Temporary caging results in reduced levels of circulating melatonin in migratory robins

Sara Lupi[a,b], Virginie Canoine[c], Paolo Pedrini[d], Leonida Fusani[b,e*]

[a] Department of Live Sciences and Biotechnology, University of Ferrara, Via Borsari 46, 44100 Ferrara, Italy;

[b] Department of Interdisciplinary Life Sciences, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Savoyenstrasse 1a, 1160 Wien, Austria;

[c] Department of Behavioural Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

[d] Sezione Zoologia dei Vertebrati, MUSE, Museo delle Scienze, Corso del Lavoro e della Scienza, 3, 38122 Trento, Italy;

[e] Department of Cognitive Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

[*]Corresponding author: leonida.fusani@vetmeduni.ac.at

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Summary statement

Laboratory studies had shown that melatonin modulates migratory restlessness in passerine birds. Here we show that nocturnal melatonin is lower in caged birds than in those flying at night.

Abstract

The hormone melatonin, a main component of the avian circadian system, plays an important role in the physiological transitions that accompany the activation of the migratory phenotype in passerine birds. Most small passerines migrate at night when circulating concentrations of melatonin are elevated. Previous work measured nocturnal melatonin levels of migratory birds only in captive animals, because free-living individuals are usually caught at day time. In this study, we compared nocturnal melatonin levels of European robins (*Erithacus rubecula*) caught during the day and held in cages overnight with those of birds that were caught at night and sampled immediately. We found that circulating melatonin at night was lower in birds held in cages compared to birds that were actively migrating. This result suggests that temporary caging affects the melatonin system and that in nature melatonin levels could be generally higher than those previously described by studies on captive birds.

Introduction

Most migratory passerines are nocturnal migrants, in that they have usually diurnal patterns of activity but become active at night during migration. The reasons for this peculiar phenotype are still unknown, but several hypotheses have been proposed ranging from improved thermoregulation to reduced predation risk (Berthold, 2001).

The diurnal-nocturnal transition is a major behavioural and physiological switch and at present very little is known about its control. In the last two decades, we have conducted a series of studies that illustrated that the hormone melatonin plays an important role in migratory physiology, especially in the expression of nocturnal locomotor activity, which in captive birds is called nocturnal restlessness or Zugunruhe. Melatonin is a well-known actor in the physiology of day-night rhythms and has been called "the hormone of darkness' because its levels are higher during the night than during the day, independently of the pattern of activity of the species (Reiter, 1991). Furthermore, this hormone plays an important role in controlling avian circadian activity. The main gland that releases melatonin, the pineal, possesses photoreceptors and is a main component of the circadian system (Gwinner et al., 1997).

Previous work showed that Zugunruhe and melatonin levels are inversely associated, higher melatonin levels are typically seen when Zugunruhe is low or absent (Fusani and Gwinner, 2001; Fusani and Gwinner, 2004; Gwinner et al., 1993). When Zugunruhe in blackcaps (*Sylvia atricapilla*) is experimentally reduced through a food deprivation protocol, circulating melatonin increases (Fusani and Gwinner, 2004). The association seems to be specific, because blackcaps of resident populations (Cape Verde) may show some levels of nocturnal locomotor activity but these are not correlated with a reduction in melatonin levels (Fusani and Gwinner, 2001).

We hypothesized that melatonin controls the switch between non-migratory and migratory status, namely, from purely diurnal to diurnal/nocturnal. We tested this hypothesis in garden warblers (*Sylvia borin*) during spring migration by using transcutaneous application of exogenous melatonin (Goymann et al., 2008). However, we found no effects of the melatonin treatment on Zugunruhe (Fusani et al., 2011). This outcome might depend on the time of the year. In fact, previous work of our lab showed seasonal differences in the responsiveness of melatonin to experimental manipulation (Fusani and Gwinner, 2004), and a number of studies had shown that migratory behaviour is less sensitive to disruptive factors in spring than in autumn (Stanley et al., 2012; Wingfield et al., 1990). When we repeated our experiment during

autumn migration, we obtained a strong reduction of Zugunruhe in blackcaps and garden warblers following the treatment (Fusani et al., 2013).

In sum, our previous studies demonstrated an involvement of melatonin in the physiological transitions from migratory to non-migratory status. A major methodological limitation of all previous studies is that melatonin levels were measured in birds that underwent temporary captivity. In fact, wild migratory birds are generally caught during the day, when melatonin levels are low or undetectable. Thus, to date we do not know how the levels measured in captive birds, even if only temporarily, compare to those found in birds that are actively migrating at night. Here, we addressed this question by taking advantage of a ringing station in the Italian Alps, Bocca di Caset, where migratory birds are trapped day and night. We aimed at understanding how melatonin levels measured in birds temporarily hosted in cages compare to those found in birds that are actively migrating. To achieve this objective, we compared birds that had been caught at the stopover site during the day and then hosted in cages overnight with birds that had been caught at night during active migration and bled immediately after capture.

Materials and Methods

Study site

The study was conducted in September 2014 at the Bocca di Caset Ringing Station in the Italian Alps (45°51' N, 10°41' E; altitude: 1.618 meters). During post breeding migration, Bocca di Caset is an important pass for passerine birds crossing the Alps and flying southwards towards their wintering grounds (Pedrini et al., 2008). The ringing station has 7 m high mist-nets that allow the capture of nocturnally migrating birds that at Bocca di Caset fly close to the ground but higher than the reach of standard mist-nets (3 m).

Experimental procedure

We trapped European Robins (*Erithacus rubecula*), the most common alpine nocturnal migrant at Caset, at day and night. Upon capture, robins were ringed following standardized EURING methods, including scoring subcutaneous fat (0–8 scale) and recording body mass to the nearest 0.1 g. We used 113 robins to compare nocturnal levels of melatonin between migrating birds caught at night (hereafter NET) and individuals caught during daytime and kept in captivity overnight (CAGE). To measure melatonin levels we blood sampled (150-200 µl) birds between 2300 and 0300 hours. To reduce the risk that exposure to white light at night affects melatonin

production (de Jong et al., 2016), we used red safelight during sampling. The NET group consisted of 61 birds caught during nighttime between 2300 and 0300 hours and immediately bled. The CAGE group consisted of 52 birds caught during daytime until 2 hours before the civil sunset, and placed in individual custom-built fabric cages (50 x 30 x 30 cm) with water ad libitum under natural photoperiod. CAGE and NET birds were bled at night in parallel. We assessed fat score and measured body mass at bleeding of all birds. We could house a maximum of 6 individuals per night and we centred blood sampling time at 0000. Blood sampling procedures were completed by 0100 hours for CAGE birds and by 0300 hours for NET birds. In each cage, an infrared sensor connected to a PC recorded locomotor activity as number of movements during 2-min periods. We calculated the intensity of diurnal and nocturnal activity as the average number of movements per 2-min interval from 1200 to sunset (diurnal activity) and from sunset to sunrise (nocturnal activity) based on civil twilight times (Greenwich mean time +1). We used the extent of nocturnal migratory restlessness or Zugunruhe, as an indicator of migratory disposition (Fusani et al., 2009). Our cages are optimized for short-term housing of small migratory birds and have been used in a number of studies (Fusani et al., 2011; Fusani et al., 2013).

Melatonin measurement

We determined plasma concentration of melatonin using a commercial radioimmunoassay kit (Melatonin Serum Direct RIA, LDN, BA R-3300RIA). Beforehand, a liquid extraction was conducted overnight with 3 ml dichloromethane. The organic phase was freeze-decanted and then dried in a water bath at 43°C under nitrogen stream. We resuspended the extracts in phosphate-buffered saline and let them stabilize at 4 C overnight. Samples were then processed following the instruction of the company. All samples were assayed in duplicate. The detection limit was 3 pg/ml, the intra-assay coefficient of variation was < 12 % and the inter-assay CV was < 13%.

Data analysis

We performed all statistical analyses with R v.3.5.0 (R Core Team, 2016) using a significance level of $\alpha < 0.05$. We calculated a scaled mass index at time of bleeding as described in Peig and Green (2009) (equation 2). For CAGE birds, we calculated an index of body mass loss between capture and blood collection as (body mass at bleeding – body mass at capture) / body mass at capture. We subdivided the variable time of bleeding into hourly intervals for all birds.

We used a general linear model (GLM) to compare melatonin levels between captive and actively migrating birds in the period 2300 to 0100 controlling for the influence of scaled mass index at bleeding. We chose to include only samples collected during the period 2300 to 0100 to have comparable times of bleeding between NET and CAGE birds (time of bleeding: CAGE = 2300 to 0100 hours; NET = 2300 to 0300 hours). The intensity of Zugunruhe (number of movements/ 2 min) in CAGE birds showed a large variability (mean = 1.6, first quartile = 0.478, third quartile = 2.351; range 0 to 9.549). This variability reflects different dispositions to migrate among CAGE birds. To take this into account, we set a threshold of 1 for the intensity of Zugunruhe to subdivide the CAGE group in two groups: birds that would likely migrate in the wild (CAGE ACTIVE, N = 24) and birds that would likely prolong stopover (CAGE INACTIVE, N = 28). In the GLM we compared melatonin levels between the three groups NET, CAGE ACTIVE and CAGE INACTIVE.

In addition, we studied the variation of melatonin in the CAGE and NET datasets independently. We performed GLMs to relate the levels of melatonin to time of bleeding, body condition indices, and, for CAGE birds, locomotor activity and body mass loss. We excluded all CAGE birds that were housed after 1200 hours (N = 12) as the measurement of diurnal locomotor activity in these birds was limited to a small number of hours. Furthermore, a more homogeneous time of caging across individuals allowed us to include body mass loss as an explanatory variable. To check for departures from regression model assumptions and fit, we used diagnostic plots (i.e. Residuals vs Fitted values, Scale-Location and Normal QQ plots). We conducted model evaluation and selected the models that best fit our data following the Akaike information criterion (AIC) (Akaike, 1974) and conducting backwards selection of insignificant terms. For CAGE birds, we additionally studied the correlations between locomotor activity (diurnal and nocturnal = Zugunruhe) and nocturnal melatonin concentrations. Zugunruhe was not normally distributed and could not be normalized with data transformation. Diurnal activity was not normally distributed and was normalized by logarithmic transformation. Therefore, we calculated non-parametric (Spearman) correlation coefficients for the relationship between Zugunruhe and melatonin levels, and parametric (Pearson) correlation coefficients for the relationship between diurnal activity and melatonin levels.

Results and discussion

The variation in melatonin was best explained by a model including only the group (NET, CAGE ACTIVE and CAGE INACTIVE). Scaled mass index at bleeding was non-significant. An analysis of variance (ANOVA) on melatonin values yielded significant variation among groups (F = 3.244, p = 0.045, AIC = 895.05, Fig. 1). Specifically, NET birds showed higher melatonin compared to CAGE ACTIVE birds (Tukey test: *p = 0.030*, Fig. 1). The difference between NET and CAGE INACTIVE birds showed a clear tendency to significance (Tukey test: *p = 0.077*, Fig. 1). The lack of a statistical significant difference is the result of one high melatonin value in the group CAGE INACTIVE. Melatonin levels did not differ between the two CAGE subgroups (Tukey test: *p = 0.894*, Fig. 1).

In CAGE birds, the variation in melatonin levels was best explained by a model including only the variation in body mass at capture. Time of bleeding, Zugunruhe and diurnal activity were excluded from the best-fitting model. Although body mass at capture did not show a significant effect on melatonin levels (F = 0.572, p = 0.454, AIC = 444.67), there was a clear tendency for melatonin levels to be lower when body mass at capture was larger (Fig 2). In fact, the negative correlation becomes significant with the exclusion of one data point (melatonin value: 436.2 pg/ml; body mass = 18.8 g) (F = 7.205, p = 0.011, AIC = 419.51). We did not find any significant correlations of Zugunruhe and diurnal activity with nocturnal melatonin concentrations (Zugunruhe: r_s = 0.065, n = 50, p = 0.652; diurnal activity: r_p = 0.077, n = 50, p = 0.597). In NET birds, none of the predictor variables explained variation in melatonin concentrations.

In summary, we found higher nocturnal levels of melatonin in robins caught at the nets and sampled immediately compared to robins that were caught earlier during daytime and hosted overnight in cages. All captive birds showed similarly low levels of melatonin regardless of the extent of nocturnal locomotor activity. Because nocturnal melatonin levels strongly depended on the captivity status (hosted in cages vs free-living) but not on the extent of migratory disposition, we conclude that in robins temporary captivity is associated with a decrease in the circulating levels of melatonin.

The results of the present study suggest that actual levels of melatonin in migrants could be generally higher than those reported by previous studies with captive birds. In most studies, the hormone was measured in samples taken from birds that were hosted in cages. Thus, the inverse

association between melatonin and migratory status found in several studies (Fusani and Gwinner, 2001; Fusani and Gwinner, 2004; Gwinner et al., 1993) might be stronger in free living birds because of the lack of dampening effects of captivity. The lack of a correlation between melatonin levels and nocturnal activity in our cage sample is not surprising, as previous studies showed that such correlation is not observed among animals in a similar migratory state despite variations in the extent of nocturnal restlessness (Fusani et al., 2011).

Effects of captivity have been described for other hormones such as testosterone (Dufty Jr. and Wingfield, 1986) and corticosterone (reviewed by Calisi and Bentley (2009)). The physiological changes consequent to transfer to captivity are quite complex, but in most cases they involve the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Corticosteroids released by the adrenals are well known to affect the activity of other endocrine systems such as the hypothalamic-pituitary-gonadal (HPG) axis and thus are probably responsible for the reduced testosterone profile in captive male birds (Calisi and Bentley, 2009).

In a detailed study designed to address this question, Dickens et al. (2009) found that wild chukars (*Alectoris chukar*) entered a state of chronic stress during the first 5 days of captivity, after which they started recovering to reach 'normal' condition by the 9th day. Another study examined the effect of stress on corticosterone and melatonin in ring doves (*Streptopelia risoria)*, and found that melatonin levels at night were reduced in birds subject to stress which had elevated circulating levels of corticosterone (Barriga et al., 2002). We did not measure corticosterone levels in our study, however it appears likely that captivity affected melatonin levels in caged birds because of the activation of the HPA axis.

Nocturnal melatonin levels can be reduced by exposing birds to artificial light (Dominoni et al., 2013). However, the room in which our birds were hosted had no artificial light and there was no significant source of light nearby. Nevertheless, the amount of light to which the birds hosted in cages and those which were moving were exposed might have been different and an effect of illumination on melatonin levels cannot be excluded.

In conclusion, differences in the nocturnal circulating levels of melatonin between robins that had been caught during daytime and then hosted in cages and robins that had been caught at night may be explained by captivity-induced stress. As birds appear to adapt to captivity after a few days, or at least to recover from the initial period of chronic stress, it is likely that such effects will be more evident in birds that have been just caged compared to those kept in cages for longer periods.

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Competing interests

No competing or financial interests declared

Author contributions

Study design: L.F.; Data collection and analysis: S.L.; Hormonal analysis: V.C., S.L.; Writing - original draft: L.F., S.L.; Writing - review & editing: L.F., S.L., V.C., P.P; Funding acquisition: S.L., L.F., P.P

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Data availability

Our data will be available on Dryad after acceptance.

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Figures

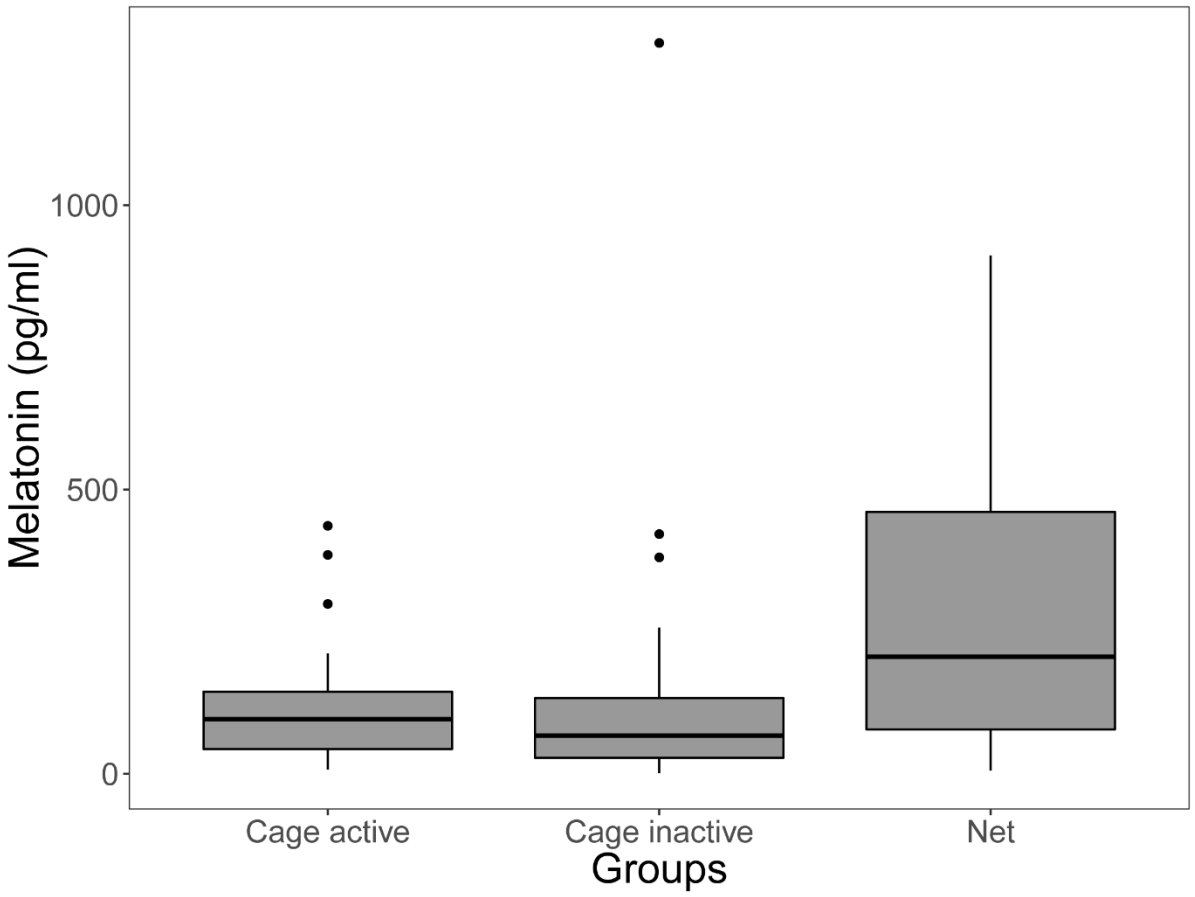

Figure 1. Nocturnal melatonin levels in migrating robins kept in cages (CAGE: ACTIVE and INACTIVE) or sampled directly at the nets (NET).

Sampling period: 2300-0100. Melatonin levels were higher in NET birds compared to CAGE ACTIVE birds, and did not differ between CAGE ACTIVE and INACTIVE birds ($F_{1, 42}$ = 3.244, p = 0.045; CAGE: N = 24, median = 96.2, interquartile = 101; CAGE INACTIVE: N = 26, median = 67.2, interquartile = 105; NET: N = 22, median = 205.9, interquartile = 382).

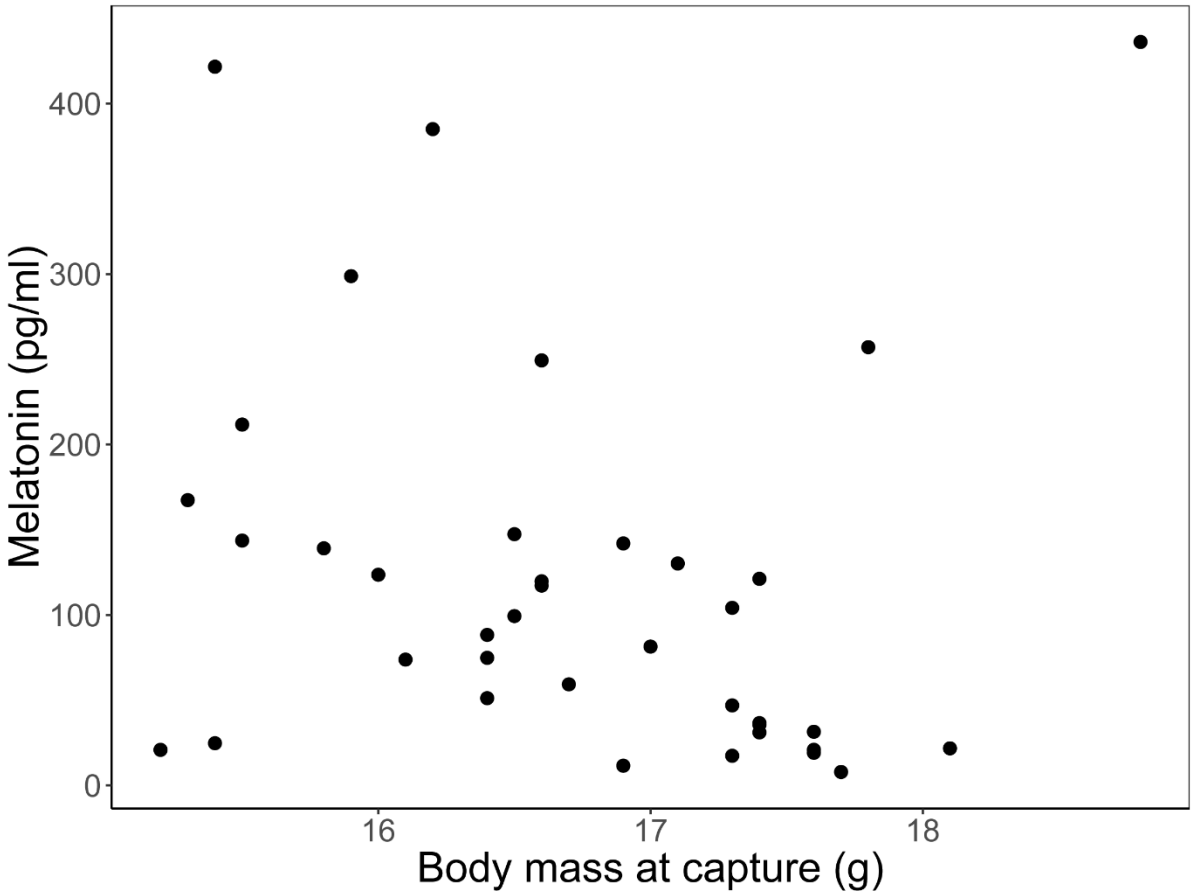

Figure 2. Melatonin levels plotted against body mass at capture in robins temporarily hosted in cages.

Body mass at capture did not show a significant effect on melatonin levels ($F_{1,35} = 0.572$, $p = 0.454$, N = 37). The exclusion of one data point (melatonin value: 436.2 pg/ml; body mass = 18.8 g), however, returns a significant negative correlation between the two variables (F = 7.205, $p = 0.011$, N = 36).