of the differences can be found in the sequence flanked by the PAS A and the PAS B motifs [10], where lPer2 is shorter by 2 amino acids compared with sparrow, quail, mouse and human Per2 proteins.

Next, we studied the expression of *lPer2* in the SCN. *lPer2* cycles in a circa 24 hour rhythm at 29 °C in LD 12:12 as well as in DD (Fig.1). Maximal expression can be seen at ZT6 and CT6/CT12, respectively (one-way ANOVA, Bonferroni post-hoc test: $p<0.05$ for LD and $p<0.01$ for DD). These results correlate with locomotor activity recordings (Fig.2A), which show a diurnal activity of ruin lizards in LD and during the subjective day in DD. To investigate the effect of low temperature on the clock in lizards we transferred animals from 29 °C to 6 °C. No apparent rhythmic expression of *lPer2* can be observed at 6 °C in both LD and DD conditions. Unexpectedly, *lPer2* expression is significantly higher at all time points at 6 °C compared to 29 °C (two way ANOVA, $P<0.0001$).

We then determined whether significant circadian expression of *lPer2* was re-established when lizards are re-exposed to 29 °C after a period at 6 °C in DD conditions. In the first

day at 29° C *lPer2* mRNA levels decrease, reaching a minimum at CT6 and then increase within the same day (Fig. 2B). The mean amounts of *lPer2* mRNA in the SCN during the transition day are higher than those of the first full day at 29 °C (one way ANOVA, Bonferroni post-hoc test: $0.001<p<0.05$ for the different points, asterisks not shown to avoid graphical confusion). Maximal levels of *lPer2* mRNA are reached at CT24 in the first day at 29 °C and maintained until CT6 of the second day, after which expression levels decrease again until CT24. Mean amounts of *lPer2* mRNA in the SCN during the first full day at 29 °C are not significantly different from those observed on the second day. However, the difference between CT6 and CT24 on the second day is statistically significant and resembles the difference found in lizards normally kept at 29 °C in DD (Fig.1B). On the third day at 29 °C, *lPer2* expression resumes its normal rhythmic profile again with maxima at CT6 and CT12.

4. Discussion

The cycling of *Per2* mRNA in the SCN of lizards supports the hypothesis that their SCN contain a molecular clockwork similar to that found in other species. The pattern of expression of lizard *Per2* (*lPer2*) at a temperature of 29°C is similar to that in the SCN of the house sparrow, quail and mouse [8, 10-12]. The comparison of homologies of the PAS domains of the *Per2* gene between the lizard, sparrow, quail and mouse reveals that the reptile sequence is closer to the bird than to the mammalian sequence as one would expect. Interestingly, however, the neuroanatomy of the lizard SCN resembles more the mouse SCN [13] than the bird SCN which seems to be made up of a medial and lateral part [14]. This lateral part of the bird SCN appears not to be present in the lizard. However, the similarity in the PAS domain between lizards and other vertebrates is high despite the fact that the ruin lizard is an ectotherm.

Ectothermy makes the lizard a good model for temperature studies. The aim of our work was to test the effects of low temperature on the circadian clockwork by looking at *lPer2* expression. Our results demonstrate that *lPer2* mRNA levels are constant over 24 hours at 6 °C compared to its cycling expression at 29° C (Fig. 1). The higher levels at 6 °C could be due to a slower degradation rate of the mRNA at lower temperatures. Alternatively, the efficiency of protein degradation or transport could be altered [15] thereby interrupting or attenuating the molecular feedback loop that regulates *lPer2* circadian expression. Hence, an extended cold exposure would bring down the levels of *Per2* mRNA or/and protein eventually, unless transcription continues at the same rate at low temperature as it does at high temperature. Keeping in mind that lizards do not maintain a constant body temperature, it is reasonable to assume that regulation of

mRNA, protein stability and localization are affected by low temperature. Interestingly, regulation of mRNA plays an important role in temperature acclimatization in *Drosophila*. Its circadian clock prepares itself to seasonal decreases in temperature through thermosensitive splicing of the 3' untranslated region of the mRNA from the *per* gene [16]. Whether the transcription rate is maintained in lizards at low compared to high temperatures is not known. The most obvious interpretation is that degradation of transcript is slowed or eliminated. This fits most data from previous studies [17].

lPer2 cycling requires two days of adaptation to regain its normal amplitude and robust rhythmicity becomes evident only from the "third day at 29 °C" (Fig. 2B). The gradual recovery of the rhythm of *lPer2* expression in the first two days at 29 °C after a period of time at low temperature may track the slow adaptation of the clock to the new external conditions and the return to the high-temperature-limit cycle. The return to the high-temperature limit cycle is marked by a pattern of *lPer2* expression coupled to the behavioral activity (Fig.2, "second day at 29 °C) with a peak of *lPer2* expression during the subjective day. Hence the molecular mechanism of the circadian clock is locked again to the behavioral rhythm after the arousal from hypothermia. Another interpretation of figure 2B is that in the cold, transcription is halted. Warming increases degradation of transcript, but there is not an immediate supply of new transcript to replace it. As a result, the relative level falls very low and only rises again through the first day. Taken together, the present behavioral and molecular results indicate that it takes the clock three days in the lizard to recover from hypothermia. The high mRNA levels of *Per2* at low temperature provide evidence (but not proof) that the clock might be stopped in the cold.

In the present work we measured molecular and behavioral responses of the circadian clock to low temperature in the lizard. We provide evidence that temperature influences *Per2* gene expression and that it takes three days for the clock to establish normal *lPer2* gene expression. It remains to be seen what consequences this has on the functional status of the clock. Further studies should be aimed at analyzing the expression levels of other clock components under different temperature paradigms to elucidate the influence of temperature on the molecular clockwork. This would lead to a better understanding of the relationship between the circadian clock and the annual rhythms of temperature changes.

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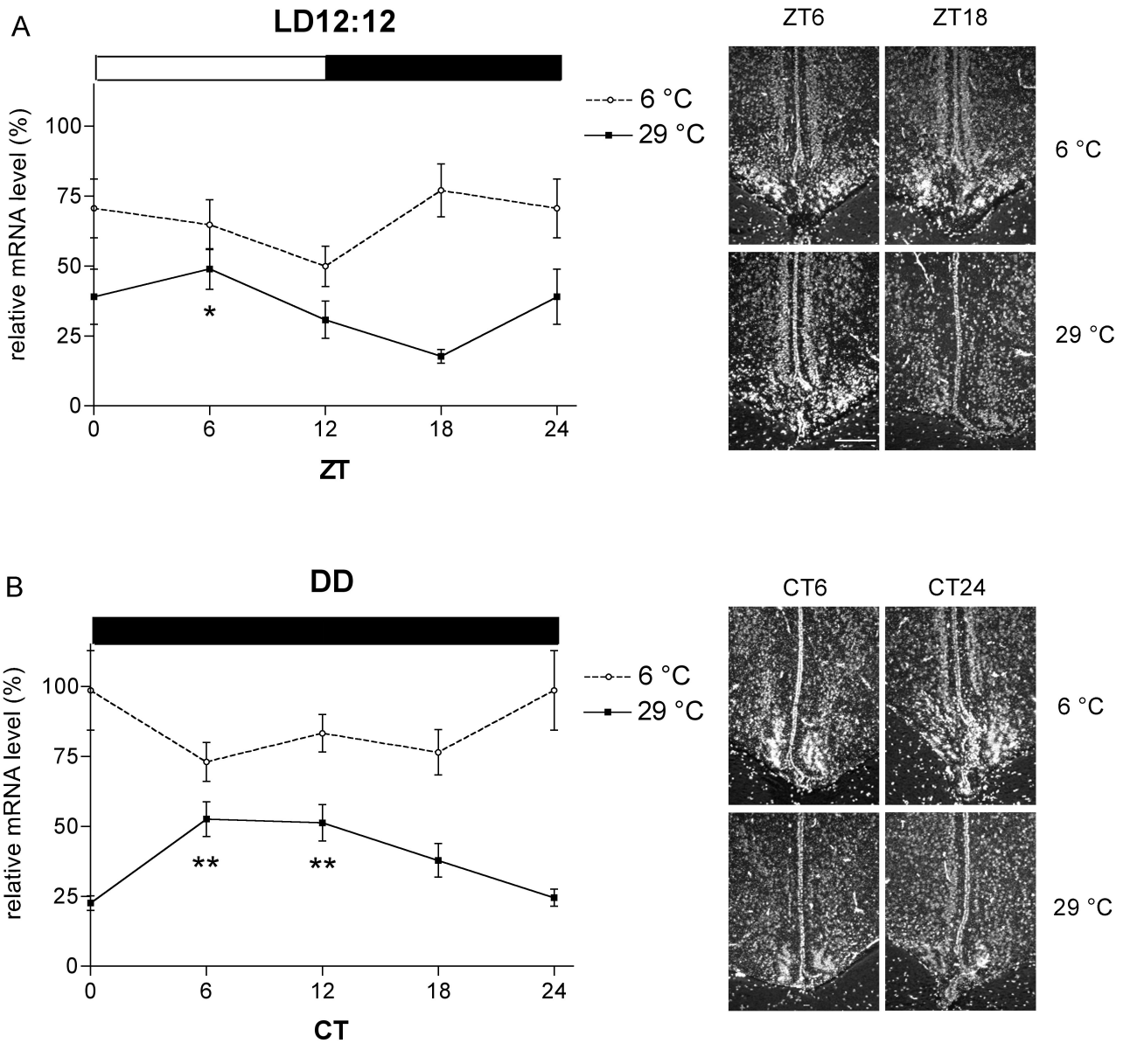
.

Figure legends

Fig. 1: Pattern of *lPer2* expression in LD 12: 12 (A) and in DD (B) at 29°C and at 6°C. (A) *lPer2* expression at ZT6-29°C is significantly higher than at ZT18-29°C (one way ANOVA, $p<0.05$, n=4 for each time point). The expression of *lPer2* is higher at 6°C than at 29°C (two-way ANOVA, $p<0.001$). The white and black bar on the top of the plot indicate the light and the dark phase of the LD cycle, respectively. The panels on the right depict representative microphotographs of *lPer2* expression in the SCN. (B) *lPer2* expression at CT6-29°C and at CT12- 29 °C are significantly higher than at CT0 ($p<0.01$, n=5 for each time point). The expression of *lPer2* is higher at 6°C than at 29°C (two-way ANOVA, $p<0.001$). The black bar on the top of the plot indicates constant darkenss. The panels on the right depict representative microphotographs of *lPer2* expression in the SCN. The values at ZT /CT 24 are a re-plot of the values from ZT/CT 0. Scale bar 100 μm.

Fig. 2: Pattern of *lPer2* expression in DD during the transition from 6°C to 29°C. (A) Representative actogram of one of the 48 lizards (≠15) used in the experiment. Each horizontal line is a record of one day's activity, and consecutive days are mounted one below the other. Lizards were entrained to a 12 :12 LD cycle at 29 °C, released into DD-29 °C for three days and then exposed to low temperature (6 °C) for four days in DD. After four days at low temperature, where they were in hypothermia, lizards were warmed up at their projected CT18. Asterisks indicate the circadian time when the lizard ≠15 was exposed to low temperature and subsequently put back to 29 °C. During the four days in DD 6 °C locomotor activity disappears. The day when the lizards were warmed

up is indicated as « transition day » and shows typical masking activity which lasts 5-6 hours. On this day lizards were sacrificed before the transition time (CT12 and 17) and shortly after (CT19 and 22). The three following days are indicated as « first », « second » and « third day at 29 °C ». Brains were sampled at 6, 12, 18 and 24 hours after this CT0 (hence CT24 corresponds to CT0 of the second day). (B) Relative *lPer2* expression during the transition from 6 °C to 29 °C and in the first three days after hypothermia. Lizards (n=48) were sacrificed at the indicated time points (compare legend in A) and the brains were processed for in situ hybridization (n=3 for each time point). *lPer2* expression during the transition day (CT12, 17, 19, 22) are not significantly different among themselves, but higher than the values during the first, second and third day at 29 °C (one-way ANOVA, Bonferroni post-hoc test: $0.001<p<0.05$, asterisks not shown). The gray shaded rectangle indicates the time at 6°C. *lPer2* expression at the indicated times during the first day at 29 °C are not significantly different among themselves nor from the second day at 29 °C (one-way ANOVA, Bonferroni post-hoc test). During the second day at 29 °C *lPer2* expression is significantly higher at CT6 than at CT24. The third day at 29 °C the normal circadian expression of *lPer2* resumes. The black bar on top of the graph represents constant darkness. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (one-way ANOVA, Bonferroni post-hoc test).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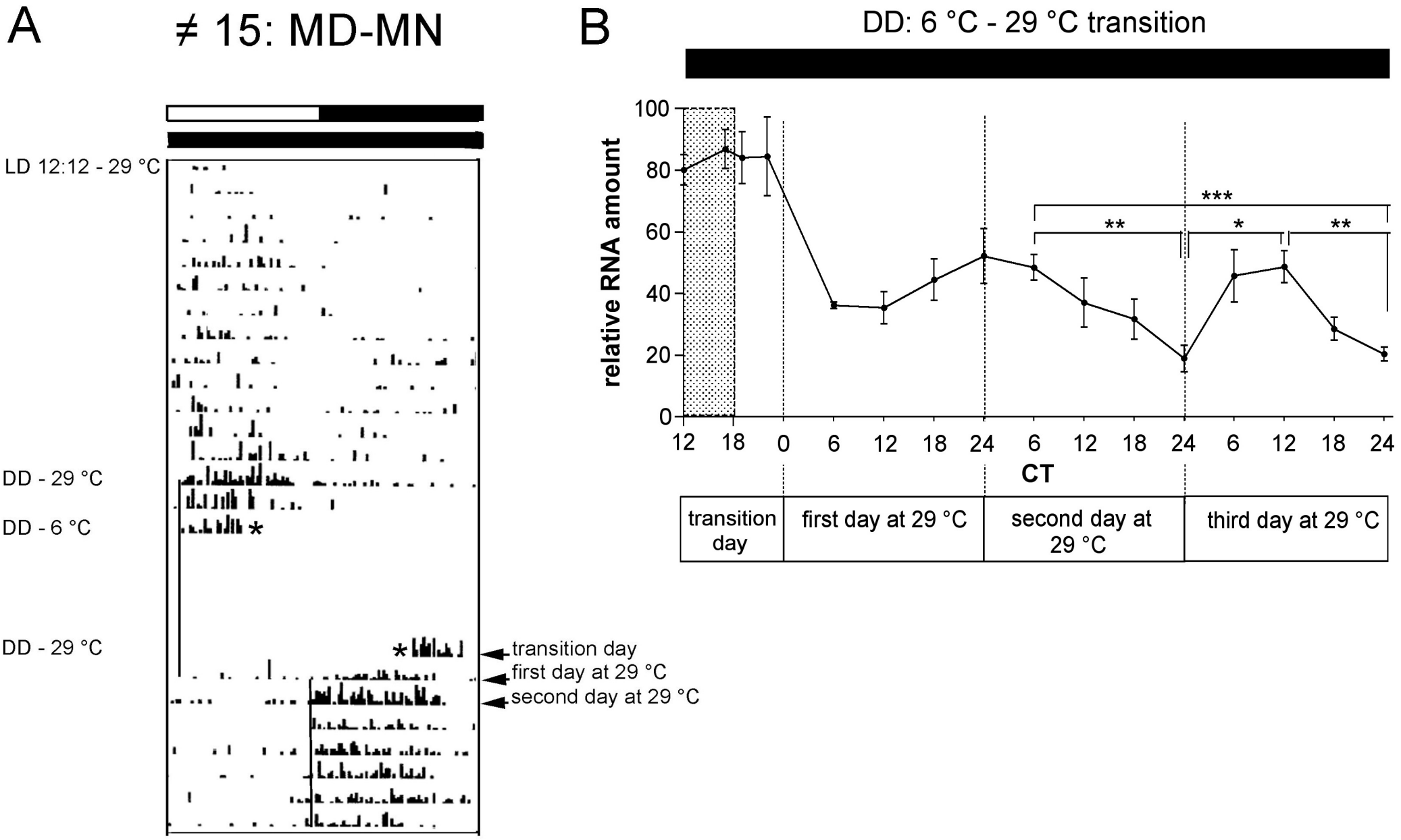
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