Food availability but not melatonin affects nocturnal restlessness in a wild migrating passerine

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A B S T R A C T

A large number of passerine species migrate at night, although most of them are diurnal outside the migratory seasons. This diurnal-to-nocturnal transition is a major life-history event, yet little is known about its physiological control. Previous work showed that during the migratory periods captive birds showing nocturnal migratory restlessness (Zugunruhe) have reduced concentrations of circulating melatonin at night compared to non-migratory periods. This suggested that the hormone melatonin, a main component of the avian circadian system, is involved in the expression of Zugunruhe. Other studies demonstrated that the relationship between low melatonin levels and Zugunruhe is not a seasonal correlation. When Zugunruhe was interrupted by exposing birds to a fasting-and-refeeding protocol, melatonin levels increased. Here we studied whether melatonin and food availability influence the intensity of Zugunruhe in wild migrating garden warblers (*Sylvia borin*) at a stopover site. Birds were held in recording cages overnight, with or without food available, and either bled to determine melatonin concentrations or treated transdermally with melatonin. We found that melatonin levels at night were correlated with the intensity of diurnal locomotor activity and with condition, but were not correlated with Zugunruhe. Similarly, the melatonin treatment did not have effects on Zugunruhe, whereas food availability increased it. Our study shows that the nocturnal melatonin levels in migrating warblers depend on food availability and are correlated with condition. In addition, it suggests that melatonin does not control Zugunruhe and might rather be involved in energy conservation and/or clock synchronization during migration.

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Introduction

A large proportion of birds are nocturnal migrants, i.e. they are usually diurnal but migrate mainly or exclusively at night (Berthold, 2001). The mechanisms controlling nocturnal migration are largely unknown. Early studies suggested an involvement of the circadian system. The pineal gland and its hormone, melatonin, are major components of the avian circadian system (reviewed in Cassone et al., 2009; Gwinner et al., 1997). The pineal gland contains an autonomous circadian oscillator that controls the rhythmic release of melatonin (reviewed in Gwinner and Brandstaetter, 2001; Takahashi et al., 1980). Melatonin release is high at night and low during the day, irrespective of whether the species is diurnal or nocturnal (Arendt, 1998; Kumar et al., 2000).

In captivity, nocturnal migrants show 'Zugunruhe' or nocturnal migratory restlessness, which is a measure of migratory disposition. In garden warblers that are in migratory condition, melatonin secretion

remains high at night and low during the day (Gwinner et al., 1993). However, the peak of night melatonin levels is lower during migratory periods than during the winter quiescent phase (Gwinner et al., 1993). This reduction of melatonin amplitude during migratory periods could simply reflect a seasonal correlation or be a consequence of the fact that the birds are awake at night (see discussion in Gwinner, 1996). Alternatively, low melatonin might be a proximate factor facilitating nocturnal activity.

Follow-up studies on the blackcap *Sylvia atricapilla*, a closely related warbler species, investigated whether the decrease in the night amplitude of melatonin was specifically related to nocturnal migration. This was done by comparing seasonal changes in the nocturnal melatonin profile between resident (non-migratory) and migratory blackcaps in the laboratory (Fusani and Gwinner, 2001). Blackcaps from migratory populations showed patterns of melatonin similar to those described for garden warblers (Gwinner et al., 1993), i.e. the melatonin amplitude was lower during the migratory period, when bird showed nocturnal activity, than at other times of the year, when birds did not show nocturnal activity (Fusani and Gwinner, 2001). On the contrary, resident birds did not show significant seasonal changes in the amplitude of melatonin (Fusani and Gwinner,

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2001). This within-species comparison suggested that changes in the night melatonin amplitude during migratory periods were directly related to migratory behaviour (Fusani and Gwinner, 2001). The study also indicated that the presence of nocturnal activity per se did not induce changes in melatonin amplitude, because resident birds showing nocturnal activity did not have different melatonin peaks compared to night-inactive birds of the same population (Fusani and Gwinner, 2001).

Another experiment tested whether the correlation between Zugunruhe and melatonin was a functional one. Studies on spotted flycatchers (*Muscicapa striata*) (Biebach, 1985) and garden warblers (Gwinner, I. Schwabl-Benzinger, and H. Schwabl, unpublished results, reported in Gwinner, 1996; Gwinner et al., 1985, 1988) had shown that a short period (2–3 days) of food deprivation followed by one day of ad libitum food temporarily suppresses Zugunruhe. This phenomenon presumably mimics the situation of a bird that has been fasting during a long migratory flight and interrupts migration upon reaching a suitable re-fuelling site. Using this experimental 'fasting and re-feeding' protocol we tested whether the experimental suppression of Zugunruhe was accompanied by an increase in the nocturnal peak of melatonin, i.e. if the behavioural and the hormonal effects were functionally associated. Indeed, the protocol induced a reduction of Zugunruhe in the first night following food reintroduction which was accompanied by an increase in the nocturnal peak of melatonin (Fusani and Gwinner, 2004) to levels observed during non-migratory periods (Fusani and Gwinner, 2001).

Altogether, the above studies suggested an involvement of melatonin in controlling nocturnal activity in migrants (reviewed in Fusani and Gwinner, 2005, in press). Recently, we showed that in wild migrating warblers condition, and fat reserves in particular, determines the intensity of Zugunruhe (Fusani et al., 2009) and the likelihood of leaving from a stopover site (Goymann et al., 2010). Thus, melatonin could be the link between the nutritional and physiological condition of a migrant and its migratory behaviour.

In this study, we tested whether melatonin influences migratory behaviour in free-living, migrating birds. Garden warblers were caught at a stopover site and held in recording cages for one day. First, we studied whether night melatonin levels were correlated to Zugunruhe in untreated birds. Then, we manipulated nocturnal melatonin levels by treating birds with melatonin administered transdermally to the skin just before dark. The melatonin treatment was done both in the presence and absence of food. Our results shows that melatonin levels are related to physiological conditions rather than to nocturnal activity, and that while food availability has a strong influence on Zugunruhe, experimental elevation of nocturnal melatonin levels does not affect migratory behaviour.

Materials and methods

Study site and housing

The study was conducted on Ponza, a small Italian island in the Tyrrhenian Sea (40°55′ N, 12°58′E) located along one of the main Mediterranean migratory routes. A ringing station, directed by M.C., has been active on the island since 2002. The experiments were conducted between May 1st and May 15th in 2006, 2007 and 2008 on garden warblers caught with mist-nets during routine ringing operations. We describe here the procedures which were common to all experiments. Birds were caught before 12:00 h (GMT + 1) and a single observer (M.C.) scored the subcutaneous fat on a 0–8 scale, the size of the pectoral muscles on a 0–3 scale, and measured the length of the 3rd primary cover and the body mass following standardized European methods (Bairlein, 1994). By 13:00 h the birds were set in custom-built individual fabric cages so that they were visually isolated from each other. Each cage was equipped with an infrared activity sensor connected with an activity recorder which recorded locomotor

activity within the cage in 2-min intervals. The room accommodated 20 cages and received natural illumination from a large door. The birds were left undisturbed until the following morning unless they received the melatonin treatment or a blood sample was taken (see below). All birds were released the following morning after 07:00 h. All experimental procedures were authorized by the competent authorities (Regione Lazio and Italian Institute for Environmental Protection and Research — ISPRA) with respect to Italian laws.

Experimental procedure

In 2006, we used 87 birds to study the relationships between melatonin, physiological conditions, diurnal activity and Zugunruhe. The birds received a full diet consisting of 10 meal worms and 20 g of a mixture of dry insect food, banana, and boiled egg (Fusani and Gwinner, 2004). Water was available ad libitum. A 100 µl blood sample was collected at 24:00 h ± 30 min from the jugular vein with a 1-ml syringe and a heparinized 30-gauge needle. After centrifugation the plasma was separated and frozen at −10 °C and, 14 days later, it was transferred to −80 °C until analysis.

In 2007 and 2008 we performed experimental manipulations of melatonin by spreading a cream containing melatonin onto the skin of the birds, following a protocol that had been previously tested and validated for garden warblers (Goymann et al. 2008). Briefly, we dissolved 13 mg of melatonin (Sigma M 5250) in 200 µl of ethanol and then mixed this solution with 30 g Eucerin (*Eucerinum anhydricum*, Bayersdorf AG, Germany), resulting in a dilution of 430 µg melatonin/g Eucerin cream. The melatonin birds were treated with 100 µl melatonin cream, containing 39 µg melatonin. Control birds were treated with 100 µl Eucerin. The cream was applied to the area of naked skin between the dorsal feather ridge, the wing, and the neck. Such a treatment elevates significantly plasma concentrations of melatonin within the physiological range for at least 4 h following the application of the cream (Goymann et al. 2008). The treatment was conducted at 18:45 h ± 30 min (GMT), i.e. 1 h before civil sunset. We started with randomly selected birds and analysed daily the results of each experimental night (i.e. 20 birds) to evaluate the effects of the treatment. In 2008, thanks to the exceptional number of captures, we were able to perform experiments selecting birds with intermediate fat scores (i.e. 2–4; see Results). In total, the melatonin treatment experiment was done on 196 birds which received either food as described above (N = 57) or no food (N = 139). In both cases water was always available *ad libitum*. From a subset of birds belonging to both food groups (food, N = 20; no food, N = 39) we collected a blood sample at 24:00 h ± 30 min as described above to monitor the effects of the treatment on plasma concentration of melatonin.

Melatonin measurement

The plasma concentration of melatonin was determined by direct radioimmunoassay (RIA) (details in Goymann et al., 2008). The standard curve and sample concentrations were calculated with Immunofit 3.0 (Beckman Inc., Fullerton, CA), using a four parameter logistic curve fit. The lower detection limit of the standard curve was determined as the first value outside the 95% confidence intervals for the zero standard (B_{max}) and was 3.3 pg/ml. The intra-assay coefficient of variation was 3.3%. Melatonin concentrations were adjusted for individual recoveries (mean ± std recoveries 86.0 ± 0.03%).

Data analysis

The activity recording system measured the number of times the infrared sensor was activated for each 2-min periods. From these values we calculated the average activity during a 2-min interval for the period 13:00–19:30 (day) and 19:30–04:30 (night; GMT + 1). The beginning and end of the night period was defined according to civil

Table 1

Spearman's correlation coefficient (rho) between physiological and behavioural variables of migratory garden warblers. Significant correlations are shown in bold face.

		Melatonin	Day activity	Zugunruhe	PC condition
Day activity	Rho	**0.299**			
	P	**0.018**			
	N	**62**			
Zugunruhe	Rho	−0.198	**−0.496**		
	P	0.123	**0.001**		
	N	62	**71**		
PC condition	Rho	**−0.334**	**−0.491**	**0.637**	
	P	**0.008**	**0.001**	**0.001**	
	N	**62**	**71**	**71**	
Body mass loss until blood sampling	Rho	**0.360**	0.043	−0.050	−0.145
	P	**0.004**	0.728	0.690	0.182
	N	**62**	67	67	87

twilight averaged for the period 1–15 May. For birds handled for the melatonin treatment we excluded from the analysis the period from when we entered the experimental room to 20 min after we left the room. For birds used for blood sampling we excluded from the analysis all data following the moment in which we entered the experimental room (24:00 h).

In unmanipulated birds (2006), we studied the correlations between physiological parameters, diurnal and nocturnal (Zugunruhe) activity, and plasma melatonin concentrations. We also calculated an index of body condition extracted by applying principal component analysis (PCA) to body mass, fat score, and muscle score. Explorative analyses showed that activity data were not normally distributed. Standard data transformation methods did not normalize the data; therefore, we calculated non-parametric (Spearman) correlation coefficients.

For the melatonin manipulation experiments of 2007 and 2008, we first performed a global analysis including all experimental birds. To account for non-linear effects of fat on individual levels of Zugunruhe (Fusani et al., 2009), we used generalized additive models (GAMs, Hastie and Tibshirani, 1990) to analyse the effect of melatonin treatment, diet, fat score, and the interactions between melatonin treatment and diet on individual levels of Zugunruhe. A natural cubic spline curve (Wood, 2006) was used to model the effect of fat as a smoother, while melatonin treatment and diet were modelled as factors. Because 182 out of 196 birds had fat scores between 2 and 4, a GAM was also fitted using only individuals with a fat score between 2 and 4 to reduce the effects of the few birds with extreme fat scores. In this second model the fat was modelled as a factor, because there were not enough categories (less than 5) to allow for the use of a smoother. Data distribution could not be normalized using standard transformation methods, therefore we used a quasi-Poisson distribution (Minami et al., 2007) with variance proportional to the mean and a log-link function to constrain the estimates to be positive. This quasi-likelihood approach assumes that the scale parameter Φ of the distribution is unknown and can consequently account for more over-dispersion than the classical Poisson distribution (Wood, 2006). Residuals were analysed using graphical methods (Cleveland, 1993) for homogeneity of variance, violation of normality assumptions and departures from the model assumptions or other anomalies in the data and in the model fit. Significance was set at $P < 0.05$ for all analyses used here.

Results

Relationships between Zugunruhe, melatonin, and physiological conditions

Table 1 reports the correlations between plasma melatonin concentration at 24:00 h, diurnal locomotor activity between 13:00 h and 19:30 h, nocturnal activity (Zugunruhe) between 19:30 and 23:30, body mass loss between capture and blood sampling, and
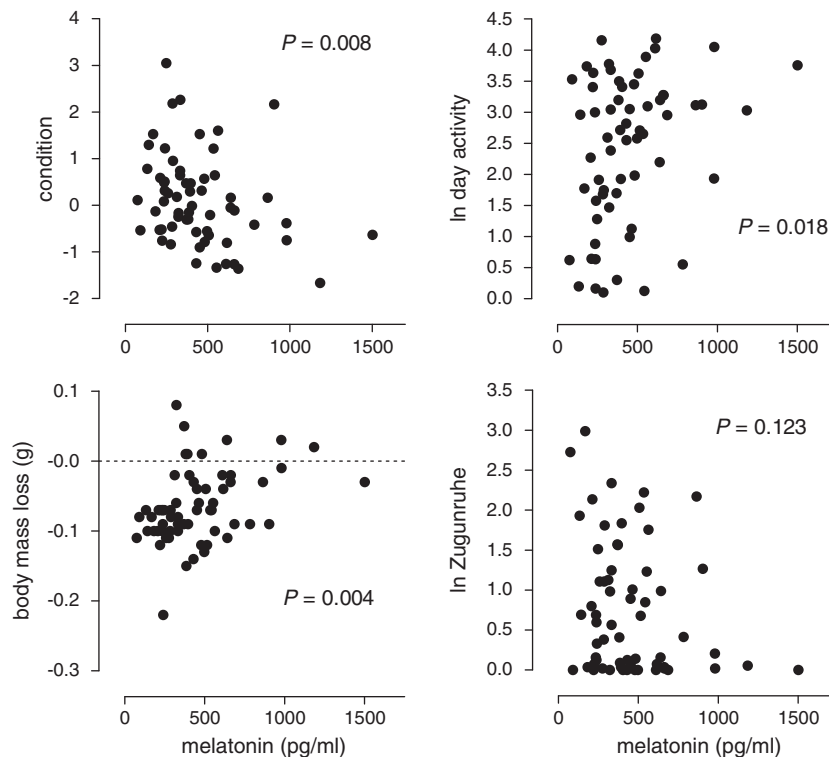

Fig. 1. Scatterplots of night melatonin concentration vs. condition, body mass loss, day activity, and Zugunruhe in migrating garden warblers caught at a stopover site. Melatonin concentration was positively correlated with body mass loss from time of capture to blood sampling (bottom left) and with day activity (top right), and negatively correlated with condition (top left). Melatonin was not significantly correlated with Zugunruhe (bottom right). See Table 1 for statistical details.
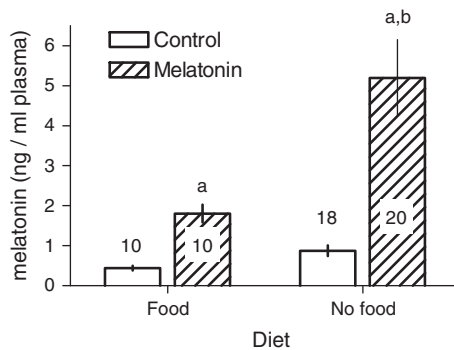
Fig. 2. Mean ± S.E.M. plasma melatonin concentrations in garden warblers at 24:00 h after transdermal treatment with a melatonin cream at 18:45 h (39 μg melatonin/100 μl cream). The treatment significantly increased melatonin levels in treated bird (a = p<0.0001), and the diet had a significant interaction with the treatment, i.e. birds which received no food expressed higher concentrations of melatonin than those that did receive food (b = p<0.001).

the factor 'condition'. This factor was the single, robust principal component extracted by principal component analysis of body mass, muscle score, and fat score, which had an Eigenvalue of 2.185, explained 72.8% of the variance, and was positively associated with body mass (0.877), fat (0.882), and muscle (0.798). Given the large proportion of variance explained and the strong positive association with the non-standardized variables, we concluded that the extracted factor provided a good measure of the physiological condition of the animals.

We found significant correlations between condition and locomotor activity: a positive correlation between condition and Zugunruhe and a negative one between condition and day activity (Table 1 and Fig. 1). Condition was also negatively correlated with plasma melatonin levels. We found no significant correlation between melatonin and Zugunruhe, however melatonin was positively correlated with day activity and with the loss of body mass between capture and blood sampling (Table 1 and Fig. 1). Results did not change when a serial Bonferroni's correction for multiple comparisons was applied to the correlation matrix (Rice, 1989).

Effects of the melatonin treatment on plasma melatonin concentrations

The melatonin treatment successfully elevated plasma melatonin concentration (Fig. 2). The mean melatonin concentration in treated birds was about 4 ng/ml plasma, within the high physiological range for *Sylvia* warblers (Fusani and Gwinner, 2004; Fig. 1). Visual inspection of the data suggested an effect of the diet on melatonin concentrations in treated birds. Therefore, we run an ANOVA with treatment as mean factor, and tested also the interaction with diet and fat score. There was a highly significant effect of the melatonin treatment ($F_{1,50} = 35.825$, $P<0.00001$) and a significant interaction between the treatment and the diet ($F_{2,50} = 8.363$, $P<0.001$), whereas the interaction with fat was not significant ($F_{4,50} = 1.933$, 0.119).

Table 2
Generalized additive model analysis of factors affecting Zugunruhe based on quasi-Poisson distribution with log-link response. All birds (N = 196) are included. Factors with a significant effect are highlighted in bold.

Factor		Coefficient	P
Melatonin treatment		− 0.207	0.073
Diet		**0.385**	**0.042**
Melatonin treatment x diet		− 0.180	0.484
Smooth term	DF	F	P
Fat score	1.77	**4.345**	**0.011**

Effects of the melatonin treatment on Zugunruhe

In a generalized additive model analysis, the diet and the fat score had significant effects on Zugunruhe (Table 2). When the 16 birds which belonged to the extreme fat classes (≤1 and ≥5) were removed from the analysis, the effect of the diet remained significant (Table 3). Thus, Zugunruhe was more intense in birds which were given food compared to those which received no food, independently of the treatment. The results of the GAM analysis are better understood by looking at the data in Fig. 3, where the Zugunruhe shown by the birds are plotted twice, either grouped by diet or by fat score.

Discussion

Our results confirm that condition and diet are major factors in controlling migratory behaviour, but do not support the hypothesis that reduced melatonin levels in migrants are involved in modulating the expression of nocturnal activity. In migratory garden warblers caught at a stopover site and held in recording cages overnight, the intensity of Zugunruhe was correlated with the physiological condition but not with mid-night melatonin concentrations. The experimental elevation of nocturnal levels of melatonin had no effects on Zugunruhe, whereas the availability of food increased the amount of Zugunruhe independently of the melatonin treatment. Thus, our results provide evidence that the physiological condition of birds arriving at a stopover site and the availability of food at the same site are the main factors controlling Zugunruhe and thus migratory disposition in garden warblers, and do not support the hypothesis that these effects are mediated by the hormone melatonin.

The hypothesis that melatonin may directly influence Zugunruhe originated from previous work on garden warblers and blackcaps (reviewed in Fusani and Gwinner, 2005, in press). In these species, the night melatonin peak is lower during migratory periods compared to other life-history stages (Gwinner et al., 1993) (Fusani and Gwinner, 2001). These seasonal changes did not depend on the photoperiod, which was kept constant at 12D:12L in these experiments. In addition, blackcaps from Cape Verde, which do not migrate (Berthold et al., 1990), showed no seasonal changes in the nocturnal melatonin peak (Fusani and Gwinner, 2001). Some birds of the latter population did show some unspecific nocturnal activity ('Nachtunruhe'; Berthold, 1988) which was, however, unrelated to melatonin levels (Fusani and Gwinner, 2001). Follow-up studies showed that in birds in which a temporary interruption of Zugunruhe was induced by means of a diet protocol, the night melatonin peak increased significantly compared to control birds (Fusani and Gwinner, 2004; Gwinner et al., 1988). The above studies altogether suggested three alternative hypotheses: 1) melatonin is involved in controlling Zugunruhe; 2) the variation in melatonin levels is a consequence of changes in locomotor activity; or 3) both melatonin and Zugunruhe are responding to other unknown factors associated with the migratory disposition (Fusani and Gwinner, 2005a,b). The present work does not support either of the first two hypotheses and suggest that melatonin and Zugunruhe respond to common regulatory factors associated with migration.

Table 3
Generalized additive model analysis of factors affecting Zugunruhe based on quasi-Poisson distribution with log-link response. Only birds with fat score 2, 3, or 4 (N = 182) are included.

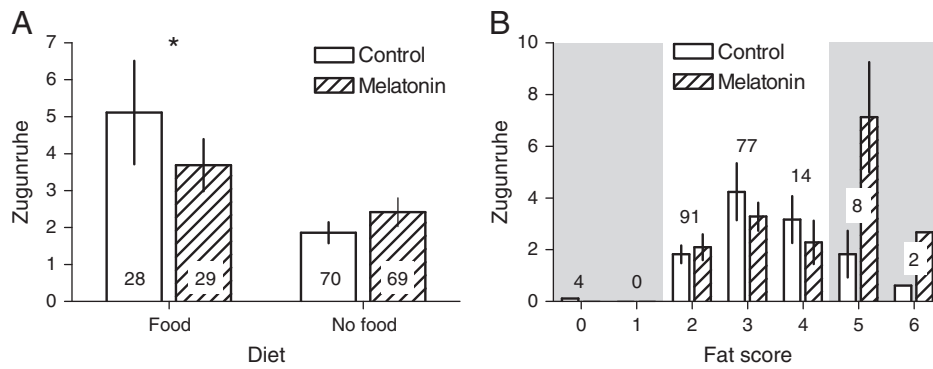| Factor	Coefficient	P
Melatonin treatment	− 0.0128	0.937
Diet	**0.412**	**0.028**
Melatonin treatment x diet	− 0.113	0.653
Fat	0.142	0.181

Fig. 3. Amount of Zugunruhe (mean ± S.E.M.) shown by garden warblers after experimental elevation of the circulating concentration of melatonin. (A) Data grouped by treatment and diet. (B) The same data grouped by treatment and fat score. When all birds were included in the analysis (shaded and unshaded background in panel B), there was a significant effect of the diet and of the fat score. When only birds with fat score 2–4 were included in the model (unshaded background in panel B), only the diet showed a significant effect on Zugunruhe. * = p<0.05, Food vs. No food. See Tables 1, 2, 3 and text for statistical details.

It is unlikely that the lack of significant relationships between melatonin and Zugunruhe depends on methodological aspects. The descriptive study was conducted on a quite large sample, revealing a number of significant correlations between melatonin and other variables including diurnal activity. A similar argument applies to the melatonin treatment experiment. The treatment procedure had been thoroughly validated in the same species and exactly under the same experimental conditions (Goymann et al., 2008) and the sample size was large for a hormone-manipulation experiment. Finally, we monitored the melatonin levels in the experimental animals and found significant differences between treated and untreated individuals at the time when the behavioural effects should have occurred. Therefore, our data strongly indicate that melatonin itself does not affect the expression of Zugunruhe.

The second hypothesis, that the reduction of melatonin amplitude during migration might be a direct consequence of nocturnal activity, is similarly not supported by the present study and by previous work. Some authors argued that nocturnal melatonin is reduced because of a higher exposure to nocturnal light in active birds that keep their eyes open compared to resting birds (see discussion in Gwinner, 1996; Gwinner et al., 1993). However, melatonin does not differ between night-active and night-inactive blackcaps from the non-migratory Cape Verde population (Fusani and Gwinner, 2001). The weak and non-significant relationship between nocturnal activity and melatonin in a large sample of wild, migrating birds reported here adds to the evidence against hypothesis 2. This raises the question of what is the role of melatonin in migratory activity.

A reduction in the amplitude of melatonin might allow migrating birds to synchronize faster to a rapidly changing photoperiod during latitudinal migration (Gwinner, 1996; Gwinner et al., 1997). This interpretation is supported by previous work showing higher peak night melatonin during autumn compared to spring migration, when birds experience a change in day length of several hours within a few days (Fusani and Gwinner, 2004).

Alternatively, melatonin might mediate physiological processes related to energy mobilization and metabolic rates. Melatonin is a major regulator of body temperature (Saarela and Reiter, 1994) and administration of melatonin induces a reduction of body temperature in birds (Murakami et al., 2001). Blackcaps *Sylvia atricapilla* showed a substantial reduction of body temperature at night during spring migration (Wojciechowski and Pinshow, 2009). The authors observed that two birds showing Zugunruhe kept their body temperature relatively high (Wojciechowski and Pinshow, 2009). Reduction of body temperature can lead to a significant reduction in the basal metabolic rate (McKechnie and Lovegrove, 2009), which could help to save energy resources during stopovers and/or buffer body mass and fat loss. We have observed hypothermia in garden warblers caught on Ponza during spring migration (Carere et al., 2010), thus the

phenomenon described by Wojciechowski and Pinshow (2009) might be common to other species and be part of a general 'migratory syndrome' involving a number of correlated physiological adaptations. In the present study, melatonin levels were positively correlated with diurnal activity and a reduction of body mass in captivity, and negatively correlated with condition. Melatonin could respond to factors associated to condition and/or energy expenditure and reduce body temperature when birds interrupt migration to decrease metabolic rate and thus energy consumption. Thus, low levels of melatonin in birds that show *Zugunruhe* (Gwinner et al., 1993; Fusani and Gwinner, 2004) may indicate a state of increased metabolism with higher body temperature and increased energy expenditure. Alternatively, increased metabolism may lead to a faster turnover of melatonin resulting in decreased levels of this hormone. It is suggestive that melatonin levels were lower in melatonin-treated birds which received food compared to melatonin-treated birds which did not receive food. To our knowledge, no study has addressed the effects of diet on melatonin metabolism in birds. In mice, the trend appears to go into the opposite direction (Bubenik et al., 1992). This might be linked to a decrease in extrapineal (gastrointestinal) production of melatonin, as suggested by the high levels of melatonin in the hepatic portal vein of pigs after feeding (Bubenik et al., 2000). The influence of food deprivation and refeeding on melatonin clearly deserves further studies.

Conclusions

The availability of food during the time in captivity increased the amount of Zugunruhe in birds independently of the melatonin treatment. Thus, the availability of food at the stopover site is, together with the physiological condition (Fusani et al., 2009; Goymann et al., 2010), a major factor in controlling migratory disposition and possibly stopover duration. Food intake could directly affect melatonin and Zugunruhe or act by increasing condition and/or day activity. At the same time good condition increases Zugunruhe and decreases nocturnal levels of melatonin. Low levels of melatonin may either be involved in upregulating metabolism required for nocturnal migration or be a consequence of this state of increased energy expenditure. Alternatively, low levels of melatonin during migration may allow synchronization to a changing photoperiod during latitudinal migration (Gwinner, 1996) (Gwinner et al., 1997).

Acknowledgments

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