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Development of post-hatching melatonin rhythm in zebra finches (*Poephila guttata*)

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Abstract. We examined levels of melatonin in the pineal, eyes and plasma over a 24 h period during development in the altricial zebra finch. Beginning as early as 2 days after hatching there was a distinct 24 h rhythm in melatonin in the pineal and plasma. Beginning at day seven after hatching there was also a 24 h rhythm present in the eyes. In the pineal and eyes the amplitude of the 24 h rhythm increased with age. In contrast, the amplitude of the plasma melatonin rhythm at 2 days was already within the range of adults and did not increase with age. These results confirm and expand earlier findings in the European starling and parallel those from precocial birds indicating that the circadian system is already competent at or shortly after hatching even in atricial birds.
Key words. Melatonin; ontogeny; zebra finch; circadian development.

Recent studies on the ontogeny of circadian rhythmicity have demonstrated 24 h changes in melatonin levels in the pineal gland associated with light-dark cycles in 18-day-old embryos of the precocial chicken (*Gallus domesticus*)[1]. Subsequent investigations revealed similar rhythms in the pineal, eyes and plasma, not only of one-day-old hatchlings of the precocial Japanese quail (*Coturnix japonica*), but also in the pineal and plasma of the altricial European starling (*Sturnus vulgaris*)[2]. Studies of pineal N-acetyltransferase activity have shown that rhythmic changes in this enzyme are responsible for the circadian production of pineal melatonin in 18-day-old chick embryos[3]. Therefore, in both altricial and precocial birds one important component of the circadian system, the pineal, appears to be functional at a very early age and even embryonically in the chicken. This contrasts with the situation in rats where the rhythmic production of melatonin probably begins postnatally[4] although other components of the circadian system appear functional earlier[5].

To explore these phenomena further in an altricial bird we investigated the ontogeny of rhythmic melatonin production in the zebra finch. We chose this species because it breeds readily in aviaries throughout the year. In contrast, European starlings do not readily raise their young in aviaries and reproduction is restricted to a few months of the year, thereby limiting opportunities for data collection. In addition, the starling study noted above concentrated on the development of the melatonin rhythm in the plasma and little or no information was available on the ontogeny of melatonin in the pineal and eyes.

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Material and methods

Zebra finches were kept in an aviary under the natural photoperiod of Andechs, Germany (48 °N, 11° 11′ E). Young were sampled over a 10 month period as they became available. Consequently, individuals were taken over a range of daylengths from 9.5 h to 17.5 h. Samples taken at 2000 and 0400 were taken during months when it was dark at these times. Nests were checked daily or on alternate days to determine hatching date. Blood samples from adult zebra finches were taken at four hour intervals (0400, 0800, 1200, 1600, 2000 and 2400) over a 24 hour period.

To follow the ontogeny of the melatonin rhythms, nestling zebra finches were sampled on the 2nd, 5th, 7th, 9th and 15th day posthatching at the same four hour intervals over a 24 hour period as the adults. An additional subset of one-day-old young were also sampled to determine the melatonin levels in the plasma, pineal and eyes at 2400. After a blood sample was taken and the bird killed, the pineal gland and eyes (vitreous humour and lens discarded) were collected. Blood samples were centrifuged and the plasma stored at −70 °C. Pineals and eyes were frozen on dry ice and subsequently stored at −70 °C until assayed. All samples were taken in the dark with the aid of a pen flashlight.

After volumes were made equal with distilled H_2O, plasma (from 25–120 µl depending upon age) was extracted overnight in chloroform with 1 M NaOH. The following day after pulse centrifugation, the chloroform layer was aspirated off using a Pasteur pipette, dried under nitrogen and redissolved overnight in assay buffer (0.1 M Tricine). Pineals and eyes were homogenized in assay buffer and extracted in the same manner as plasma. To determine extraction efficiency 2000 cpm of ^{3}H-melatonin were added to each extraction tube. Mean

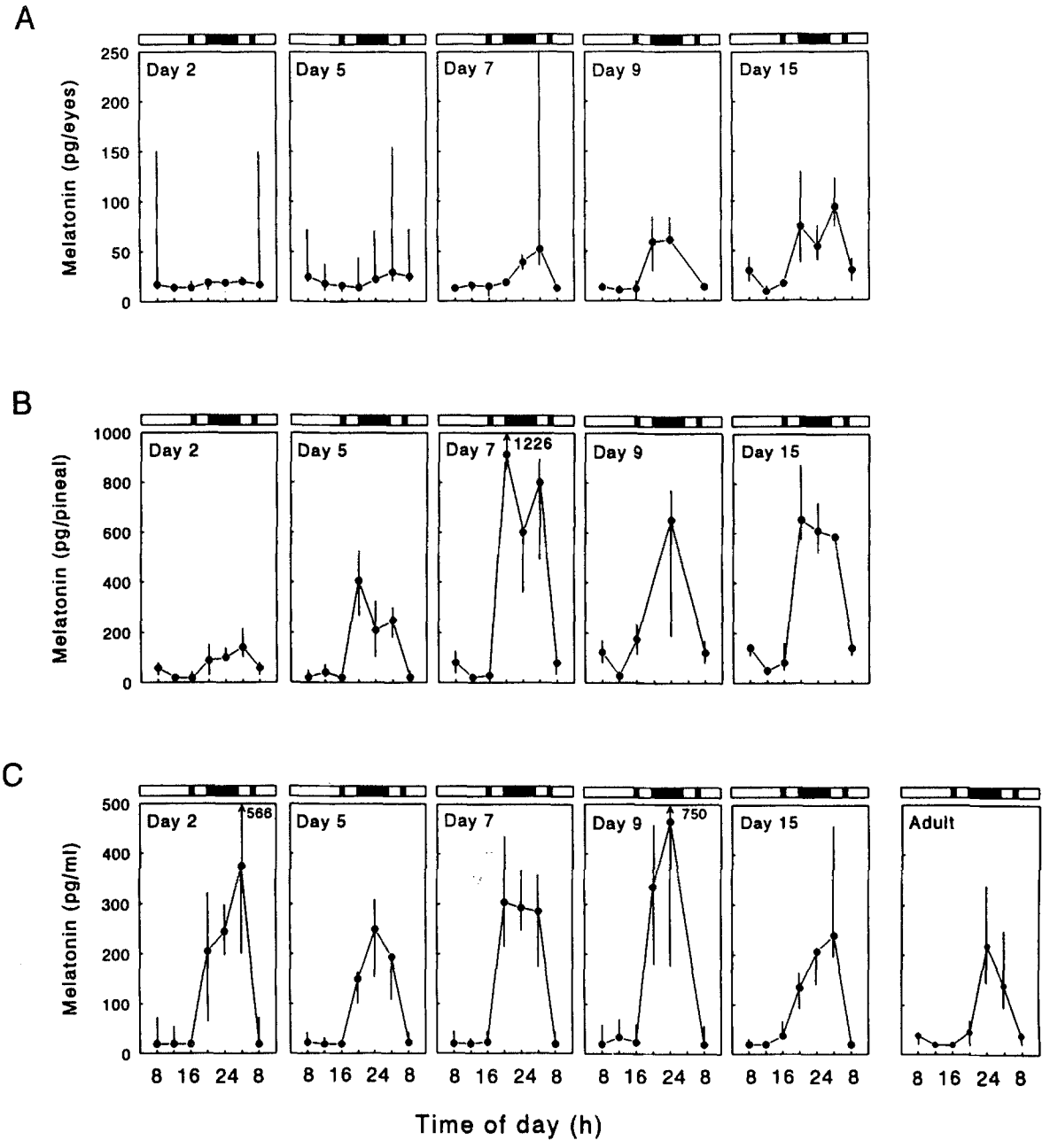

Figure 1. Post-embryonic development of ocular *A)*, pineal *B)* and plasma *C)* melatonin rhythm in zebra finches. Points with vertical lines represent medians with quartiles. Median sample size for each time point for each age is 7. The maximum (slash) and minimum (black bar) length of darkness based upon twilight at Erling-Andechs is shown above the respective figure.

recovery was 92.4% and 76.6% for plasma and tissue samples, respectively.

Melatonin was measured by radioimmunoassay in 200 ul aliquots (after extraction). Sheep anti-melatonin antiserum (G/S/704-8483) was purchased from Stockgrand Ltd., Guildford, U.K. and ^{3}H-labelled melatonin with a specific activity of 3148.7 GBq/mM from NEN, Du Pont. The mean lower detection limit of sensitivity was 18.6 pg/ml ($n = 9$ assays). Intra-assay variation (CV) was $< 3\%$ and interassay variation 6.3% for plasma control (100 pg/ml).

Statistical comparisons of melatonin levels over time within each age group were made on log transformed data using ANOVA and grouped using a modified LSD with Bonferroni adjustment (SPSS Inc.). In age groups where the data did not conform to parametric assumptions, Mann-Whitney U-tests were employed to determine significance at selected transitions between light

and dark time points. A $p < 0.05$ was considered significant.

Results

Ocular melatonin content increased significantly from day 1 to day 2 at 2400 (ANOVA, $F = 16.4$, $p < 0.001$). Median levels in the eyes were 12.0 ($n = 5$) and 28.7 ($n = 8$) pg/ml for one- and two-day-old hatchlings, respectively. Beginning at age 7 days levels of *ocular* melatonin increased significantly from 1600 to 2400 and decreased significantly from 0400 to 0800 (fig. 1A). There was a significant effect of age on melatonin level at 2400 and 0400 (ANOVA, $F = 14.1$, $p < 0.0001$; $F = 4.2$, $p < 0.01$ for 2400 and 0400, respectively) where melatonin increased with age. However, daytime levels were the same in all age groups. The development of the rhythm in the eyes coincided with the opening of the eyelids at age 6 or 7. Prior to age 7 days melatonin levels did not change over the 24 hour period and there was no difference between any of the day and nighttime melatonin levels at any point in the cycle.

There was also a distinct daily rhythm in melatonin content of the *pineal* (fig. 1B). Levels were significantly higher at 2400 than 1600 and significantly lower at 0800 from 0400 in all age groups. There was a significant effect of age on the amplitude of melatonin content at 2400 and 0400 (ANOVA, $F = 8.6$, $p < 0.0001$; $F = 27.4$, $p > 0.0001$ for 2400 and 0400, respectively) where the amplitude increased with age. In two-day-old birds the median melatonin content during darkness ranged from 88 pg/pineal at 2000 to 141 pg/pineal at 0400. Compared with day 2, melatonin levels had increased approximately two fold by age five days and six fold by age 15 days. Median melatonin levels in one- and two-day-old young at 2400 were 75 ($n = 10$) and 101 ($n = 5$) pg/pineal, respectively. However, the difference is not significant. There were no differences in melatonin content between the age groups during daylight.

A concomitant daily rhythm in *plasma* melatonin beginning as early as day 2 after hatching is shown in figure 1C. The amplitude at this age was already similar to that of an adult zebra finch and did not increase with age. Levels increased significantly from 1600 to 2400 and remained higher until 0400 when levels decreased significantly at 0800 in all age classes. Median plasma levels at 2400 were 285 ($n = 7$) and 245 ($n = 9$) pg/ml in one- and two-day-old nestlings, respectively, and are not significantly different.

Discussion

The present results widen the scope of avian species exhibiting a distinct melatonin rhythm early in ontogeny. As in the European starling, the only other altricial species studied[2], the zebra finch had distinct melatonin rhythms in the plasma and pineal that were already in the same range as adult birds as early as two days after hatching. The sample of one-day-old birds at 2400 indicates that at least at this hour, melatonin levels were similar to those of two-day or older birds.

There were also similarities between the development of the melatonin rhythm of the zebra finch and the precocial Japanese quail[2]. In both species there was a melatonin rhythm in the plasma and pineal beginning at one day posthatching. However, in the zebra finch the amplitude of the 24 h profile in plasma melatonin did not increase with age. In contrast, in Japanese quail the amplitude of the plasma melatonin rhythm increased until day seven but declined to adult values in 14-day-old hatchlings.

As in the Japanese quail, the pineal melatonin content of zebra finches increased with age. The ocular melatonin content followed a similar developmental sequence, albeit delayed, to that of pineal content. Ocular melatonin content did not rise significantly during darkness until the young were seven days old, coincident with the opening of the eyelids. In Japanese quail the amplitude of ocular melatonin also increased dramatically between day one and seven. However, in contrast to the zebra finch, the eyes of Japanese quail are fully open at hatching. The similarity between the timing of the increase in amplitude of the ocular melatonin rhythm between a species that hatches with fully open eyes and one that hatches blind suggests that production of ocular melatonin in the zebra finch is unrelated to the opening of the eyelids. Therefore, the timing of the increase in melatonin could be a consequence of an enhanced capacity of the eyes to produce melatonin during the course of post-hatching development.

The present data suggest that in the altricial zebra finch the primary source of plasma melatonin in the first days after hatching is the pineal rather than the eyes. Surprisingly, while the plasma levels of melatonin of two- and five-day-old young were in the same range as those of 15-day-old young, pineal melatonin levels were much lower. Since it is unlikely that ocular melatonin contributes to plasma levels in two- and five-day-old zebra finches (see above) the higher plasma levels could be the consequence of a slower rate of degradation of melatonin in the first week after hatching. Consistent with this, developmental changes associated with the metabolism of melatonin possibly leading to higher circulating levels of melatonin were found in rats[6]. Alternatively, it is conceivable that other sources such as the gastrointestinal tract contribute to plasma melatonin[7]. However, there is no evidence that gastrointestinal melatonin contributes to circulating levels.

Like the European starling, zebra finches are altricial. They hatch nearly naked and require almost constant brooding. However, despite being altricial both species resemble the precocial Japanese quail and domestic fowl

in terms of post-hatching ontogeny of the plasma and pineal melatonin rhythm. The function of the rhythmic melatonin during the first days of life is not yet clear but the similarity between the altricial and precocial birds suggests that it could be involved in the timing of hatching or entrainment of physiological processes that are common to both groups.

It is not known whether the melatonin rhythms of the nestling zebra finches are endogenous or simply imposed upon the birds by the environment. In either case the birds must be susceptible to some external factor(s) that undergo 24 h changes. Our zebra finches nested in small domed baskets in which grasses are tightly woven. Hence, little light penetrated the nest. Still, it is possible that light is a factor responsible for the pronounced 24 h melatonin rhythm, but other stimuli such as temperature or mechanical stimuli resulting from egg turning may also be involved.

1 Zeman, M., Gwinner, E., and Somogyiova, E., Experientia *48* (1992) 765.
2 Zeman, M., and Gwinner, E., J. comp. Physiol. A *172* (1993) 333.
3 Binkley, S., Comp. Biochem. Physiol. *64A* (1979) 201.
4 Binkley, S., Endocr. Rev. *4* (1983) 255.
5 Davis, F. C., and Mannion, J., Am. J. Physiol. *255* (1988) R439.
6 Weinberg, U., and Gasparini, F. J., in: Melatonin Rhythm Generating Systems, Developmental Aspects, p. 193. Ed. D. C. Klein. Karger, Basel 1982.
7 Huether, G., Experientia *49* (1993) 665.