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Exogenous melatonin accelerates re-entrainment: attenuation of the circadian rhythm of metabolic rate in the canary, *Serinus canaria*

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Abstract. Administration of melatonin in the drinking water (200 µg/ml in 1% ethanol) decreased the time of re-entrainment of the circadian rhythm of the metabolic rate (measured as oxygen uptake) of domestic canaries (*Serinus canaria*) after 10-h delay phase shifts of the light-dark (LD) cycle by 1.3 days on average. Associated with faster re-entrainment, the amplitude of the metabolic rhythm was attenuated by 46% on average on the first day after the shift as compared with about 25% in the controls. After re-entrainment, the amplitude of the metabolic rhythm during melatonin administration was about 23% lower than in the controls. The minimum resting metabolic rate increased by ca 5% on average during treatment with melatonin. The results are consistent with the hypothesis that constant high plasma levels of melatonin act on higher levels of the circadian oscillatory system rather than by directly affecting peripheral or central photoreceptors.
Key words. Melatonin; circadian rhythms; metabolic rate; re-entrainment; birds.

The pineal organ and its hormone melatonin play an essential role in the generation and persistence of autonomous, self-sustained circadian rhythmicity in birds, primarily oscine Passeriformes[1]. Circadian rhythms are entrained to light-dark (LD) cycles by the interaction of circadian oscillators (pacemakers) and photoreceptors that are situated in the pineal gland, the eyes and in the hypothalamus[2]. Entrainment properties of the avian circadian system have been studied in various species of birds by shifting the LD cycle (zeitgeber) relative to behavioral rhythms and by determining the time and direction of re-entrainment[3–5]. Recently, it was shown that continuous administration of melatonin accelerated re-entrainment of the circadian activity rhythm in house sparrows (*Passer domesticus*)[6]. Similar effects on re-entrainment of behavioral and endocrine rhythms by continuous or periodic application of melatonin were known of rats and hamsters, as well as of humans[7–13]. It is, however, not known where melatonin acts upon the circadian system: (1) on the pacemaker itself, (2) on the photoreceptors, (3) on integrative sensory structures or (4) on the coupling between circadian pacemakers and 'secondary' (damped) oscillators that control various behavioral functions.

In most studies on effects of light, hormones or other factors upon the avian circadian system, locomotor activity was measured as overt rhythmic function. Locomotor activity in a caged bird, however, may reflect different motivational states, including activities related to feeding, preening and so forth, as well as spontaneous activity[14,15]. Its quantitative determination depends on the experimental methods, which involve such diverse recording devices as movable perches, motion detectors and photocells, as well as on the location in the cage where activity is being measured. By measuring metabolic rate (e.g. oxygen uptake), a quantitative determination of total activity and basal (resting) metabolic rate can be obtained, and possibly a closer representation of the effects of melatonin on the underlying oscillatory system.

In the present study, re-entrainment of the circadian rhythms of metabolic rate after 10-h phase shifts of the LD cycle, either by lengthening of light time (L) or dark time (D), was studied in the domestic canary (*Serinus canaria*) to determine the effects of oral administration of melatonin on the circadian system of a passerine bird.

Materials and methods

Male domestic canaries were held individually in chambers (30 × 20 × 38 cm) with free access to food (canary seed mixture) and water. Continuous oxygen uptake was measured using an open-flow system with a paramagnetic oxygen analyzer and a mean flow rate of 40 l/h[16]. The metabolic chamber was made of Plexiglas. Its upper half was divided into two compartments: one with a perch and the water reservoir, the other with the feeder and a grid floor. To feed, the bird had to pass an opening in the dividing screen. Spilled seeds and husks were collected in a drawer below the grid floor. The bottom of the chamber was covered with paper and a sand dish containing additional minerals. Feeding activity was measured by a photocell in front of the feeder, and the data were processed on an event recording system (ERS, University of Groningen). Pure water,

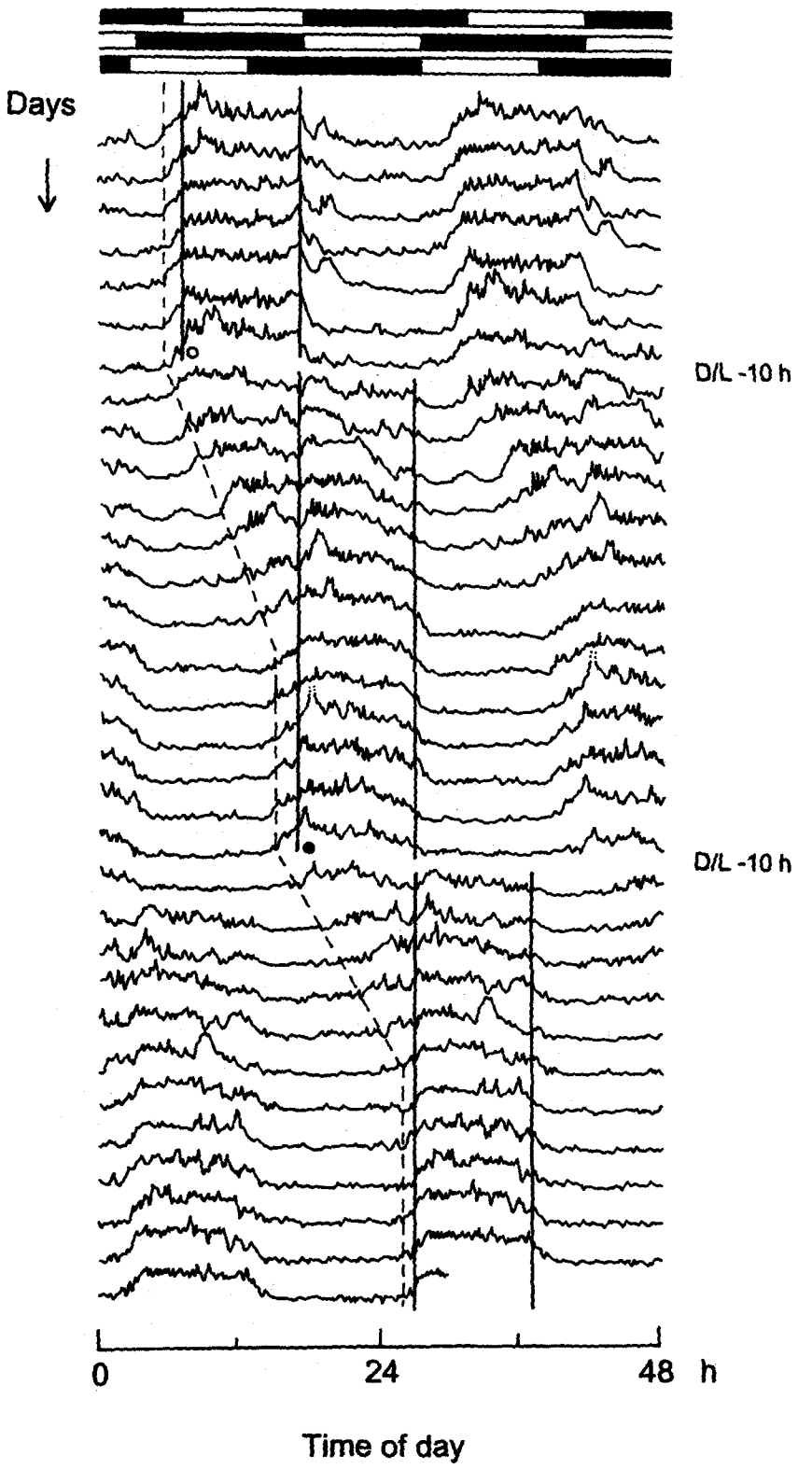

Figure 1. Double-plotted recording of oxygen uptake of canary no. 1 kept in LD 10:14 (ca 30:1 lux) and subjected to two 10-h delay phase shifts of the LD cycle by lengthening of D. Open circle below curve: time of administration of 1% ethanol in the drinking water (control shift); closed circle below curve: administration of melatonin (200 µg/ml dissolved in 1% ethanol). Light time (L) is shown by two vertical lines (the three consecutive LD regimes are shown on top of the figure). Broken lines indicate mean phases of daily increase in metabolic rate and the duration of re-entrainment (t_r) until the new steady-state phase relation between rhythm and LD cycle is achieved.

1% ethanol, or melatonin (200 µg/ml dissolved in 1% ethanol) were supplied by a peristaltic pump from outside the soundproof cabinet. Fluorescent white light illuminated the metabolism chamber from the top. Light intensities measured above the chambers were ca 30 lux in L and ca 1.0 lux in D with an FLMX 007 luxmeter (Optronik, Berlin) and a silicon cos V (λ) receptor. The cages were cleaned at two-to-three-day intervals at different times of day when the birds were active.

After re-entrainment of the circadian rhythms of metabolic rate and feeding following the first phase shift of the LD cycle (control test), the birds were subjected to the same phase shift while melatonin was administered in the drinking water. Melatonin was first given shortly after lights-on of the day before the shift until a new stable phase relationship between rhythm and zeitgeber was attained for about one week.

Data analysis. The time of re-entrainment (t_r) was determined (1) by calculating the mean phase of increase in oxygen consumption from mean resting level relative to onset of L (ϕ_o) for six to seven days before the phase shift; (2) by fitting a line from the last ϕ_o before the shift to first ϕ_o of the new stable phase relation between rhythm and zeitgeber after the shift; and (3) by measuring the days between the two phases (examples shown in figs 1 and 2).

For determining the metabolic parameters, original chart recordings of oxygen uptake were photographically reduced to an appropriate size. Resting metabolic rate (RMR) was measured by averaging the minimum values of oxygen uptake over at least 2 h during rest time. The amplitude of the rhythm of oxygen consumption was determined using RMR as minimum and the average of the values measured over the first 5 to 6 h of activity time as maximum. A second peak shortly before onset of D or rest time was not used for determining the amplitude.

Parametric as well as non-parametric tests were used to evaluate differences in t_r of the rhythms of oxygen consumption and feeding activity, in amplitude of the metabolic rhythm and in RMR between the control and the experimental condition. *t* Tests for pair differences and Wilcoxon signed-rank tests were applied to all parameters. Two-way analysis of variance (ANOVA) was used for testing possible differences in the effects of melatonin on the various parameters between the two types of phase-shift experiments (D/L–10 h and L/D–10 h).

Results

In the first experiment, six canaries were tested from January to March under LD 10:14 by shifting the LD cycle by a single 10-h lengthening of D. As an example, the original recording of oxygen uptake of canary no. 1 is shown in figure 1. Following the first phase shift with 1% ethanol in the drinking water (control test), the metabolic rhythm reached the new steady-state phase relationship between rhythm and zeitgeber (ψ_o) after eight transient cycles. When treated with melatonin (second phase shift), t_r lasted only six days. All canaries re-entrained by delay transients, and five of the six birds shifted their metabolic rhythms at a faster rate during treatment with melatonin (see table). Re-entrainment was associated with a reduction of the amplitude of the metabolic rhythm on the first day after the control shift, and the amplitude was even further reduced when melatonin was administered (see table). After the control phase shift, the mean amplitude reached its initial value, while during administration of melatonin it maintained a level below that of the controls. After pure water replaced melatonin, the mean amplitude gradually increased.

In the second experiment, eight canaries kept in LD 12:12 were subjected to 10-h phase shifts by lengthening of L during March and April (n = 4) and during November and December (n = 4). Two of the eight birds were first subjected to melatonin treatment and then to the control conditions. Original recordings of oxygen uptake of two canaries (nos 9 and 10 in table) that were tested in reverse order are shown in figure 2A and B. Following the phase shifts, the circadian

Table. Summary of the results on the effects of melatonin on the time of re-entrainment (t_r), on the amplitude of the metabolic rhythm and on RMR after phase shifts of the LD cycle. Percentage differences in metabolic parameters refer to the following states: A = entrained state before first shift; B = first day after control shift (1% ethanol); C = entrained state after control shift and before shift with melatonin; D = first day after shift with melatonin; E = entrained state after shift with melatonin. Negative values indicate longer t_r, lower amplitude or lower RMR during the second state as compared with the first one. Positive values refer to the opposite relations.

Phase shift	Bird #	t_r (Δd)	Amplitude			RMR C/E (Δ/%)
			A/B (Δ%)	C/D (Δ%)	C/E (Δ%)	
D/L–10 h	1	−2	−21	−37	−13	+10
	2	−2	−28	−34	−26	+11.5
	11	+1	−43	−63	−36	+4.5
	12	−2	−40	−43	−25	+5
	13	−3	−15	−51	−31	−5
	14	−1	−29	−59	−31	+5
L/D–10 h	3	−3	−36	−57	−27	+4
	4	0	0	−42	−29	+7
	5	−2	−19	−36	−12	0
	6	0	0	−56	−39	0
	7	−1	−18	−53	−5	0
	8*	0	−32	−16	0	0
	9	−1	−42	−62	−33	+17
	10*	−2	−23	−41	−14	+10
Mean	n = 14	−1.3	−24.7	−46.4	−22.9	+4.9
SE		0.3	3.7	3.5	3.2	1.6

*Melatonin test before control test.

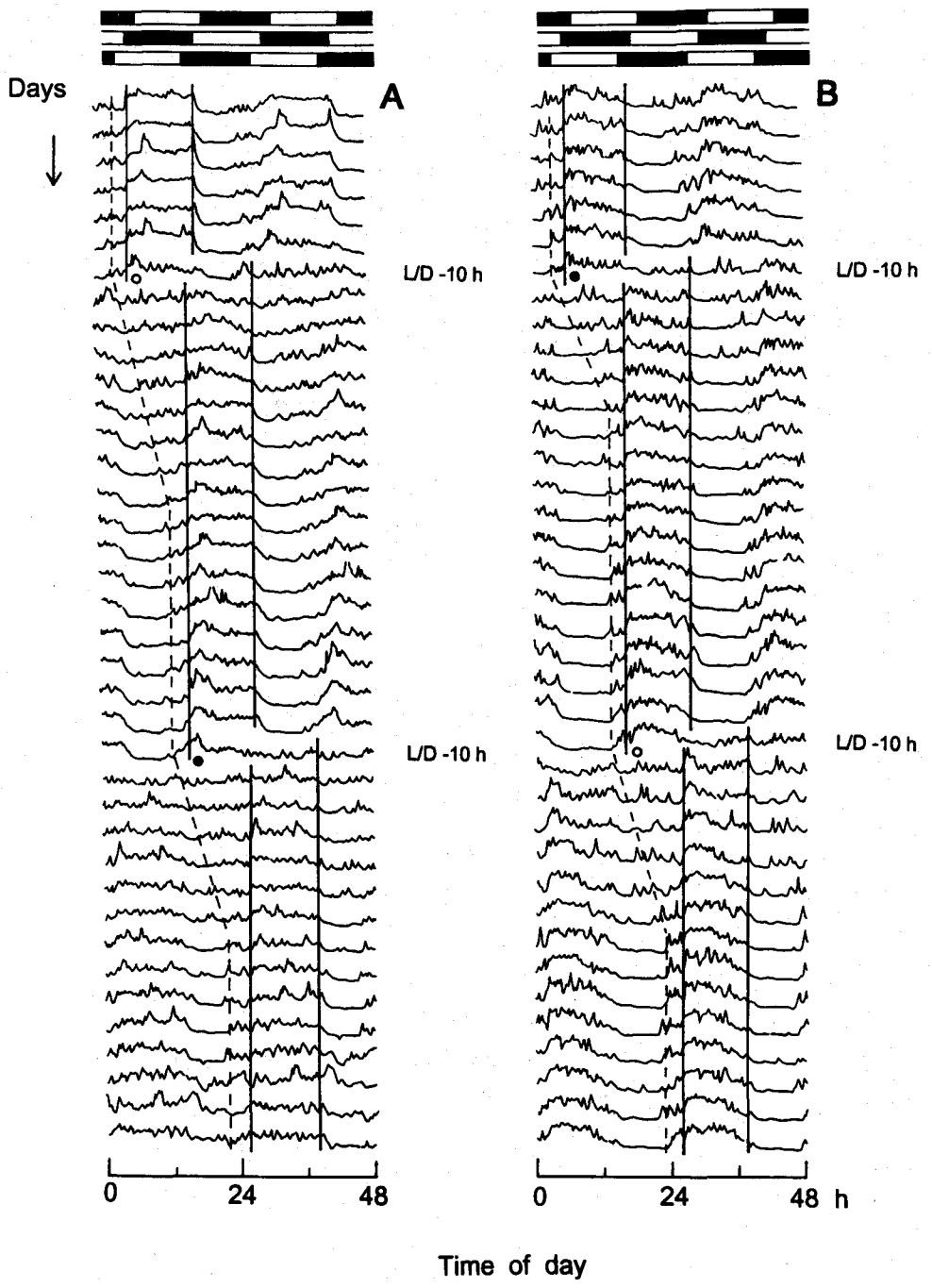

Figure 2. Double-plotted recordings of oxygen uptake of canaries no. 9 (*A*) and no. 10 (*B*) kept in LD 12:12 (ca 30:1 lux) and subjected to two 10-h delay phase shifts by lengthening of L. Further details as in figure 1.

rhythms of all birds re-entrained by delay transients. In five of the eight birds, t_r was faster during treatment with melatonin than in the control test.

The effect of melatonin on t_r of the metabolic rhythm was evaluated over all birds tested in both experiments, since no significant differences were found between the experiments (two-way ANOVA). Re-entrainment was faster by 1.3 days on average when melatonin was administered (see table) (t test for pair differences: $t = 3.99$, df 13, $p < 0.01$; Wilcoxon signed-rank test: $T^* = 2.5$, $n = 11$, $p < 0.01$).

In figure 3A, the mean amplitudes of the metabolic rhythms of all birds that were tested in the same order are shown for consecutive days during the two experiments. Since no significant differences were found between the experiments (two-way ANOVA), the data were combined for statistical treatment. Significant differences were found (1) between controls before the phase shift and on the first day after the shift ($t = 2.79$, df 13, $p < 0.02$; $T^* = 0$, $n = 12$, $p < 0.001$), (2) between the re-entrained state and first day after the shift with melatonin ($t = 10.1$, df 13, $p < 0.001$; $T^* = 0$,

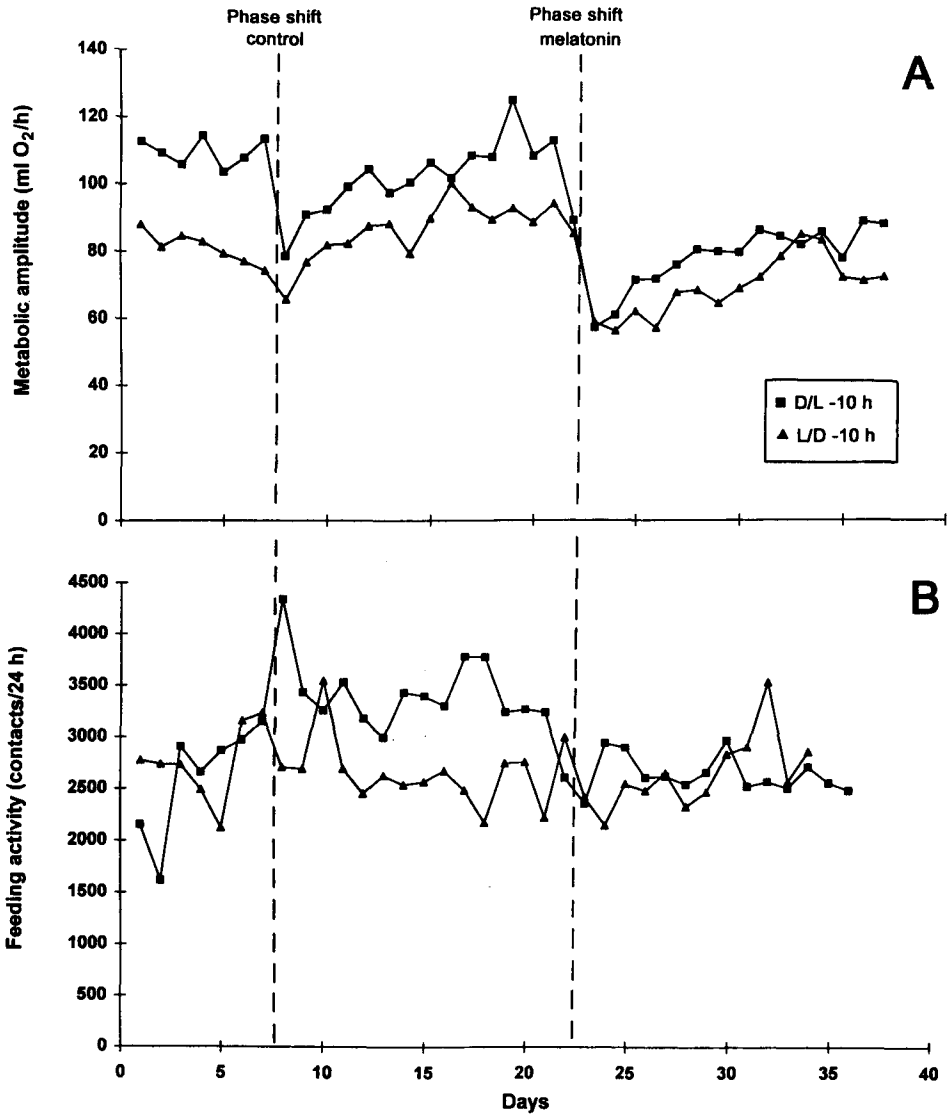

Figure 3. (*A*) Mean amplitudes of the metabolic rhythm of canaries in two experiments with phase shifts by lengthening of D (D/L–10 h) or L (L/D–10 h) of the LD cycle. Control phase shift with 1% ethanol in the drinking water. Each mean represents five to six birds. (*B*) Mean daily amounts of feeding of the same birds in the two experiments as in (*A*).

$n = 14$, $p < 0.001$); (3) between re-entrained states before and after the shift with melatonin ($t = 6.36$, df 13, $p < 0.001$; $T^* = 0$, $n = 14$; $p < 0.001$). Significant differences were also found between the amplitudes during the first days after the phase shifts and the following entrained states.

RMR between the entrained states without and with melatonin increased by 4.9% on average. Since no significant differences were found between the two experiments (two-way ANOVA), the data were combined. The increase in RMR was significant at the 95% level ($t = 2.66$, df 13, $p < 0.05$; $T^* = 4$, $n = 10$, $p < 0.05$).

Re-entrainment of the feeding rhythm was also faster during treatment with melatonin in the majority of the birds tested in both experiments. With the paired t test, the difference in t_r was significant at the 95% level ($t = 2.29$, df 10, $p = 0.045$), while no significance could be detected with the non-parametric signed-rank test. The amount of feeding per 24 h was not systematically influenced by either the phase shift or melatonin treatment (fig. 3B).

Discussion

The present study shows that administration of melatonin in the drinking water accelerated re-entrainment of the circadian rhythms of metabolic rate after 10-h delay phase shifts of the LD cycle by 1.3 days on average. Preliminary radioimmunoassay analyses suggest that plasma concentration of melatonin was constantly high during the 24-h period. Since the changes in amplitude were similar in the two experiments (see fig.

3) and all birds re-entrained by delay transients, the following assumptions are based on the data of all 14 individuals. Associated with re-entrainment during treatment with melatonin was an attenuation of the amplitude of the metabolic rhythm of almost 50% on the first day after the shift as compared with only ca 25% in the controls. After re-entrainment, the mean amplitude was reduced by 23% on average during melatonin administration (see table). In 10 of the 14 canaries (71%) tested in the two experiments, an acceleration of re-entrainment was associated with a significant reduction of the amplitude of the metabolic rhythm on the day after the phase shift and/or after re-entrainment when melatonin was administered. In only 3 birds (nos 4, 6 and 11 in table), no such relationship between changes in amplitude of the rhythm and t_r was observed. When being entrained to an LD cycle, a dose-dependent reduction in amplitude of the rhythm of perch hopping was found after implantation of melatonin in intact house sparrows[17]. In the present study, a reduction in locomotor activity could account for the decrease in maximum metabolic rate immediately after the phase shift of the zeitgeber and for that caused by melatonin. It is, however, remarkable that the amplitude of the feeding rhythm was not influenced by melatonin, which supports the hypotheses that different behavioral functions, such as locomotion and feeding, are differently influenced by endogenous (e.g. hormonal) or exogenous factors (e.g. light), and that they are controlled by separate pacemakers[18,19].

Constant high plasma levels of melatonin may have a tonic effect, either by influencing light perception in the retinae or by modulating sensory input at the level of retinorecipient structures in the supra-chiasmatic nuclei (SCN) or analogues in the avian brain[20,21]. However, instead of (or besides) tonic influences, the absence of phasic effects by periodic release of melatonin from the pineal may itself have profound effects on the properties and stability of the circadian oscillatory system. A specific model interprets the diversity of avian circadian systems with regard to the degree of interaction of various oscillatory components[18]. In case of a sparrow, the absence of a periodic input from the pineal to an extra-pineal pacemaker system (EPPS) would reduce the amplitude of the EPPS oscillation and weaken the coupling strength between EPPS and 'secondary' oscillators. A reduced amplitude of the pacemaker may be associated with a higher 'amplitude' of the phase response curve (PRC) to light pulses, which characterizes the responsiveness of the circadian system to changes in light intensity[22]. In previous studies on birds and mammals, it was documented that the degree of persistence of circadian rhythmicity was inversely related to the degree of responsiveness of the system to periodic light signals[5] or even to periodic injections of melatonin[23]. In a recent study on the mammalian circadian system, Warren and Cassone[24] claimed that the pineal gland, via its rhythmic secretion of melatonin, may be involved in coupling different components of the circadian system. Since several circadian functions of pinealectomized rats were disrupted in constant bright light in a similar way, the authors suggested that melatonin acts at a higher level at the SCN, either on coupling among multiple SCN oscillators or between SCN oscillators and multiple outputs.

An important finding of this study was that RMR increased substantially ($\geqslant 10\%$) in some birds after melatonin was administered, though in the majority of the birds RMR was not considerably influenced (see table). The circadian rhythm of oxygen uptake represents at least two independent functions: a rhythm of resting metabolic rate and a rhythm of activity metabolism[25]. A simultaneous reduction in activity metabolism and increase in RMR reduces the amplitude of the metabolic rhythm by a considerable amount. The increase in RMR cannot be attributed to a direct effect of melatonin on basal metabolic rate and/or body temperature, since a decrease in both functions would have been expected when plasma levels of melatonin are constantly high[26–28]. It is therefore conceivable that the observed increase in RMR represents changes in the mean level and/or amplitude of oscillatory units (pacemakers) underlying the rhythm of the basal metabolic rate.

An alternative hypothesis is based on the assumption that constant high plasma levels of melatonin may enhance the sensitivity of the circadian system to light changes either by way of direct effects on the retinae or other photoreceptive structures, or by modulating sensory input at higher levels of the light information processing system[29,20,21]. Melatonin is known to be involved in adaptation of the retina to dark, probably by modulating dopaminergic neurotransmission[30–32]. It is also well known that adaptation to dark decreases the intensity discrimination of the eyes of invertebrates as well as of vertebrates. In the canary, a decrease of the mean level of illumination (e.g. 10 vs 1 lux) does not change the minimum amplitude of the light-dark cycle to which the circadian system can entrain (unpubl. data). Since an increase of light responsiveness in the retinae by melatonin would be in contradiction to these findings, at the present state of knowledge effects of melatonin on higher levels of the circadian system are more probable.

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