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SEASONAL VARIATIONS OF PINEAL INVOLVEMENT IN THE CIRCADIAN ORGANIZATION OF THE RUIN LIZARD *PODARCIS SICULA*

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Summary

To establish whether the effects of pinealectomy on circadian locomotor rhythmicity vary with season, we examined, in constant temperature and darkness, the locomotor behaviour of ruin lizards *Podarcis sicula* collected and subjected to pinealectomy at different times of the year. Changes in the freerunning period in response to pinealectomy were found to be significantly greater in summer than in winter, spring and autumn. Circadian activity time changed significantly in response to pinealectomy only in spring and summer. Furthermore, while pinealectomy was effective in altering the locomotor rhythms of all individual lizards tested in summer, the same surgery was found to leave locomotor rhythmicity of many lizards tested in autumn and winter completely undisturbed. These results demonstrate for the first time in a non-mammalian vertebrate that the pineal gland is centrally involved in determining circadian organization in some seasons and is only marginally involved in others.

Key words: lizard, locomotor activity, seasonality, pinealectomy, circadian rhythms, *Podarcis sicula*.

Introduction

The daily pattern of locomotor activity of the ruin lizard *Podarcis sicula* in its natural environment changes from unimodal in spring (with only one activity peak per day) to bimodal in summer (with two well-separated activity peaks per day), becoming unimodal again in autumn (Foà *et al.* 1992*c*; Tosini *et al.* 1992). Seasonal differences in pattern have been unequivocally found to have an endogenous component, as most lizards retain, under constant conditions in the laboratory, the locomotor pattern they showed in the field during the same season (Foà *et al.* 1994). Laboratory experiments in constant conditions further revealed that seasonal differences in locomotor pattern are associated with systematic differences in both the freerunning period of locomotor rhythms (τ) and the circadian activity time (α). While the bimodal locomotor pattern expressed by lizards between June and August is typically associated with a short τ and long α, the unimodal pattern expressed in the remaining months is typically associated with a long τ and short α (Foà *et al.* 1994). The seasonal reorganization of the circadian system that occurs in *P. sicula* may either result from the after-effects of the different photoperiods and thermoperiods experienced by the lizards in different seasons or, alternatively, reflect the existence of true circannual cycles of circadian rhythms (Gwinner, 1975; Pittendrigh and Daan, 1976*b*; Foà *et al.* 1994).

Since pinealectomy was the only treatment known to induce significant changes in both τ and α in *Podarcis sicula* (Foà, 1991), other investigations have been carried out to examine whether the pineal gland played a role in the control of the seasonal changes in circadian parameters observed in this lizard. Pinealectomy was found to induce a sudden transition from the circadian locomotor behaviour typical of summer (bimodal pattern, short τ and long α) to the circadian locomotor behaviour typical of autumn and winter (unimodal pattern, long τ and short α) (Innocenti *et al.* 1994). Furthermore, the behavioural effects of chronic implants of exogenous melatonin (in silastic capsules) 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 τ and shortening in α (Foà *et al.* 1992*b*). Robust circadian rhythms of blood-borne melatonin expressed by intact *Podarcis sicula* in late summer become heavily disrupted or abolished in response to either pinealectomy or melatonin implants (Foà *et al.* 1992*a*). Taken together, these results strongly support the view that the transition from a summer locomotor pattern to an autumn–winter one induced both by pinealectomy and by melatonin implants is due to the concomitant suppression of circadian melatonin rhythms in the blood. Accordingly, the transition from a summer locomotor

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pattern to an autumn–winter one that occurs in intact lizards is likely to depend on a natural, dramatic damping of rhythmic melatonin levels from one season to another. This implies that in autumn–winter the effects of pinealectomy on circadian locomotor behaviour may be substantially reduced with respect to those we have found in summer.

The present investigation was aimed at testing whether the behavioural effects of pinealectomy vary depending on season. For this purpose, we examined, in constant temperature and darkness, the locomotor behaviour of lizards collected and subjected to pinealectomy at different times of the year.

Materials and methods

Ruin lizards *Podarcis sicula campestris* (adult males only; 6.5–8 cm snout–vent length) were collected on sand dunes along the coast of the Ligurian Sea (Marina di Vecchiano, Pisa, Italy) at four different times of the year: February 1994; N=18 (winter); May 1994; N=25 (spring); July 1994; N=30 (summer); and October 1994, N=20 (autumn). After capture, each lizard was carried to the laboratory and immediately put into an individual tilt-cage (30 cm×15 cm×11 cm) for monitoring locomotor activity. Some tilt-cages were connected to an Esterline Angus event recorder, and others to a computer-based data acquisition system (DataQuest III, MiniMitter, Sunriver, OR, USA). All tilt-cages were placed inside environmental chambers kept in constant darkness (DD) and at a constant temperature (29 °C). Pinealectomy (PIN-X) and sham pinealectomy (SHAM) were always performed during the lizard's subjective day. Cold anaesthesia, PIN-X and SHAM were performed as described in Foà (1991). Postexperimental histological evaluation of the brain of PIN-X lizards showed that the pineal gland was completely removed.

Each seasonal group of lizards was allowed to freerun in constant conditions for 20–30 days, after which PIN-X or SHAM was performed (Table 1). Locomotor activity in DD was recorded for several weeks after surgery.

Estimates of the freerunning period (τ) and the circadian activity time (α) were made for the 10-day segment just before surgery and a 10-day segment 3–6 weeks after surgery. τ was estimated to the nearest 0.1 h by fitting by eye a straight line through the onsets of activity for each 10-day segment. α was estimated to the nearest 0.5 h by measuring the interval between an eye-fitted straight line connecting the onsets and another connecting the offsets of activity for each 10-day segment (eye-fitting method: pp. 226–227 in Pittendrigh and Daan, 1976*a*).

Table 1. *Numbers of animals in each seasonal grouping subjected to pinealectomy (PIN-X) or sham pinealectomy (SHAM) in the month following capture*

Season	Month of capture; recording initiated	SHAM *N*	PIN-X *N*
Winter	February	5	13
Spring	May	5	20
Summer	July	6	24
Autumn	October	5	15
Totals		21	72

Results

In all sham pinealectomized lizards (representative examples are given in Fig. 1), the changes in τ and α in response to surgery were negligible ($|\Delta\tau| \leq 0.2$ h and $|\Delta\alpha| \leq 1.0$ h). No seasonal differences in $|\Delta\tau|$ and $|\Delta\alpha|$ were found (one-way analysis of variance, ANOVA): $F_{3,17}$=0.56, P>0.50 for $|\Delta\tau|$ and $F_{3,17}$=0.88, P>0.20 for $|\Delta\alpha|$), and so the data for the four seasonal groups of SHAM lizards (N=21) were pooled for further comparisons. Overall, the absolute changes in τ induced by pinealectomy were significantly greater than those induced by sham surgery (one-way ANOVA: $F_{4,88}$=12.11, P<0.0001). The same is true for $|\Delta\alpha|$ ($F_{4,88}$=15.53, P<0.0001). There are, however, seasonal differences. As regards the absolute changes in τ, differences between PIN-X and SHAM are statistically significant in all seasons, but the levels of significance of the observed differences are highest in summer and lowest in winter (Table 2; see Fig. 4). As regards the absolute changes in α, differences between PIN-X and SHAM lizards are statistically significant only in spring and summer (Table 2; see Fig. 4). A direct statistical comparison among seasonal groups of PIN-X lizards has confirmed that the effects of pinealectomy on both τ and α changed significantly depending on season (one-way ANOVA; $F_{3,68}$=4.49, P<0.007 for $|\Delta\tau|$; $F_{3,68}$=8.14, P<0.0005

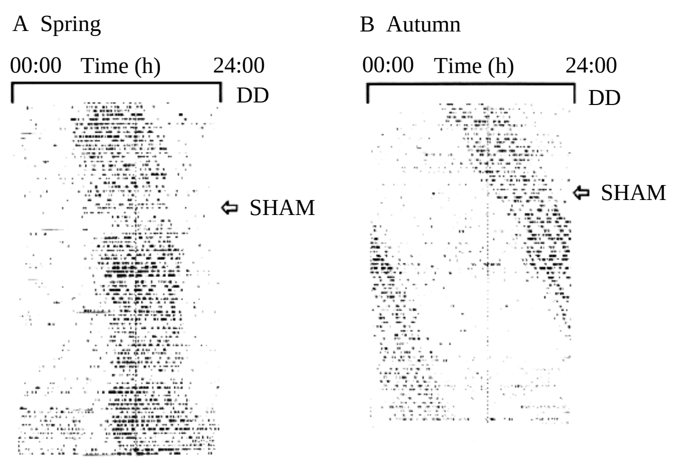

Fig. 1. Locomotor activity records of two lizards kept in constant temperature (29 °C) and darkness (DD) and subjected to sham pinealectomy (SHAM) in spring (A) and autumn (B). Each horizontal line is a record of the activity over a single day, with consecutive days mounted, below each other. Arrows indicate the day of surgery. Irrespective of season, sham pinealectomy did not affect locomotor rhythms. Representative examples of the lack of any effect of sham surgery on circadian locomotor behaviour in summer are reported in Fig. 1 of Innocenti *et al.* (1994).

Table 2. *Seasonal differences in the effects of pinealectomy on τ and α: multiple comparisons with respect to sham pinealectomy*

	$\|\Delta\tau\|$	$\|\Delta\alpha\|$
SHAM *versus* PIN-X winter	$P<0.05$	$P>0.10$
SHAM *versus* PIN-X spring	$P<0.005$	$P<0.001$
SHAM *versus* PIN-X summer	$P<0.001$	$P<0.001$
SHAM *versus* PIN-X autumn	$P<0.01$	$P>0.05$

Dunnet's test was used to compare seasonal means of $|\Delta\tau|$ and $|\Delta\alpha|$ of PIN-X lizards with means of pooled $|\Delta\tau|$ and $|\Delta\alpha|$ of SHAM lizards.

Probability value (P) for each comparison is given.

For further explanation, see text.

Table 3. *Seasonal differences in the effects of pinealectomy on τ and α: multiple comparisons among groups of lizards pinealectomized in different seasons*

	$\|\Delta\tau\|$	$\|\Delta\alpha\|$
Summer *versus* Winter	$P<0.005$	$P<0.0001$
Summer *versus* Autumn	$P<0.005$	$P<0.0005$
Summer *versus* Spring	$P<0.05$	$P>0.10$
Spring *versus* Winter	$P>0.10$	$P<0.01$
Spring *versus* Autumn	$P>0.10$	$P<0.05$
Autumn *versus* Winter	$P>0.10$	$P>0.10$

Multiple comparisons were made among pairs of seasonal means of $|\Delta\tau|$ and among pairs of seasonal means of $|\Delta\alpha|$ of PIN-X lizards by using the GT2 method (Sokal and Rohlf, 1981).

Probability value (P) of GT2 for each comparison between two seasonal means is given.

for $|\Delta\alpha|$). Specifically, multiple comparisons revealed that the absolute changes in τ induced by pinealectomy are significantly greater in summer than for all other seasons (Table 3; Figs 2, 4), but no further seasonal differences were found. The absolute changes in α induced by pinealectomy are significantly greater in spring and summer than in winter and autumn (Table 3; Figs 2, 4) and, in addition, there is a seasonal change in the numbers of pinealectomized animals whose locomotor rhythms are affected by the surgery (Table 4). Furthermore, the main bout of activity became diffuse (virtually arrhythmic) in response to pinealectomy only in spring (one lizard) and summer (two lizards) (Fig. 3). In summer, pinealectomy induced a significant lengthening in τ ($P<0.01$, Student's paired t-test) and shortening in α ($P<0.01$). Furthermore, the bimodal locomotor pattern typical of summer was abolished after surgery (Fig. 2C). In spring, there was a significant tendency for α to shorten ($P<0.05$) in response to pinealectomy (Fig. 2B), while there was no trend in the direction of changes in τ ($P>0.10$). No trend in the direction of changes in τ was observed after pinealectomy in autumn and winter (Fig. 2A,D).

Discussion

The present results demonstrate marked seasonal variations

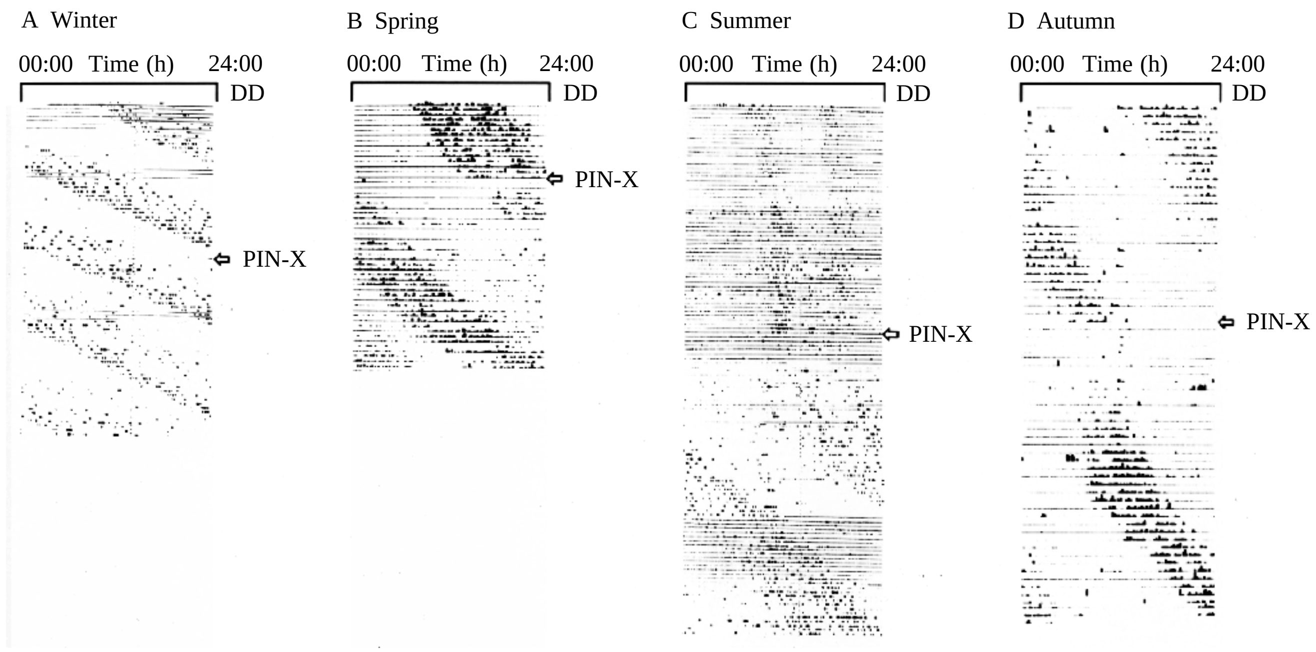

Fig. 2. Locomotor activity records of four lizards subjected to pinealectomy (PIN-X) in winter (A), spring (B), summer (C) and autumn (D). The examples are representative of the seasonal differences in the effects of pinealectomy on circadian locomotor rhythmicity of *Podarcis sicula*. In autumn and winter, changes in the freerunning period (τ) in response to pinealectomy are weak (D) or absent (A). (B) Besides changing τ, pinealectomy in spring induces a significant shortening in the circadian activity time (α). (C) In summer, pinealectomy markedly lengthens τ, shortens α and abolishes the bimodal locomotor pattern typical of the season.

in the effects of pinealectomy on the circadian rhythms of locomotor activity in ruin lizards *Podarcis sicula*. Although pinealectomy was effective in altering the freerunning period (τ) in all seasons, changes in τ were significantly greater in summer than in winter, spring and autumn (Tables 2, 3; Fig. 4). Circadian activity time (α) was found to change significantly in response to pinealectomy only in spring and summer (Table 2; Fig. 4). Furthermore, while pinealectomy was effective in altering the locomotor rhythms of all individuals tested in summer (not only by markedly changing τ and/or α, but also, in two cases, by virtually abolishing circadian rhythmicity), the same surgery was found to leave locomotor rhythmicity of many lizards (10 out of 28) tested in autumn–winter completely undisturbed (Table 4 and a representative example in Fig. 2A). These data demonstrate for the first time in a non-mammalian vertebrate that the pineal gland is centrally involved in determining circadian organization in some seasons and is only marginally involved in others.

Seasonal differences in the behavioural effects of pinealectomy have been reported in only one other non-mammalian species, the burbot *Lota lota* (Kavaliers, 1980). In the burbot, pinealectomy was found to induce a drastic lengthening in τ in winter and a comparably drastic shortening in τ in summer. Hence, unlike the case of ruin lizards, the pineal gland in burbots is centrally involved in determining circadian organization both in winter and in summer. It seems, however, that the only seasonal difference in this case is the direction of the changes in τ in response to pinealectomy.

Previous investigations have demonstrated that the pineal gland is involved in the seasonal reorganization of the

Table 4. *Numbers of lizards whose locomotor rhythms have been affected by pinealectomy in each different season*

	Number of animals subjected to pinealectomy			
	Winter 13	Spring 20	Summer 24	Autumn 15
Effects on τ	3	1	1	2
Effects on α	1	4	3	2
Effects on both τ and α	5	12	18	5
Arrhythmia	0	1	2	0
Total showing an effect	9	18	24	9
Total showing no effect	4	2	0	6

Effects of pinealectomy on τ are only the absolute changes in τ greater than 0.2 h (0.2 h = maximum $|\Delta\tau|$ in response to sham surgery).

Effects of pinealectomy on α are only the absolute changes in α greater than 1.0 h (1.0 h = maximum $|\Delta\alpha|$ in response to sham surgery).

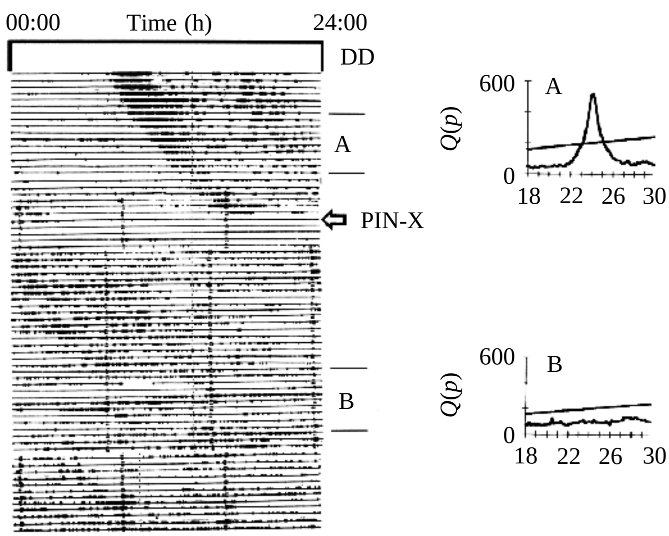

Fig. 3. Locomotor activity record of one lizard whose locomotor rhythms were abolished after pinealectomy (PIN-X) in summer. The presence of circadian periodicities was tested for using a χ^2 periodogram, as reported in Minutini *et al.* (1995). A and B mark sections of the locomotor record that were separately subjected to χ^2 periodogram analysis (plots in the right-hand panels A, B). In each χ^2 periodogram, an index of rhythmicity [$Q(p)$, in ordinate] calculated as in Sokolove and Bushell (1978) is plotted over the tested period in hours (abscissa). Confidence limits were chosen at the 99 % level. As shown in A, periodogram analysis before pinealectomy shows the presence of a circadian periodicity which is abolished after pinealectomy (B). In addition to this lizard, only two other lizards (one in summer and one in spring) developed arrhythmic locomotor patterns in response to pinealectomy.

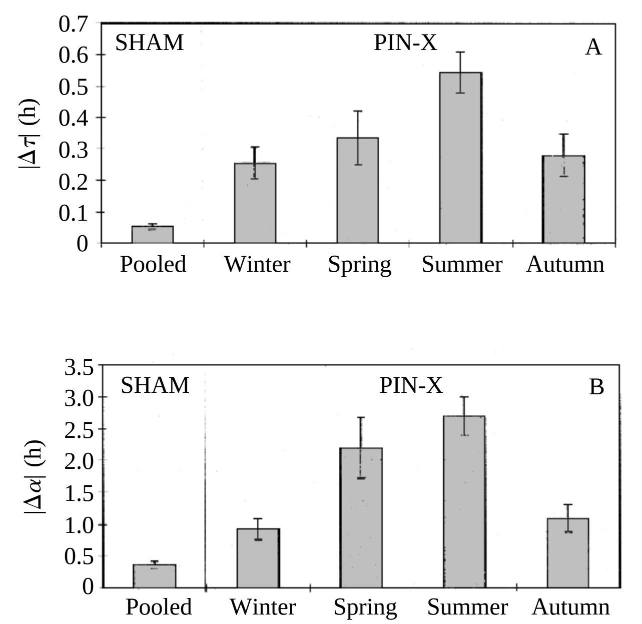

Fig. 4. Summary of the results: means ± S.E.M. of the absolute changes in the freerunning period of locomotor rhythms ($|\Delta\tau|$, A) and circadian activity time ($|\Delta\alpha|$, B) induced by pinealectomy (PIN-X, N=72) in different seasons and by sham pinealectomy (SHAM, N=21). Since no seasonal differences in $|\Delta\tau|$ and $|\Delta\alpha|$ were found among the four seasonal groups of SHAM, the data were pooled. Levels of significance of the statistical comparisons among the groups are reported in Tables 2 and 3.

circadian system that occurs in *P. sicula* (Foà *et al.* 1994; Innocenti *et al.* 1994). Pinealectomy in summer was found to induce a sudden transition from the circadian locomotor behaviour typical of summer (bimodal pattern, short τ and long α) to that typical of autumn–winter (unimodal pattern, long τ and short α) (Innocenti *et al.* 1994).

Besides confirming these findings, the present results make it clear that the primary role of the pineal in the circadian system of *P. sicula* is that of establishing and maintaining the circadian locomotor pattern typical of the summer where τ is typically short (pinealectomy markedly lengthens τ), α long (pinealectomy markedly shortens α) and the locomotor pattern bimodal (pinealectomy abolishes bimodality). In spring, there was no trend in the direction of the changes in τ after pinealectomy. This suggests that the role of the pineal in maintaining τ short is delayed until summer. In spring, however, the pineal apparently contributes to the long α (pinealectomy significantly shortens α) that usually precedes the appearance of bimodality (A. Foà, A. Innocenti and C. Bertolucci, unpublished observations). In autumn and winter, irrespective of the presence of the pineal, τ is long (changes in τ after pinealectomy are small and without directional trend), α is short (α does not change significantly after pinealectomy) and the locomotor pattern is unimodal (pinealectomy never induces a change in pattern). The small changes in τ induced by pinealectomy in autumn and winter seem to indicate that during these seasons the pineal merely contributes to the stability of locomotor rhythms.

Previous evidence suggests that the disappearance of the typical summer locomotor pattern in response to pinealectomy is due to the concomitant withdrawal of rhythmic changes in melatonin levels in the blood. Robust circadian rhythms of blood-borne melatonin expressed by intact *Podarcis sicula* in late summer were abolished after pinealectomy (Foà *et al.* 1992*a*). Chronic administration of exogenous melatonin (in silastic capsules), another treatment that virtually abolishes melatonin rhythms in the blood, induces the same behavioural effects as pinealectomy in summer so that after melatonin administration the typical summer locomotor pattern immediately disappears (Fig. 1C in Foà *et al.* 1992*b*). Remarkably, after blood-borne melatonin rhythms have been abolished in summer (in response to either pinealectomy or melatonin implants) the locomotor pattern typical of autumn–winter immediately appears (Foà *et al.* 1992*b*; Innocenti *et al.* 1994). Hence, in contrast to the situation in summer, in autumn and winter rhythmic changes in levels of blood-borne melatonin do not seem to be required for the expression of the locomotor pattern typical of these seasons. This explains why the behavioural effects of pinealectomy in the present study were found to be so weak (or absent) only in autumn and winter. Overall, these data strongly support the view that seasonal differences in the degree of pineal involvement in the circadian organization of *P. sicula* may depend on profound seasonal variations of melatonin profiles in the pineal and the blood. Seasonal changes in rates of pineal melatonin synthesis have been found to occur in other non-mammalian vertebrates, such as the tortoise *Testudo hermanni* (Vivien Roels *et al.* 1979) and the lamprey *Lampetra japonica* (Samejima *et al.* 1995).

The observation that the pineal of *P. sicula* is not usually necessary for the persistence of behavioural circadian rhythmicity demonstrates the existence of a primary pacemaker for locomotor rhythms elsewhere in the system. Recently, lesions to the suprachiasmatic nuclei of the hypothalamus (SCN) of *P. sicula* have been shown invariably to abolish circadian rhythms of locomotor activity (Minutini *et al.* 1995). This strongly supports the view that the SCN contain the primary pacemaker(s) that drives locomotor rhythms in this lizard. Alterations of locomotor rhythms in response to pinealectomy, when present, suggest the possibility that the pineal acts as a coupling device between SCN oscillators (Underwood, 1983, 1992; see Minutini *et al.* 1995 for a detailed discussion). If this were the case, then the effectiveness of this coupling action is likely to change in parallel with seasonal variations in melatonin profiles and to be maximal in summer, as shown by the present results.

Previous investigations have indicated that the role played by the pineal gland in circadian organization may vary among lizard species belonging to the same taxonomic family (Iguanidae): pinealectomy abolishes circadian rhythms of locomotor activity in *Anolis carolinensis*, while it produces marked changes in τ and α in *Sceloporus occidentalis* and has no behavioural effects in *Dipsosaurus dorsalis* (Underwood, 1981, 1983; Janik and Menaker, 1990). However, owing to the seasonal differences in the behavioural effects of pinealectomy we have found in *P. sicula*, it seems reasonable to doubt that the differences among the Iguanidae are completely interspecific in nature. Instead, they may, at least in part, depend on the particular season in which the behavioural effects of pinealectomy have been examined in each different species. The Iguanidae, as well as the lacertid lizard *P. sicula*, inhabit temperate zones, i.e. zones in which seasonal changes in circadian organization are likely to have evolved in response to the regular seasonal fluctuations in photoperiod and/or thermoperiod experienced by the lizards throughout the year (Underwood, 1992; Foà *et al.* 1994). Hence, before deciding about interspecific differences, one should verify whether, for instance, *A. carolinensis* and *S. occidentalis* were tested during a season when the behavioural effects of pinealectomy are maximal and whether *D. dorsalis* was tested during a season when these effects are minimal.

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