

TOWARD A SEASONAL MODEL OF THE CIRCADIAN SYSTEM: THE CASE OF RUIN LIZARDS

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1. ABSTRACT

The pineal gland plays a role in the seasonal reorganization of the circadian system that occurs in Ruin lizard *Podarcis sicula*. In fact, either pinealectomy or melatonin implants induce an immediate transition from the circadian locomotor pattern typical of summer to the circadian locomotor pattern typical of spring or autumn. Furthermore, the degree of involvement of melatonin and the pineal in circadian organization changes dramatically with season: 1. The effects of pinealectomy, as well as those of melatonin implants on circadian locomotor rhythms are strong in summer and weak in the other seasons; 2. Daily melatonin injections entrain locomotor rhythms in summer, but not in other seasons; 3. A phase response curve for melatonin was generated in summer, but not in winter; 4. Daily injections of luzindole, an antagonist for melatonin receptors alter locomotor rhythms in summer, but not in other seasons; 5. A robust pineal-dependent circadian rhythm of circulating melatonin exists in summer, but not in spring or in autumn. This confirms that the central role of the pineal in determining circadian organization in summer and the marginal role of the pineal in either spring or autumn are respectively due to appearance (summer) or disappearance (spring, autumn) of a circadian melatonin signal in the circulation.

2. INTRODUCTION

Circadian rhythms are controlled by endogenous clocks that have been localized to discrete neural anatomical structures within the central nervous system. In vertebrates these structures are the suprachiasmatic nuclei (SCN) of the hypothalamus, the lateral eyes and the pineal complex. This set of organs constitutes what is now called the "Vertebrates Circadian Axis" (1). In lizards the pineal gland is considered the neuroendocrine transducer of variations in environmental conditions, and such action is likely to be mediated *via* the hormone melatonin. Investigations in the lizards *Anolis carolinensis* and *Tiliqua rugosa* actually demonstrated that 24-h cycles of both light and temperature can entrain the pineal melatonin rhythm

and that differences in length of daily photoperiod or thermoperiod affect the phase, amplitude and duration of this rhythm (2-4). Hence, the current ambient lighting and temperature conditions (and their seasonal changes) are readily translated into an internal cue in the form of the pineal melatonin rhythm. This cue can be used to regulate both the daily and annual physiology of lizards (5). In our model animal, the Ruin lizard *Podarcis sicula*, dramatic seasonal changes in the daily pattern of locomotor activity occurring in the field were shown to be incorporated in the circadian system and, at least in part, mediated by the pineal gland (6-9). The present paper is focused on the seasonal changes of the circadian system that occur in Ruin lizards, their adaptive function, and the role of melatonin and the pineal in such a seasonal reorganization of the system.

3. SEASONAL CHANGES OF THE ACTIVITY PATTERN IN THE MODEL ANIMAL

Many studies showed that the Ruin lizard is a well-suited reptile model animal to study seasonal changes of circadian locomotor rhythms. Ruin lizards are diurnal, heliotherm and actively foraging animals (10-13). Field observations showed that the daily pattern of locomotor activity of Ruin lizards changes dramatically throughout the year (figure 1). In spring and autumn the pattern of locomotor activity is unimodal (one activity peak per day, without interruptions). In summer activity becomes bimodally distributed, with an early or mid-morning peak and a late afternoon peak, separated by a period of time during which activity is dramatically reduced (12-13). Since lizards are ectotherms, which can be active only within a limited range of body temperatures, it is clear that under favorable thermal conditions, as in spring, locomotor activity is more intense and without substantial interruptions throughout the day. In summer, during the hottest part of the day lizards retreat to the shade or into their burrows to avoid overheating, so that locomotor activity is dramatically reduced. These seasonal differences

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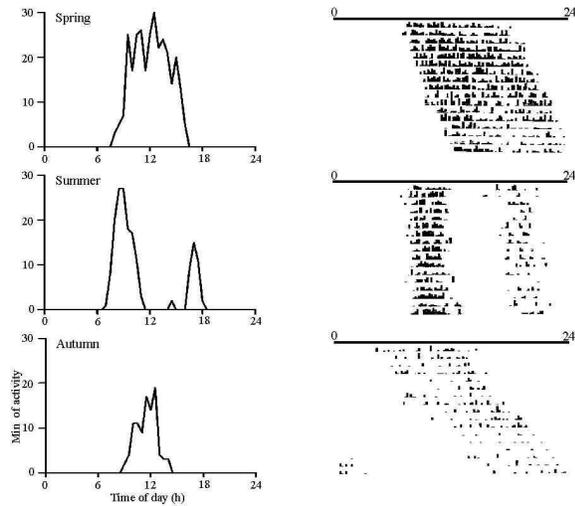


Figure 1. Locomotor activity of three individual Ruin lizards recorded both in the field and in the laboratory. Each lizard was tested in a different month. For each lizard the left panel shows one day of locomotor activity in the field, while the right panel reports two weeks of locomotor activity under constant temperature (29° C) and darkness in the lab. Left panels: Minutes of activity per each half-hour interval from dawn to dusk are plotted over 24-h. Right panels: each horizontal line is a record of 1 day's activity, and consecutive days are mounted one below the other. Each lizard retained in the lab the same locomotor pattern previously shown in the field. Clearly, the locomotor pattern is different depending on season.

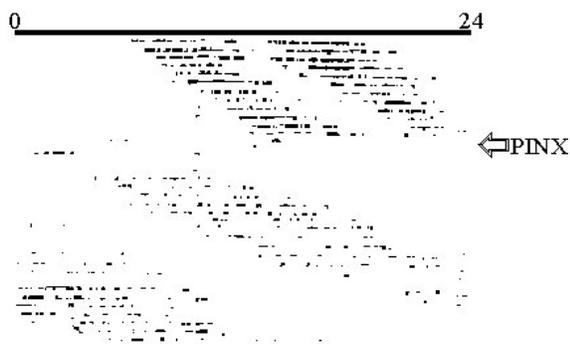


Figure 2. Locomotor activity record of one Ruin lizard subjected to pinealectomy in summer. Before surgery this lizard expresses the bimodal locomotor pattern typical of summer. The arrow indicates the day of pinealectomy (PINX). Pinealectomy abolished the bimodal locomotor pattern and induced a marked lengthening in freerunning period and a shorting of circadian activity time.

in activity patterns have been generally interpreted as a direct behavioral response of lizards to related changes in solar radiation and ambient temperature. However, different experiments showed that seasonal changes in activity patterns have an endogenous component (7): Ruin lizards held in constant temperature (29° C) and darkness (DD) retain the locomotor pattern they showed in the field during the same season (figure 1). Furthermore, the

bimodal locomotor pattern in constant conditions expressed by most lizards in summer is typically associated with a short freerunning period and a long circadian activity, while the unimodal locomotor pattern expressed in the remaining months is typically associated with long freerunning period and short circadian activity (7). Altogether, the data are compatible with the idea that both the interdependent changes of freerunning period and circadian activity time and the changes in locomotor pattern occurring seasonally in the circadian activity rhythms of Ruin lizards would depend on changes in the phase relationship between mutually coupled oscillators which drive these rhythms (14).

4. ROLE OF THE PINEAL AND MELATONIN IN DETERMINING THE SUMMER ACTIVITY PATTERN

In Ruin lizards pinealectomy does not abolish circadian locomotor rhythmicity, but produces marked changes of both freerunning period and circadian activity time (15). Further investigations have been carried out to examine whether pineal and melatonin played a role in the control of the seasonal changes in circadian parameters observed in these lizards. In constant temperature and DD, pinealectomy was shown to induce an immediate transition from the typical circadian locomotor behavior of summer (characterized by a marked bimodal pattern, a short freerunning period and long circadian activity) to the typical circadian locomotor behavior of spring and autumn (characterized by an unimodal pattern, a long freerunning period and a short circadian activity) (figure 2; 8). Furthermore, the behavioral effects of chronic administration of exogenous melatonin (melatonin implants) were found to be the same as those of pinealectomy in summer: the abolition of the bimodal pattern after application of the implants was always associated with a lengthening in freerunning period and shortening in circadian activity (16). These data suggest that the pineal plays a central role in establishing and maintaining summer bimodality. However, in summer transplantation of pineals from bimodal donors to hosts previously rendered unimodal by pinealectomy did not restore a bimodal locomotor pattern in the hosts (17). Since transplanted pineals, differently from intact ones are deprived of their stalks containing pineal nerves, it is reasonable to assume that these nerves carry crucial information to either establish or recovery a bimodal locomotor pattern typical of summer. Taken together, these results demonstrate that the pineal and melatonin play a central role in the seasonal reorganization of the circadian system that occurs in Ruin lizards, although the appearance of bimodality is not exclusively pineal-dependent (8, 17).

5. SEASONAL CHANGING ROLE OF PINEAL AND MELATONIN IN THE CIRCADIAN SYSTEM

The results of investigations which compared systematically the effects of pinealectomy on circadian locomotor behavior of Ruin lizards at different times of the year showed the existence of marked seasonal changes in the role of the pineal in their circadian organization (9).

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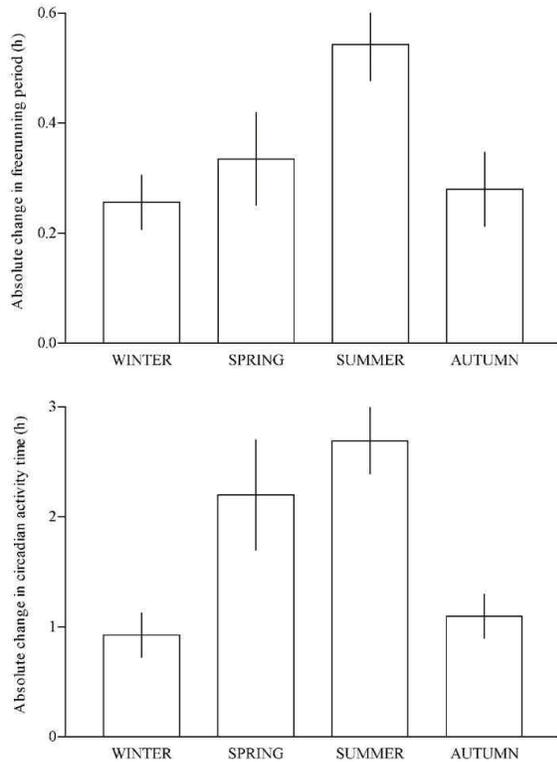


Figure 3. Absolute changes in freerunning period and circadian activity time induced by pinealectomy in different seasons. Changes in freerunning period (panel above) were significantly greater in summer than in winter, spring and autumn. Circadian activity time (panel below) was found to change significantly in response to pinealectomy only in spring and summer.

Changes in the freerunning period in response to pinealectomy were found to be significantly greater in summer than in winter, spring and autumn, and circadian activity time changed significantly only in spring and summer (figure 3). Furthermore, while pinealectomy was effective in altering the locomotor rhythms of all individual lizards tested in summer, the same surgery left locomotor rhythmicity of many lizards tested in autumn and winter completely undisturbed (9). Similarly, chronic implants of exogenous melatonin were found to change significantly freerunning period of locomotor rhythms and circadian activity time in summer, but were ineffective in autumn-winter (unpublished data; figure 4). Other studies were aimed at testing whether daily injections of exogenous melatonin are capable of entraining circadian locomotor rhythms of Ruin lizards in some seasons and not in others. For this purpose, the locomotor behavior of lizards collected and subjected to daily melatonin injections at different times of the year was examined in constant temperature and DD (18). Remarkably, while in summer the locomotor rhythms of most Ruin lizards entrained to the 24-h period of melatonin injections, in spring, autumn and winter the locomotor rhythms of these lizards did not entrain to the period of the injections (18). Further studies aimed at exploring the mechanisms of entrainment of circadian rhythms of Ruin lizards to melatonin showed that

in summer administration of exogenous melatonin at different phases of the circadian cycle of lizards freerunning in constant temperature and DD allows to generate a phase response curve (PRC) for melatonin (figure 5; 19). The existence of a PRC for melatonin confirms that daily melatonin injections actually entrain locomotor rhythms of Ruin lizards and do not merely cause masking of the underlying oscillation (18-21). In autumn-winter, however, no phase of the lizards' circadian cycle was shifted in response to melatonin injections, so that it was impossible to generate a PRC for melatonin in these seasons (figure 5). Hence, there is no PRC for melatonin in the same seasons – autumn-winter – in which daily melatonin injections were shown to be ineffective in entraining locomotor rhythms. Finally, it became clear that a PRC for melatonin exists only in summer, i.e. the same season in which daily melatonin injections are effective in entraining locomotor rhythms (18-19). Although the pineal is not necessary for the persistence of behavioral circadian rhythmicity in Ruin lizards, the data reported above made it clear that this gland plays a central role in the maintenance of circadian organization of these lizards in summer, and only a marginal role (if any) during the rest of the year. Endocrinological studies showed the existence in summer of robust circadian rhythms of plasma melatonin in Ruin lizards, which become abolished in response to pinealectomy (22). Hence, the pineal is the only source of the circadian rhythms of plasma melatonin measured in Ruin lizards during summer. Most recently, we decided to test whether the seasonal differences in the degree of pineal involvement in the circadian organization of Ruin lizards reported above were due to seasonal differences in plasma melatonin profiles. These studies actually showed that 24-h profiles of plasma melatonin concentration in Ruin lizards under constant temperature and DD vary significantly depending on season (23). While in summer plasma melatonin profiles were rhythmic even after three days of DD, both in spring and autumn plasma melatonin profiles in DD were shown to be completely arrhythmic (figure 6; 23). These results strongly support the view that the central role of the pineal in determining circadian organization in summer and the marginal (or absent) role of the pineal in either spring or autumn are respectively due to appearance (summer) or disappearance (spring, autumn) of a circadian melatonin signal in the blood circulation. In other words, the dramatic effects either of pinealectomy or melatonin implants on circadian locomotor rhythms in summer are attributable to the withdrawal of rhythmic changes in plasma melatonin levels; in winter, spring and autumn the behavioral effects of pinealectomy are weak or absent simply because during those seasons there are no rhythmic changes in melatonin levels to be withdrawn. In general, the fact that robust circadian locomotor rhythmicity persists also in intact Ruin lizards tested in spring and autumn-winter, when circadian rhythms of circulating melatonin are clearly absent demonstrates that during these seasons melatonin rhythms are not required to maintain circadian organization. Furthermore, endocrinological investigations revealed that the amount of melatonin produced over 24-h varies dramatically with season. In spring the amount of melatonin is greater than in summer, and the amount found in autumn smallest. Since there is no correlation between

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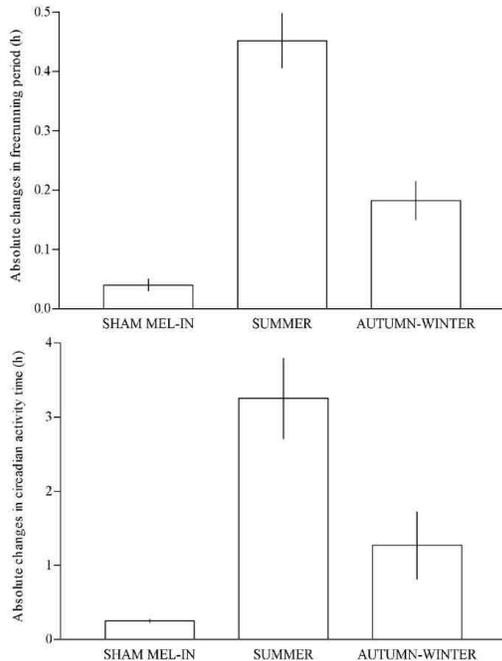


Figure 4. Absolute changes in freerunning period and circadian activity time induced by melatonin implants (MEL-IN) and sham melatonin implants (SHAM MEL-IN) in different seasons. Changes in freerunning period (panel above) in response to melatonin implants were significantly greater in summer than in autumn-winter. Also changes in circadian activity time (panel below) were significantly greater in summer than in autumn-winter.

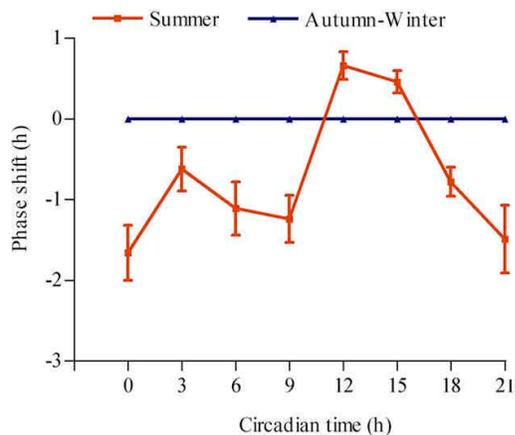


Figure 5. Phase-response curve (PRC) for melatonin in summer and autumn-winter. To induce phase shift of activity onsets, lizards freerunning in constant temperature and DD were injected subcutaneously biweekly with 3 micrograms of melatonin in 10 microliters of 1% ethanolic solution. Summer: average (\pm SEM) phase shifts are plotted in function of the phases of the melatonin injections, relative to activity onsets (red squares). The area under the delay parts of the curve is much greater than the area under the advance part of the curve and there is no dead zone. Autumn-winter: no one phase of the ruin lizards' circadian cycle was shifted in response to melatonin injections (blue triangles).

melatonin amounts and presence of circadian melatonin rhythms, the data available so far suggest that the striking seasonal differences in daily amounts of plasma melatonin should be ascribed to physiological processes generally subjected to seasonal changes, such as, for instance, reproduction and emergence from hibernation (23).

6. TARGET SITES OF MELATONIN IN THE CIRCADIAN SYSTEM: SEASONAL CHANGES

Daily melatonin injections were found to entrain circadian locomotor rhythms of pinealectomized Ruin lizards in summer, but not in spring (figure 7). Besides confirming that only in summer melatonin injections are capable of entraining the activity rhythm, these data demonstrated that the pineal is not the primary target site of melatonin in the circadian system of Ruin lizards (18). Since the hypothalamic SCN are the primary circadian pacemaker in Ruin lizards (SCN ablation invariably abolishes circadian locomotor rhythmicity), we thought the SCN were the best candidate as target sites for the modulating action of melatonin within the circadian system (18, 24). To test this, we compared in constant temperature and DD the behavioral response to daily melatonin injections of two different groups of summer lizards, one of which underwent bilateral ablation of the SCN (SCN-X) and the other unilateral ablation of the SCN (USCN-X). SCN-X lizards became behaviorally arrhythmic, and daily melatonin injections did not restore rhythmicity in SCN-X lizards. USCN-X lizards, however, remained behaviorally rhythmic, and their locomotor rhythms did entrain to the 24-h period of melatonin injections (18). Besides demonstrating that the presence of one SCN is sufficient to achieve entrainment of locomotor rhythms to melatonin injections, these data indicate the SCN as the primary extrapineal target sites of melatonin in the circadian system of Ruin lizards. The fact that circadian locomotor rhythms of intact, pinealectomized and USCN-X lizards are entrainable to melatonin only in summer strongly supports the contention that the SCN of Ruin lizards undergo a dramatic seasonal change in responsiveness to melatonin secreted by pineal into the blood, with high responsiveness in summer and low or no responsiveness in other seasons. Such a situation suggests the existence of marked seasonal variations of melatonin receptors concentration at the level of SCN. To test whether melatonin receptors concentration varies seasonally, we used a behavioral approach. We examined whether daily injections of luzindole, a well-known antagonist for melatonin receptors (25) would affect circadian locomotor behavior of Ruin lizards in constant temperature and DD, and further compared the results of experiments carried out in spring, summer and autumn. Luzindole injections were found to change significantly freerunning period and circadian activity time of locomotor rhythms in summer, but were ineffective in spring and in autumn (unpublished data). This result confirms that melatonin receptors concentration is higher in summer than in every other season. Future summer studies should try to explain why daily injections of luzindole induce changes in freerunning period and circadian activity time similar to those elicited by melatonin implants instead of entraining the lizard locomotor rhythm to the 24-h period of the injections.

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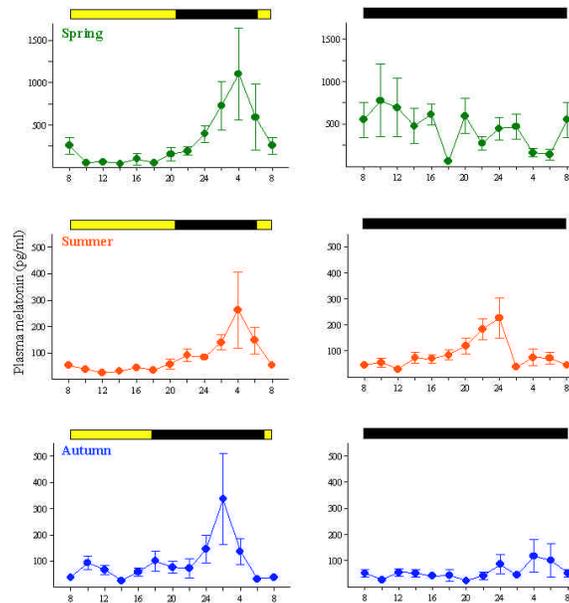


Figure 6. Seasonal variations of 24-h plasma melatonin profiles in Ruin lizards. In all lizards blood sampling was carried out in LD and then in DD during spring (green), and both LD and DD tests were repeated in summer (red) and autumn (blue). Each point represents mean \pm SEM value of plasma melatonin concentration based on the blood samples from 13 lizards. Yellow and black bars indicate light and dark phases of 24-h cycles. The results in LD (left panels) showed the presence of robust daily rhythms of plasma melatonin in all seasons. The results in DD (right panels) showed a clear circadian rhythm of plasma melatonin only in summer. No circadian rhythms of plasma melatonin were found either in spring or autumn.

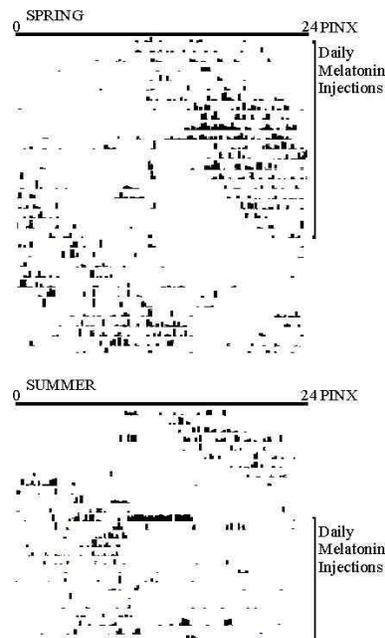


Figure 7. Locomotor records of two pinealectomized Ruin lizards subjected to daily melatonin injections in spring (top) and summer (bottom) while freerunning in constant temperature and DD. Pinealectomy (PIN-X) was carried out before starting with locomotor recording. Ruin lizards were injected subcutaneously every 24-h at 12:00 with 3 micrograms of melatonin in 10 microliters of 1% ethanolic solution. Starting and ending of melatonin treatment are indicated by a vertical line drawn on the right of each record. Top: Melatonin injections did not entrain the activity rhythm of the PINX lizard tested in spring. Bottom: This record was double-plotted on a 48h time scale to aid in interpretation. Melatonin injections actually entrained the activity rhythm of the PIN-X lizard tested in late summer. Besides confirming that only in summer melatonin injections are capable of entraining the activity rhythm, these data show that the pineal is not the primary target site of melatonin in the circadian system of Ruin lizards.

7. PERSPECTIVES: PROGRESS TOWARD A MODEL

In Ruin lizards the SCN warrant expression, persistence and stability of circadian rhythms of locomotor activity all the year around. As regards spring, autumn and winter no adjustments of the system via circadian rhythms of circulating melatonin are required in these seasons to maintain the proper circadian locomotor pattern. Summer, however, is characterized by appearance of a pineal-dependent circadian melatonin signal into the blood, that allows Ruin lizards the achievement of the temporal organization most adaptive for the season, particularly the bimodal locomotor pattern. To achieve bimodality the rhythmic melatonin signal works through the SCN, but this is clearly not the whole story. The fact that in summer transplantation of a pineal gland from a donor lizard expressing a bimodal activity pattern to a host previously rendered unimodal by pinealectomy does not restore a bimodal locomotor pattern in the host demonstrates that bimodality is not a phenomenon exclusively pineal-dependent (17). It seems reasonable that the pineal receives an afferent neural input from extrapineal sites of the system that allows the gland changing temporal pattern of melatonin synthesis and secretion in such a way, that a bimodal locomotor pattern is readily achieved. (17). Interestingly, the circadian system of birds was proposed to work as a neuroendocrine loop, with pineal oscillators being influenced by rhythmic sympathetic input driven by the SCN, and the SCN in turn being influenced by the rhythmic melatonin signal from the pineal (26). The existence of such a loop in the circadian system of Ruin lizards would necessarily imply that each component of the loop undergoes quite dramatic seasonal changes in function. In the detail, the seasonal loop of Ruin lizards may work in several ways. For instance, the pineal may be unable to generate a circadian rhythm of melatonin autonomously (a so called "slave oscillator" in the sense of 27) until summer, when neural inputs from SCN change in a way that enables pinealocytes to synthesize melatonin rhythmically. Alternatively, the pineal may contain a perfectly working circadian clock coupled to melatonin synthesis, but in spring, autumn and winter its clock function may become inhibited or suppressed by specific neural signals received during those seasons from the SCN. A comparison between *in vivo* and *in vitro* 24-h melatonin profiles of Ruin lizards in the four seasons will be useful to start answering the questions above.

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