

# Photoperiodic information acquired and stored *in vivo* is retained *in vitro* by a circadian oscillator, the avian pineal gland

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Endogenous circadian rhythms have been described in a wide range of organisms from prokaryotes to man. Although basic circadian mechanisms at the molecular level are genetically fixed, certain properties of circadian rhythms at the organismic level can be modified by environmental conditions and subsequently retained for some time, even in organisms shielded from 24-hr environmental variations. To investigate the capacity of animals to acquire and store photoperiodic information, we examined activity and melatonin rhythms in house sparrows during synchronization to two different photoperiods and during subsequent prolonged darkness. Under constant environmental conditions, intact animals continued to have long feeding activity times when previously exposed to long days and short feeding activity times when previously exposed to short days. Correspondingly, significantly different durations of elevated melatonin in the plasma directly reflected the differences in night length during synchronization as well as during prolonged darkness. Additionally, we found a significant difference in the amplitude of the nocturnal melatonin signal, which also was conserved in prolonged darkness. To investigate whether the photoperiodic experience of an intact animal can be “memorized” by an isolated component of its circadian pacemaking system, we have investigated *in vitro* melatonin release during continuous darkness from explanted pineal glands of house sparrows after *in vivo* synchronization to two distinct photoperiods. Differences in the durations of elevated melatonin occurred during the first two cycles in culture and a difference in melatonin amplitude was detectable during the first night in culture. Our data indicate that photoperiodic patterns imposed on sparrows during *in vivo* synchronization can be maintained as an internal representation of time within the isolated pineal gland. Hence, the pineal gland, as one of the most significant components of the songbird circadian pacemaker, not only has the capacity to autonomously produce circadian rhythms of melatonin release but also is capable of storing biologically meaningful information experienced during previous cycles.

Daily rhythms of endogenous origin, persisting with a period near 24 h for many cycles even in the absence of environmental timing cues, are known to control a great variety of biochemical, physiological, and behavioral functions in organisms from bacteria to man (1, 2). These rhythms are based on the activity of cells that contain particular molecular gears, ultimately resulting in the expression of physiological or behavioral rhythms at the organismic level: All known clock cells use molecular loops that close within cells and, thus, do not require cell–cell interactions to produce a circadian rhythm. However, to be effective for the organism, these molecular oscillations have to be transduced within the clock cell to change its activity and then outside the cell to induce daily changes in behavior or general physiology (3–5).

Under natural conditions, rhythmicity is synchronized to certain environmental factors acting as “external Zeitgebers”, but under constant conditions (e.g., continuous darkness) circadian rhythms “free-run” with periods slightly deviating from 24 h (1, 2). The expression of circadian rhythms at the organismic

level can be influenced by individual experience, and certain characteristics of an animal’s previous synchronized state can be retained for some time, even when the environmental situation changes. An example of this kind of “history-dependence” is provided by the “circadian time–place association”, i.e., the capacity to store information about the time and place of daily food availability within the circadian system and to recall it during subsequent cycles (6, 7). The so-called “carry-over” effect describes the phenomenon that the avian pituitary gland retains a pattern of gonadotrophin secretion characteristic of a stimulatory photoperiod for 1 or more days after transfer to a nonstimulatory photoperiod (8). Similarly, photic inducibility of c-Fos expression in the rat suprachiasmatic nuclei, i.e., the circadian “light responsiveness” of the major mammalian pacemaker, depends on the previous photoperiodic experience of the animals (9, 10). Such photoperiodic “aftereffects” suggest that information characterizing a particular daylength can be stored within the organism and subsequently used to control photoperiodic reactions for some time (11). This capacity also is expressed in activity aftereffects: Day-active animals previously exposed to long photoperiods retain longer activity times ( $\alpha$ ) of feeding or locomotion when transferred to constant light or darkness than animals previously exposed to short photoperiods (11–13).

If it is indeed one of the major functions of circadian systems to store daily recurring environmental information and to recall it on a daily basis (14), animals could construct their daily routine from an array of time and place predictions, based on the repetition of individually acquired information and intrinsic reinforcement of their successful “behavioral yesterday.”

What is the physiological basis of this kind of memory, and where is it localized? Answers to these questions are likely to be found through the analysis of components known to be part of the circadian pacemaking system, particularly after isolation from the animal when systemic feedback mechanisms are excluded. Recently, rhythms of multiunit neuronal activity in slices of the suprachiasmatic nucleus from hamsters entrained to distinct light/dark (LD) cycles were demonstrated to show photoperiod-dependent durations of high activity (15). In comparison to mammals, the avian circadian pacemaking system seems to be more complex, being composed of at least three major components containing autonomous circadian oscillators: the pineal gland, the retina, and a central nervous hypothalamic component possibly equivalent to the mammalian suprachias-

Abbreviations: LD, light/dark; DD, continuous darkness.

See commentary on page 11677.

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matic nucleus (16–18). The relative contribution of these components to circadian pacemaking varies among species, but in many passerine birds the pineal organ and its periodic melatonin production are of prime importance (19). In house sparrows kept in constant conditions, pinealectomy usually eliminates overt circadian rhythms (20). Rhythmicity can be restored with the appropriate phase if the pineal of a conspecific is implanted into an arrhythmic pinealectomized bird (21), or if melatonin is periodically applied (22, 23). These and other results, indicating that the sparrow pineal gland is both a circadian oscillator and a major component of the circadian pacemaking system, qualify this organ as a potential substrate of circadian memory phenomena. To investigate this possibility, we have asked whether the differential patterns of melatonin secretion characteristic for birds kept under long or short photoperiods are maintained in explanted pineal glands.

## Methods

**In Vivo Synchronization.** Two groups of adult male house sparrows were kept in individual activity-recording cages for 4 weeks, group 1 being synchronized to an LD schedule of 16/8 h ( $n = 20$ , “short-night”), and group 2 to an LD schedule of 8/16 h ( $n = 20$ , “long-night”). Daylight intensity was 100 lux. Food and water were provided ad libitum.

**Photoperiodic Conditions.** Both groups had identical “light off” times at 6 p.m. Because of the different night lengths, “light on” times were different by 8 hours, occurring at 2 a.m. in the short-night group and at 10 a.m. in the long-night group. When released into constant conditions, “light on” was omitted. Thus, “continuous darkness” started at the time at which light had been turned off during the preceding exposure to LD cycles. In contrast, the onset of “prolonged darkness” is defined as the time at which lights had been turned on during the previous LD exposure.

**Recording of Activity Rhythms.** Feeding activities were recorded during 4 weeks of synchronization in all animals. Activity times ( $\alpha$ ) were calculated as the intervals between activity onsets and offsets of feeding, and periods ( $\tau$ ) as the intervals between sequential onsets of feeding activity detected with the help of CHRONO II software (24). After 4 weeks of synchronization, six animals from each group were released into continuous darkness (DD) for 1 week to allow additional monitoring of activity during free-running conditions.

**In Vivo Melatonin Rhythms.** To obtain 24-h melatonin profiles during synchronization to the two distinct photoperiods, 12 blood samples were taken within 24 consecutive days from another 12 animals. Blood was sampled every second day at particular Zeitgeber times (ZTs) such as to scan the 24-h day with a 2-h sampling interval (ZTs 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 h). Thereafter, melatonin profiles under constant environmental conditions were determined: Animals were released into DD for 2 days, bled twice during prolonged darkness with a minimum time lag of 12 hours between bleedings, and subsequently resynchronized to their previous LD conditions for 5 days. This protocol was repeated six times to obtain melatonin profiles over a full circadian cycle.

Samples of 150–200  $\mu$ l of blood were collected in heparinized capillary tubes by puncturing the wing vein. For sampling at night and during DD, a weak red light beam from a fiber-optic light source was aimed at the point of venipuncture. Blood samples were immediately centrifuged to separate the plasma and stored at  $-70^{\circ}\text{C}$  until analyzed by RIA as previously described (25, 26).

For calculations of the duration of elevated melatonin, individual data were separately plotted and the mean of three daytime values served as baseline for peak detection. GraphPad

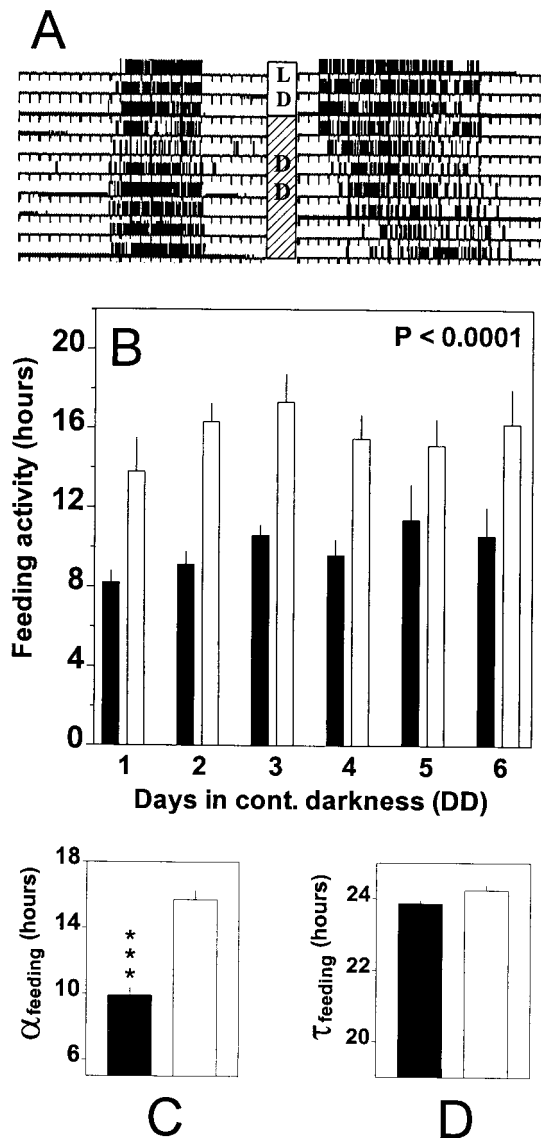
PRISM software (GraphPad, San Diego) was used to determine the times of the beginning (defined as an at least 3-fold increase of melatonin above baseline), the highest point, and the end of each peak. The durations of elevated melatonin levels were obtained by calculating the intervals between individual peak onsets and offsets. The highest points were used to determine peak amplitude levels for each individual bird.

**In Vitro Culture of Explanted Pineal Glands.** Pineal glands were explanted from eight animals of each photoperiodic group for *in vitro* culture to investigate circadian rhythmicity of melatonin release. All pineal glands were explanted at identical Zeitgeber time, 2–3 h before the end of the light period. The glands were quickly removed from the brain and transferred to individual superfusion chambers inside a purpose-built flow-through culture system placed in a light-tight box.

A constant temperature of  $40^{\circ}\text{C}$  in the culture chambers was maintained by a circulating water bath. Culture medium [consisting of Dulbecco’s minimum essential medium base (Sigma) supplemented with 50 units/liter penicillin, 0.05 mg/liter streptomycin, 2.25 g/liter glucose, 0.3% sodium bicarbonate, and 20 mM HEPES, pH 7.35, was equilibrated by continuously gassing with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (vol/vol) throughout the experiment] was delivered to the culture chambers according to a particular protocol by a multichannel peristaltic pump. The final data set was obtained by performing two independent but identical *in vitro* experiments with four glands from each experimental group per experiment to ensure the reproducibility of the results. Pineal glands were maintained under weak illumination (10 lux) until the end of the light period (the time of “light off” during *in vivo* synchronization) and subsequently cultured for 4 days in darkness. Superfusates were sampled starting with the onset of continuous darkness at 2-h intervals throughout culture.

**HPLC.** For qualitative and quantitative analysis of released melatonin, samples were automatically injected into an HPLC system with electrochemical (coulometric) detection. The mobile phase (pH 4.38) was a mixture of 20% acetonitrile and buffer containing 50 mM citric acid, 50 mM sodium acetate, and 0.05 mM EDTA. Methoxyindoleamines were separated in a spherisorb ODS II reversed phase column (100  $\times$  4.6 mm; 3  $\mu$ m) and oxidized at a potential of +550 mV versus a platinum reference electrode. Melatonin in the superfusates was identified after comparison with external standards containing authentic 5-methoxytryptamine, 5-methoxytryptophol, 5-methoxyindole acetic acid, and melatonin.

**Data Analysis.** Melatonin values obtained by HPLC originally were plotted as absolute amounts of melatonin released into the superfusate per sampling interval. To compensate for differences in the overall levels of melatonin produced by individual glands, melatonin release was normalized relative to the 4-day average release rate for each pineal gland. These normalized values were plotted as a function of time in culture. For calculations of circadian period and duration of elevated melatonin, individual data from each cycle were plotted separately as residuals from linear regressions to compensate for drifts in baseline values. The mean of at least three consecutive baseline values (successive data points that were not significantly different from each other) served as baseline for peak detection. The GraphPad PRISM software then determined the x coordinates of the beginning (defined as at least 10% of the way between the minimum and the maximum y values of the peak), the highest point, and the end of each peak. Peaks that did not reveal an at least 3-fold (300%) increase of melatonin (representing the smallest measured amplitude) from baseline to the highest point were omitted from the analysis. Periods were estimated by calculating the average time intervals between consecutive onsets and consecutive ends of the peaks. The durations of elevated

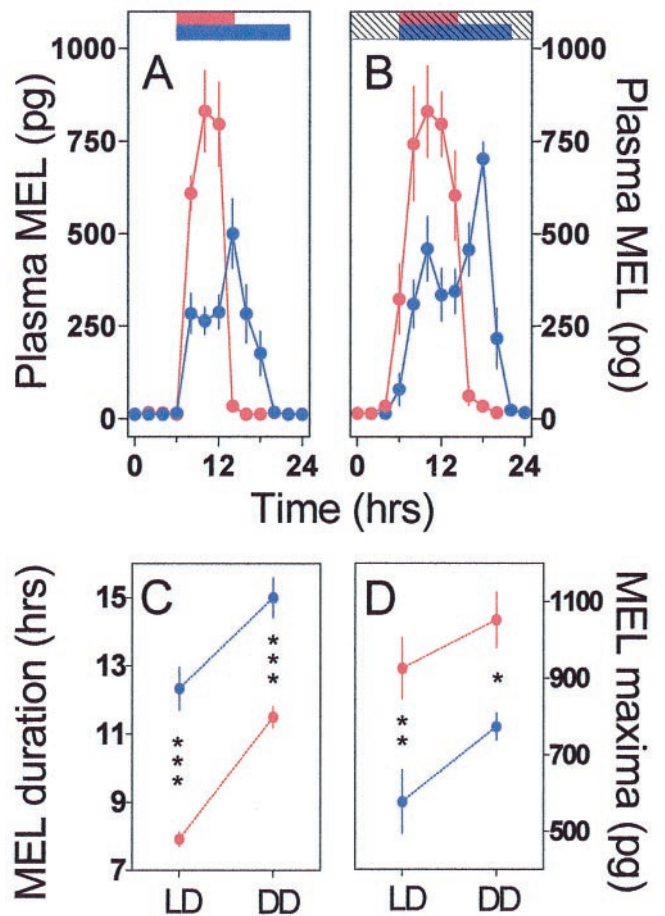


**Fig. 1.** Patterns of feeding activity of house sparrows under constant conditions after exposure to different photoperiods. (A) Activity recordings of two representative birds from the LD 8/16 group (Left) and from the LD 16/8 group (Right) during the last 3 days of synchronization (LD) and during the first week in continuous darkness (DD). (B) Mean ( $\pm$ SEM;  $n = 6$ ) daily feeding activity times during the first week in DD. (C) Mean ( $\pm$ SEM) duration of daily feeding activity ( $\alpha$ ) during the first week in DD. (D) Mean ( $\pm$ SEM) period ( $\tau$ ) during the first week in DD. \*\*\*,  $P < 0.001$ ; black bars, LD 8/16 group; open bars, LD 16/8 group.

melatonin levels were obtained by calculating the intervals between individual peak onsets and offsets. All statistical analyses were performed according to Motulsky (27).

## Results

**Activity Rhythms.** Activity rhythms of all birds of the two photoperiodic groups (LD 18/6 h and LD 8/16 h) were fully synchronized with the 24-hr LD cycles, activity being restricted to the light fraction (Fig. 1A). All animals maintained rhythmic feeding activities with significantly different daily activity times when released into DD (repeated measures ANOVA with Tukey's post test,  $P < 0.0001$ ; Fig. 1B). Average activity times during 1 week in DD were  $9.91 \pm 0.45$  h in the LD 8/16 group and  $15.71 \pm$



**Fig. 2.** Plasma melatonin during synchronization and prolonged darkness. *In vivo* melatonin profiles during synchronization with two distinct photoperiods (A) and during the first 24-h interval in prolonged darkness (B). Symbols represent mean values  $\pm$  SEM ( $n = 6$ ) of melatonin (MEL) in pg/ml plasma. Melatonin values ( $\bullet$ ), and night length (A) as well as projected dark periods (B, filled bars) are plotted in red for the short-night group and in blue for the long-night group. Hatched bar on top of B indicates prolonged darkness. Hour zero in A and B represents 12 p.m. (C) Mean ( $\pm$ SEM) durations of elevated melatonin levels in the short-night group (blue symbols) and in the long-night group (red symbols) during synchronization (LD) as well as in prolonged darkness (DD). (D) Maximum peak values of nighttime melatonin during synchronization (LD) as well as in prolonged darkness (DD). Symbols represent mean values  $\pm$  SEM of the durations of elevated melatonin levels in hours (C) and of maximum melatonin values in pg (D). \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ .

0.57 h in the LD 16/8 group ( $t$  test,  $P < 0.0001$ ; Fig. 1C). The average periods of the two groups were similar:  $23.87 \pm 0.08$  h for the LD 8/16 group and  $24.24 \pm 0.16$  h for the LD 16/8 group ( $P > 0.05$ ; Fig. 1D).

**Plasma Melatonin.** During synchronization with the two photoperiods, pronounced day/night variations of circulating melatonin were detected in all animals: Plasma melatonin levels were low during daytime and increased after the onset of darkness. Melatonin reached distinct peak values during the night phase and decreased to baseline values before the onset of light in both groups (Fig. 2A and B). Durations of elevated melatonin levels were significantly different between the two photoperiodic groups [ $7.92 \pm 0.22$  h in the short-night group (LD 16/8),  $12.34 \pm 0.63$  h in the long-night group (LD 8/16);  $P < 0.001$ ; Fig. 2C]. In prolonged darkness, durations of elevated melatonin levels increased similarly in the two groups. Thus, the differences in the durations of elevated melatonin levels were maintained



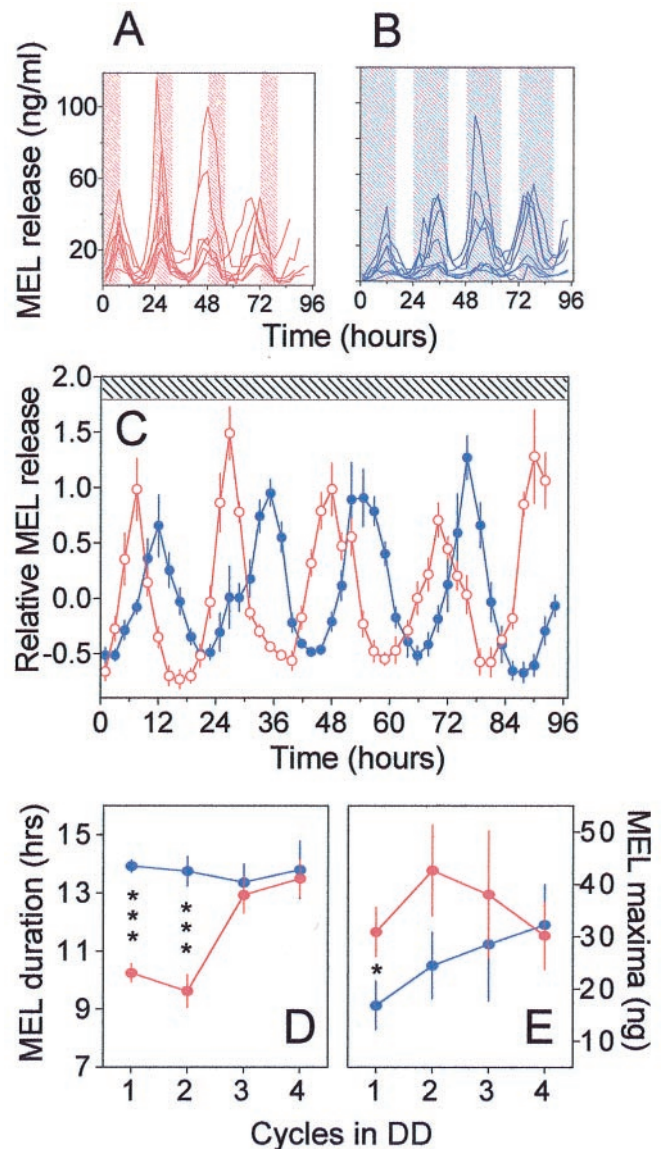
( $11.50 \pm 0.32$  h in the short-night group;  $15.00 \pm 0.58$  h in the long-night group;  $P < 0.001$ ; Fig. 2C). Melatonin peak values also were significantly different during synchronization ( $P < 0.01$ ) as well as in prolonged darkness ( $P < 0.05$ ; Fig. 2D)

**In Vitro Melatonin Release.** *In vitro* melatonin release from pineal glands of the two photoperiodic groups continued to show a free-running circadian rhythm throughout prolonged darkness, with similar circadian periods ( $21.74 \pm 0.34$  h in the long-night group and  $21.37 \pm 0.26$  in the short-night group,  $P > 0.05$ ; Fig. 3A–C). This circadian rhythmicity was characterized by significant differences in the durations of elevated melatonin levels and maximum peak values at the beginning of the experiment as well as a prominent phase difference, with the short-night pineal glands phase-advanced relative to the long-night glands throughout 4 days in culture. The initial phase of melatonin production in the cultured pineal glands corresponded to the projected dark period of the preceding LD cycles to which the intact animals were exposed (Fig. 3A and B). During the night after explanation, relative melatonin release increased in the two groups from hour zero in culture (onset of darkness and sampling-start) through hour 8. A marked difference emerged after 10 h in darkness: At that time, melatonin release from the short-night pineal glands had declined, reaching baseline levels over the following two sampling points. In contrast, melatonin release from the long-night pineal glands continued to increase over 4 more h and then declined beginning 14 h after lights off. As a consequence, the durations of elevated melatonin levels were significantly different during the first night in culture,  $10.25 \pm 0.31$  h in the short-night group and  $13.93 \pm 0.20$  h in the long-night group ( $P < 0.001$ ; Fig. 3C), directly reflecting the difference in night length to which the intact animals previously had been exposed. This difference in the durations of elevated melatonin levels was still detectable during the following night in prolonged darkness ( $9.63 \pm 0.56$  h in the short-night group, and  $13.75 \pm 0.50$  in the long-night group;  $P < 0.001$ ; Fig. 3C). Beginning with the third night in culture, the durations of the melatonin signals were similar in the two groups (Fig. 3D). A significant difference in maximum peak values of *in vitro* melatonin release could be detected in the first night in culture ( $P < 0.05$ ; Fig. 3E).

## Discussion

Our data provide evidence that certain parameters of the circadian pacemaking system of the house sparrow reflect synchronization to distinct photoperiods: Daily feeding activity times were longer and durations of elevated plasma melatonin were shorter in birds kept under short-night conditions as compared with long-night birds. Additionally, amplitudes of plasma melatonin were significantly different, high in short-night birds and low in long-night birds. These differences in activity and plasma melatonin were maintained in prolonged darkness, indicating that previously experienced photoperiodic information is stored within the circadian pacemaking system and can be retained under constant conditions.

Photoperiodic patterns imposed on house sparrows during *in vivo* synchronization additionally modulated *in vitro* melatonin release from isolated pineal glands: During the first two nights in culture, the durations of night length during *in vivo* synchronization were rather precisely reflected by the durations of elevated melatonin levels. Additionally, the difference in melatonin amplitudes found between LD 8/16 birds and LD 16/8 birds were retained during the first night in culture, and the time of “light on” seemed to be “expected,” as indicated by the decline of melatonin release at the particular times at which lights had gone on during synchronization of the donor sparrows by different LD cycles. This “light on” information (acquired *in vivo* and recalled *in vitro*) is likely to be the major reason for the



**Fig. 3.** Characteristics of the free-running circadian rhythmicity of *in vitro* melatonin release. Individual profiles of melatonin release (ng/ml per gland per sampling interval) from pineal glands of the two photoperiodic groups ( $n = 8$ ) throughout 4 days of culture in continuous darkness (A and B). Data are plotted as a function of projected Zeitgeber time. Hatched columns indicate projected dark periods. Melatonin values (lines) and projected dark periods (hatched columns) are plotted in red for the short-night group and in blue for the long-night group. Hour zero represents onset of darkness (sampling start). (C) Mean values  $\pm$  SEM ( $n = 8$ ) of relative melatonin release from pineal glands of the two photoperiodic groups indicate the prominent phase difference of the circadian oscillations. Melatonin values ( $\bullet$ ) are plotted in red for the short-night group and in blue for the long-night group. Hour zero represents onset of night (sampling start). Hatched bar on top indicates prolonged darkness. Durations of elevated melatonin are significantly different during the first two cycles in culture (D), whereas maximum peak values (E) significantly differ during the first night in culture only. Symbols represent mean values  $\pm$  SEM of the durations of elevated melatonin in hours (D) and of maximum melatonin values in ng (E). \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ .

persisting phase difference of the circadian melatonin oscillations in the two photoperiodic groups. Because our pineal glands did not receive any exogenous signal that could have acted as a Zeitgeber during *in vitro* culture and because the only difference between the glands' histories was photoperiod, our results

suggest that the phase of the circadian melatonin rhythm was set before explantation by synchronization of the circadian oscillator to a specific parameter of the photoperiod, presumably “light on,” and then maintained by the isolated pineal glands.

Theoretically, the retention of certain photoperiodic parameters by the pineal gland could be the basis for aftereffects of photoperiod at the organismic level (11, 12). However, in comparison to the retention of distinct activity times in the intact animals (Fig. 1), the photoperiodic aftereffects on the durations of elevated melatonin levels found in our isolated pineal glands are only transiently expressed. It has been hypothesized that the duration of the daily activity time in constant conditions is the result of photoperiod establishing a set of phase relationships between two or more constituent oscillators of the circadian system, which are then able to retain this temporal pattern for a certain time after transfer to constant conditions (13). Thus, the long-term retention of previous conditions in the intact house sparrows could result from the interaction of at least two components of the circadian system, e.g., the pineal gland, a hypothalamic oscillator possibly equivalent to the mammalian suprachiasmatic nucleus, and/or the retina (17, 18).

In many organisms, including the house sparrow (28), the annual change in photoperiod provides the major environmental time cue for the control of seasonal activities, notably reproduction (29–31). Animals must be able to discriminate between

short and long days to perform photoperiodic time measurement, and there is evidence that the circadian pacemaking system is involved in this process (29). Our present data indicating a retention of photoperiodic information by the pineal gland suggest that this system might be used for determining whether day length is increasing in spring (if “light on” advances in relation to what is memorized) or decreasing in autumn (if “light on” is delayed). Additionally, the transient retention of photoperiodic information by the pineal gland might allow animals to reduce the effects of photoperiodic noise resulting from day-to-day changes of weather conditions: By calculating sliding averages from day lengths measured and retained during 2 or more successive days, photoperiodic curves could be smoothed and timing relative to season could be improved.

Circadian time keeping phenomena can undoubtedly result from a variety of rather different mechanisms. We provide initial experimental evidence that part of the mechanism that stores photoperiodic information in the house sparrow is based on endogenous circadian oscillations generated in the pineal gland itself.

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