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Temperature-compensated circadian clock in the pineal of *Anolis*

(organ culture/melatonin/circadian rhythm/circadian oscillator)

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ABSTRACT **The pineal organ of the lizard *Anolis carolinensis* can be maintained for up to 10 days in superfused organ culture. During this time it synthesizes and releases melatonin into the medium flowing slowly over it. Collection of timed aliquots of medium and subsequent analysis for melatonin by radioimmunoassay reveal circadian rhythms of melatonin output by the isolated pineal. These rhythms persist for many cycles in constant darkness and at several constant ambient temperatures ranging from 22 to 37°C. The period of the rhythm is temperature compensated (Q_{10} ≈ 1.14) and the rhythm is synchronized by light–dark cycles imposed on the cultured gland. This isolated vertebrate organ displays the three major properties of circadian systems and must therefore contain one or more circadian oscillators.**

Circadian rhythms are characterized by three major properties: they oscillate under constant conditions (free-run), they can be synchronized by environmental light–dark (LD) cycles (entrainment), and their periods vary only slightly with changes in ambient temperature (temperature compensation). Here we report that all three of these properties are clearly displayed by the isolated pineal gland of the lizard *Anolis carolinensis* in its synthesis of melatonin. The gland itself must contain one or more temperature-compensated circadian oscillators coupled to photoreceptors on the input side and to melatonin synthetic pathways on the output side.

To study the pineal of *Anolis in vitro* we used the flow-through apparatus developed for work with the isolated pineal of chickens. Not only the apparatus but also the culture technique, the culture medium (we used GIBCO medium 199 with Hanks' salts and L-glutamine), and the radioimmunoassay were as reported in ref. 1. We varied the constant temperature at which the isolated glands were held by varying the temperature of the room in which the experiments were conducted (coarse adjustment) and of a water jacket in the immediate vicinity of the glands (fine adjustment). Temperatures were recorded continuously at the level of the isolated glands: they were 22, 25, 27, 30, 32, and 37°C in dark–dark (DD) and 27°C in LD. Neither in DD nor in LD did the temperature variations (no more than ±0.5°C) have periodicities at or around 24 hr. Attention to the temperature regimen is particularly important as locomotor rhythmicity in some lizards has been shown to be sensitive to entrainment by temperature cycles (2). Lizards were held on a 12:12 LD cycle at one of several ambient temperatures until late in the light period of the day on which the experiment was to begin. In most cases constant darkness was initiated in the culture chamber within hours of isolating the gland, at the time of the light to dark transition normally seen by the lizards. However, four glands held at 27°C were exposed to four LD cycles in phase with those previously seen by the lizards. The cultured glands were illuminated by a 45-W quartz-iodide bulb mounted inside a water jacket through which temperature-controlled water was pumped. Glands in individual glass chambers were arranged in a semicircle around the water jacket and each received white light at ≈1.9 mW/cm^2 (3). They were continuously superfused with medium that was collected every 90 min and assayed for melatonin by radioimmunoassay. The radioimmunoassay that we used (1) has not yet been validated for superfusate from *Anolis* pineals. In the study reported here we were primarily concerned with rhythmicity of the isolated pineal and not with the details of its biochemistry. However, the reader should bear in mind that in this report "melatonin" is used as an abbreviation for "radioimmunoassayable melatonin."

Isolated *Anolis* pineals had free-running rhythms of melatonin synthesis in constant darkness at each of the temperatures at which we assayed them (22, 25, 27, 30, 32, and 37°C). Robust rhythms, persisting for at least six cycles in DD, were recorded at the four highest temperatures, perhaps most reliably at 32°C, which is also the preferred temperature for *Anolis* (4, 5). In Fig. 1 the best (most clearly rhythmic) record obtained at each of four temperatures has been plotted (at 22°C the best record is significantly better than the next best record; this is not true at the other temperatures). In Fig. 2 estimated free-running periods of individual glands are plotted as a function of temperature. Periods were estimated by autocorrelation (6) on Minitab statistical package (7, 8). Lag intervals with the highest significant R value in the circadian range were taken as estimates of period. Period estimates and their reciprocals (frequency) were regressed against temperature on Minitab as well. The regression line has a slope of 0.00063, indicating a Q_{10} for the rhythm over this 15°C temperature range of 1.135. In the context of this initial study the exact value of the Q_{10} is not particularly important. What is significant is that (*i*) the period of the free-running rhythm does vary systematically with temperature, demonstrating that the rhythm is not being driven by an exogenous variable that we have failed adequately to control, and (*ii*) the amount of the systematic variation with temperature is small, indicating that the underlying process generating the rhythm is temperature compensated and thus truly circadian (9).

The only other demonstration of temperature compensation in an isolated organ has been accomplished in the eye of the mollusk *Aplysia* (10). In either the lizard or the slug natural selection might have achieved temperature compensation of the overall circadian system differently—e.g., by the interaction of oscillators with complementary Q_{10}s located in separate structures. Such an organization would not seem *a priori* to be unlikely, especially in view of the fact that oscillators are known in fungi that have some "circadian" properties but lack temperature compensation (11). It has long been known that the circadian rhythms of many single-celled organisms are compensated for changes in temperature (9). The finding of this

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Abbreviations: LD, light–dark; DD, dark–dark.

property in organs isolated from both invertebrates and vertebrates strengthens the widely held belief that temperature compensation is a fundamental property of circadian oscillators at the cellular level.

The rhythm of melatonin synthesis in isolated *Anolis* pineals can be entrained by LD cycles. The record shown in Fig. 3 is typical of those from the four glands exposed to a 12:12 LD cycle at 27°C. The rhythms of all four glands bear the same phase relationship to the light cycle and their periods are indistinguishable from 24 hr. The six glands measured in constant darkness at this temperature ranged in period from 25.6 to 28.2 with a mean of 26.7 (Fig. 2). The periods of the glands exposed to light cycles are thus outside the range of free-running periods expressed in constant darkness. Apparently the ambient light cycle controls the phase and period of the rhythm *in vitro*. Lizard pineal glands are known to contain photoreceptors (12) and evidently in *Anolis* at least some of these are coupled to the circadian oscillators that regulate the synthesis of melatonin.

Examination of the individual records in Figs. 1 and 3 reveals a good deal of variability in the height of the melatonin peaks and the level of the troughs both among glands and within the record of a single gland. There is a clear tendency for the amplitude of the rhythm to be greater at 27–37°C than at 22 or 25°C, but this is the only systematic trend in the variability among glands. There may well be considerable genetic heterogeneity in the wild population from which our animals were drawn (our lizards were purchased from Louisiana) and the history of individuals may vary widely, especially in diet and in reproductive activity. On the other hand, the records of single glands do vary systematically with time in culture. With few exceptions in both LD and DD at all temperatures the first peak is 4–10 times higher than the second and the second is sometimes considerably higher than the third. Third, fourth, and fifth peaks are usually about the same height, whereas from peak six on, there is often a slow decline. The most striking feature of this progression is the precipitous decline in peak height after the first (and sometimes the second) peak. Explanation awaits a more complete understanding of lizard pineal biochemistry. Does the first peak contain substances in addition to melatonin (that crossreact in the assay) that do not continue to be synthesized in culture? Does the gland "dump" these or melatonin (or both) in response to trauma? Does the cultured pineal lack important substrates or cofactors necessary for continued normal synthesis? Whatever the reason for the drastic change in the amount of synthesis it is important to note that there is no obvious associated effect on the circadian timing system. The interval between the first and second peak is not sig-

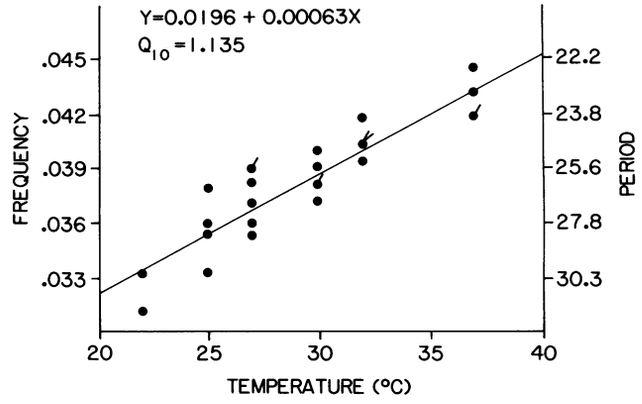

FIG. 2. Frequency and period estimates of the circadian rhythms of all *Anolis* pineals examined. As indicated, 26 glands were measured at six temperatures between 22 and 37°C. Each point is the best estimate of the period of the rhythm of a single gland that produced from 4 to 10 cycles in constant darkness. Points with tails indicate two or, in one case, three glands with these coordinates.

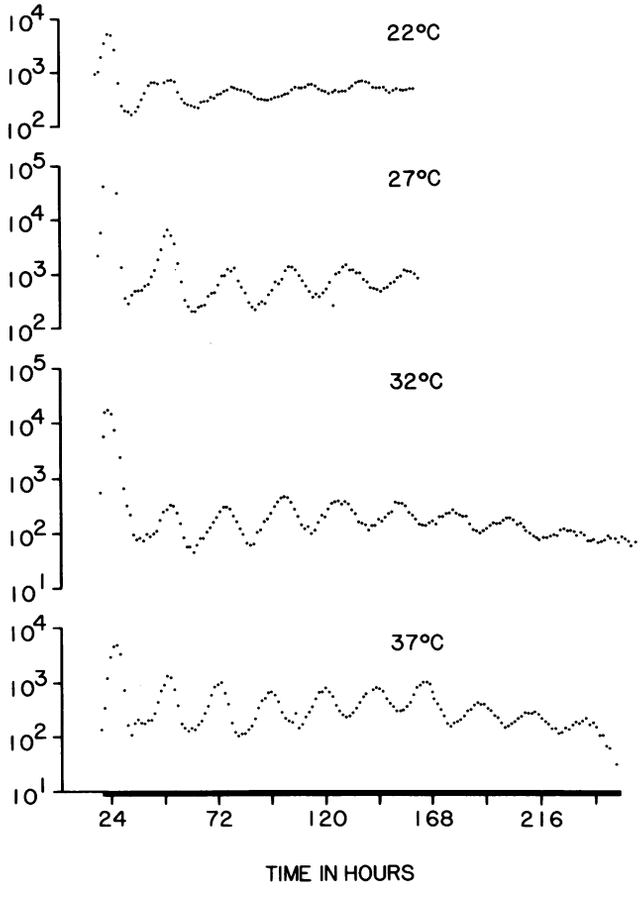

FIG. 1. Circadian rhythms of melatonin produced by four individual isolated pineal glands of *Anolis* held in constant darkness at four different temperatures. Each point was determined by radioimmunoassay of a superfusate sample. Superfusion was continuous and all medium was collected, in 90-min aliquots. On the ordinate is plotted the log of the melatonin concentration in pg/ml. Note that the periods of the rhythms are shorter at the higher temperatures.

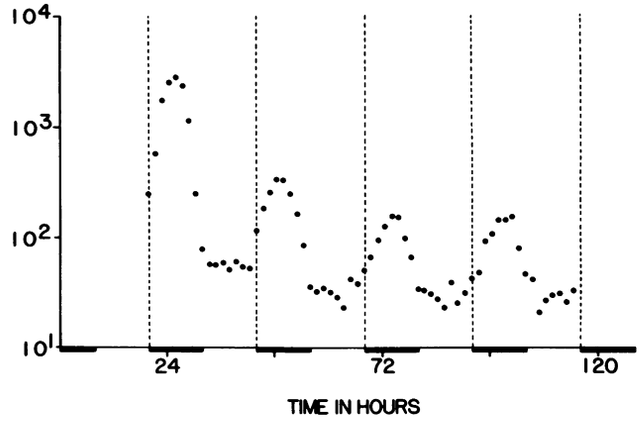

FIG. 3. Melatonin rhythm entrained *in vitro* by a light cycle (diagrammed on the abscissa). Temperature was a constant 27°C ± 0.5°C. Samples were obtained and plotted as described in the legend to Fig. 1. Note that the period of the rhythm is indistinguishable from 24 hr and that its phase relationship to the light cycle is as expected—i.e., melatonin is high during the dark.

nificantly different from that between the second and third or third and fourth.

Isolated avian pineal glands also produce melatonin rhythmically *in vitro*, although their rhythms have not yet been tested for temperature compensation (13–16). Our initial experiments with avian pineals in culture were stimulated by the results of a long series of behavioral experiments with the locomotor rhythms of house sparrows that strongly indicated that the pineal contained circadian oscillators (17–19). The same behavioral experiments when performed on chickens, quail, and starlings gave negative or weak evidence of the existence of pineal circadian oscillators (20–22). In spite of the differences among these three species, their pineals behave similarly in culture; robust rhythms of melatonin synthesis are produced by cultured glands exposed to LD cycles and in DD rhythms persist for up to four cycles but are heavily damped (3). Thus, in birds the behavior of the pineal gland in culture does not directly reflect its role in the overall circadian organization of at least some behaviors. It seems likely that all avian pineals contain circadian oscillators and that the variability among species is a consequence of differential coupling of these oscillators with the other components of the circadian system (5). H. Underwood (23) has found that pinealectomy of free-running *Anolis* renders them arrhythmic. His previous behavioral work with other lizard species (24) and the results reported here suggest that reptiles and birds may share a basic pattern of circadian organization.

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