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ORIGINAL PAPER

No evidence for melatonin-linked immunoenhancement over the annual cycle of an avian species

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Abstract The winter immunoenhancement hypothesis associates long nights and increased exposure to melatonin with enhanced immune function in winter when resource availability is low and the chances of becoming ill are high. Thus, increased exposure to melatonin in the winter could be adaptive for species facing difficult winter conditions. This idea has found some support in studies of resident mammals. In birds, the link between day length and melatonin over the annual cycle is weaker, and contributions of melatonin to seasonal timing are unclear. Furthermore, many species, especially migrants, do not experience the most difficult conditions of their annual cycle in winter. In this study, we tested whether the winter immunoenhancement hypothesis holds in an avian species, the red knot *Calidris canutus*. We found that melatonin duration and amplitude varied significantly over the annual cycle with the highest values occurring in winter. However, peaks did not correspond to the winter solstice or with annual variation in immune function. Our findings do not support the winter immunoenhancement hypothesis in knots and question whether the idea that immune function should be bolstered in winter can be generalized to systems where winter is not the most difficult time of the year.

Keywords Annual cycle · Immune function · Melatonin · Seasonal stressors · Winter immunoenhancement hypothesis

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Introduction

In many species resource availability and disease risk vary over the annual cycle (reviewed in Nelson et al. 2002; Altizer et al. 2006). If this variability is predictable, and assuming that immune defense is costly to maintain and use (Råberg et al. 1998; Schmid-Hempel and Ebert 2003; Klasing 2004), it may be adaptive for immune defenses to become adjusted to these seasonal fluctuations in resources and disease. The winter immunoenhancement hypothesis is derived from this reasoning and associates long winter nights with increased exposure to melatonin and enhanced immune function when resource availability is low and the chances of becoming ill are high (Nelson and Demas 1996; Nelson et al. 2002, also known as the melatonin-induced immunoenhancement hypothesis; Hasselquist 2007). Melatonin is an indole amine secreted mainly by the pineal gland primarily at night. In mammals the duration of melatonin secretion is proportional to night length, such that the highest exposure to melatonin occurs during short days (Prendergast et al. 2002). Because melatonin can enhance the immune system, it is proposed that melatonin functions as a proximate mechanism for winter immunoenhancement.

This could be adaptive for animals experiencing harsh winter conditions, as immunoenhancement might counteract the effects of thermal stress and low food resource availability which would otherwise suppress immunity (Nelson and Demas 1996).

The winter immunoenhancement hypothesis is based mainly on data from mammals that have tightly linked day length and melatonin rhythms, and that reside in the temperate zone year round, thus experiencing harsh conditions during the winter (Nelson et al. 2002). In birds, the link between day length and melatonin over the annual cycle is weaker, and contributions of melatonin to seasonal timing are unclear. Furthermore, many species, especially migrants, do not experience the most difficult conditions of their annual cycle in winter. In this study, we tested whether the winter immunoenhancement hypothesis holds in an avian species.

Testing the winter immunoenhancement hypothesis requires an annual profile of melatonin to assess whether melatonin exposure peaks when days are short. Furthermore, an annual profile of immune function is needed to verify that immune indices increase under short days and are associated with increased exposure to melatonin. To date such data on melatonin and immune function in birds are scarce (reviewed in Hasselquist 2007). Changes in melatonin profiles have been compared for selected seasons under temperate light conditions, but have not been tracked over the entire annual cycle (Brandstätter et al. 2001; Hau et al. 2002; Fusani and Gwinner 2005). Studies involving detailed sampling of melatonin throughout the year have focused on extreme photoperiodic situations such as Antarctic (Miché et al. 1991) and Arctic (Reierth et al. 1999) polar light, or constant equatorial day lengths (12 L:12 D, Gwinner et al. 1993). From an immune standpoint, studies tracking immune function over the annual cycle show no general pattern of either immunoenhancement or attenuation during short days (reviewed in Hasselquist 2007). Furthermore, experimental studies linking melatonin and immune function via melatonin implants or photoperiodic manipulation have produced incongruent results in terms of melatonin-linked immunoenhancement (Giannessi et al. 1992; Bentley et al. 1998; Moore and Siopes 2000; Majewski et al. 2005). Finally, no combined melatonin and immune function dataset is available for a single avian species.

In this paper, we present a detailed dataset of melatonin measured biweekly over an annual cycle in red knots *Calidris canutus*. Red knots are long-distance migrant shorebirds with distinct phenotype annual cycles that are maintained even in captivity (Cadée et al. 1996; Piersma 2007; Buehler et al. 2008a). We then examine these data in conjunction with a yearlong dataset of immune function measured monthly (Buehler et al. 2008a) to test the winter immunoenhancement hypothesis.

Materials and methods

Birds

Red knots of the subspecies *C. c. islandica*, were captured in the Dutch Wadden Sea (53°31′N 6°23′E) and brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). At capture the birds were ringed, weighed, and aged (all older than 2 years; Prater et al. 1977) and sexes were later determined using molecular techniques (Baker et al. 1999).

All birds were kept under local photoperiodic and temperature conditions. In the wild, *C. c. islandica* knots spend nearly 10 of 12 months of the year in Western Europe (Piersma 2007; Buehler and Piersma 2008). They depart for breeding grounds in Greenland and the Canadian Arctic in late May and return in early August. Thus, our birds experienced natural photoperiodic conditions for most of the year, but days were shorter than on their Arctic breeding grounds during mid summer.

The birds used to study seasonal rhythms of melatonin ($n = 6$) were captured on 6 November 1994 (all males) and on 27 October 1995 (two females and a male) and kept in outdoor aviaries ($4.5 \times 1.5 \times 2.3$ m high; Piersma et al. 2000). These aviaries were covered to protect birds from the wind and were not exposed to any sources of artificial light. Therefore, these birds experienced natural photoperiodic and temperature conditions of the northern Netherlands. The birds used to study seasonal rhythms of immune function ($n = 12$) were part a larger study examining the effect of ambient temperature on immune function over the annual cycle (Buehler et al. 2008a). For the discussion presented in this paper we included only birds in the control group (variable treatment), since the conditions experienced by these birds matched those experienced by the birds used to study melatonin. These birds were captured on 22 August and 16 September 2004 (six females and four males) or on 8 February 2005 (one female and one male), and were housed in indoor aviaries identical to the outdoor aviaries described above. Photoperiod in the indoor aviaries was set to track that of the northern Netherlands and the birds were not exposed to any other sources of artificial light. Furthermore, temperature in the indoor aviaries tracked outdoor conditions because outdoor air was constantly brought in through vents.

The birds used to study melatonin and the birds used to study immune function were fed slightly different diets (protein-rich trout pellets vs. mudsnails *Hydrobia ulvae*, respectively); however, both diets were similar in nutritional value (T. Piersma, unpublished data) and both groups had ad libitum access to food and fresh water for drinking. To verify that all study birds were healthy, we performed weekly assessments of feather condition and checked the

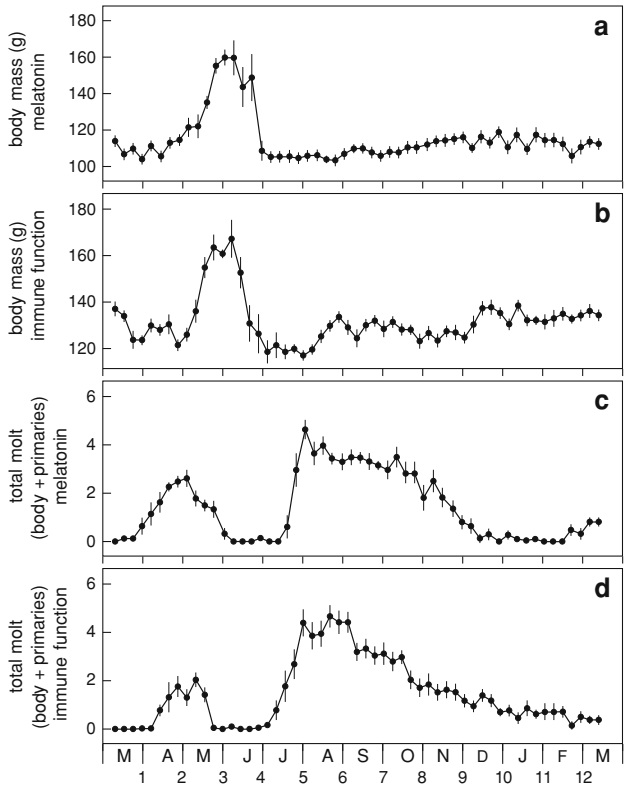

Fig. 1 Patterns in body mass and molt over the annual cycle of red knots. **a** Body mass from birds used to examine melatonin, **b** body mass from birds used to examine immune function, **c** molt from birds used to examine melatonin, **d** molt from birds used to examine immune function

skin of the feet, elbows (carpals) and keel for signs of local inflammation (e.g. bumblefoot).

All birds were weighed and scored for molt once a week, and because we were interested in molt as a whole, we calculated total molt as the sum of a breast molt index (Piersma and Jukema 1993) and the number of growing primaries (feathers in wing molt categories 1 through 4; Ginn and Melville 1983). Annual patterns of body mass and total molt showed nearly identical patterns (Fig. 1) verifying that the two annual cycles were comparable.

Sampling

The birds used to study seasonal rhythms of melatonin were sampled bimonthly from early March 1997 to late February 1998. We took samples over a 24-h session, starting and ending at midday with bleedings at 4-h intervals (12:00, 16:00, 20:00, 00:00, 04:00, 08:00 and 12:00). We collected blood samples within a few minutes of entry the aviary (always within 15 min) and any differences in handling time were semi-randomized with respect to time over the course of the day and throughout the year. Blood was collected by puncturing the brachial wing vein and siphoning 150–250 µl of blood into heparinized capillary tubes. We centrifuged the blood within 2 h of sampling (6,900*g* for 15 min) and stored the plasma at −80°C until processing.

The birds used to study seasonal rhythms of immune function were sampled monthly from March 2005 to March 2006 between 10:30 and 11:00 am (mean ± SD = 10:44 ± 5 min) and always within 20 min of entering an aviary (Buehler et al. 2008a). A previous study has verified that none of the indices of immune function used by Buehler et al. (2008a) are impacted by handling stress if blood is taken within 30 min of entry into the aviaries (Buehler et al. 2008b). We refer the reader to Buehler et al. (2008a) for further details on sampling and methodologies for the immune assays.

Quantifying melatonin

We quantified melatonin by radioimmunoassay (Van't Hof and Gwinner 1996). We extracted plasma overnight in chloroform with 1 M NaOH, the next morning the extractions were pulse centrifuged and the chloroform layer was aspirated off. Then chloroform was added again and after at least 2 h the top layer was aspirated off. The extraction was then dried under nitrogen and redissolved overnight in 0.1 M Tricine buffer. We then performed another extraction with Petroleum ether (petroleum benzine) to remove fat. We added Petroleum ether to the extractions on dry ice and then aspirated off the upper Petroleum ether phase. To determine extraction efficiency, we added 2,000 cpm of 3H-melatonin to each extraction tube. After extraction, melatonin was measured by radioimmunoassay using sheep anti-melatonin antiserum (G/S/704-8483, Stockgrand Ltd., Guildford, UK) and 3H-labelled melatonin with a specific activity of 3,148.7 (GBq/mM from NEN, Du Pont). The lower detection limit of the assay was 17 pg/ml and intra- and inter-assay variation were 13 and 14%, respectively (similar to Van't Hof and Gwinner 1998). For each individual bird over the 24-h blood sampling session we examined the duration of melatonin secretion (melatonin duration) and the highest melatonin concentration (melatonin amplitude). Melatonin duration was calculated as the number of hours between samplings with detectable melatonin. For example, if we saw melatonin greater than 17 pg/ml from 20:00 to 6:00 then melatonin duration was at least 10 h.

Statistical analysis

We used Kolmogorov–Smirnov tests and visual examination of histograms to examine response variables and model residuals for normality. Peak melatonin and melatonin duration were right skewed and were log 10 transformed to achieve normality. To examine melatonin variation over the annual cycle, we performed univariate analyses using a

linear mixed model with "week" as a fixed factor and "bird" as a random factor. We ran the models including and excluding an effect of sex. Sex was never significant and never changed the outcome of the model, thus the statistics presented are from models excluding sex. Complete univariate and principal component analyses of variation in immune function over the annual cycle are presented in Buehler et al. (2008a). Because only a portion of those data are used for discussion in this study, we calculated principal component scores again using the within-bird component loadings presented in Buehler et al. (2008a). Measures of microbial killing were not available for March and early April, thus principal component scores for that month were calculated using heterophil data only.

Results

Melatonin cycles had very low amplitudes in knots, and melatonin duration and amplitude showed similar patterns (were positively correlated) over the annual cycle (Fig. 2a–c). Both measures were lowest in late June and early July when days were long and increased as day lengths shortened. Peak melatonin duration and amplitude occurred in winter; however, contrary to predictions they did not correspond with the winter solstice. Values dropped in December when day lengths were shortest and increased sharply in January and early February as day lengths began to increase again. Seasonal variation was significant for both melatonin duration (week: $F_{19,91} = 7.00$, $P < 0.001$; bird: $F_{5,91} = 3.90$, $P = 0.003$) and amplitude (week: $F_{19,91} = 6.34$, $P < 0.001$; bird: $F_{5,91} = 2.98$, $P = 0.015$).

Fluctuations in principal component scores for immune function are presented in Fig. 2d–f (calculated from birds in the variable treatment only, Buehler et al. 2008a). Detailed graphs and statistics for individual immune indices are presented in Buehler et al. (2008a).

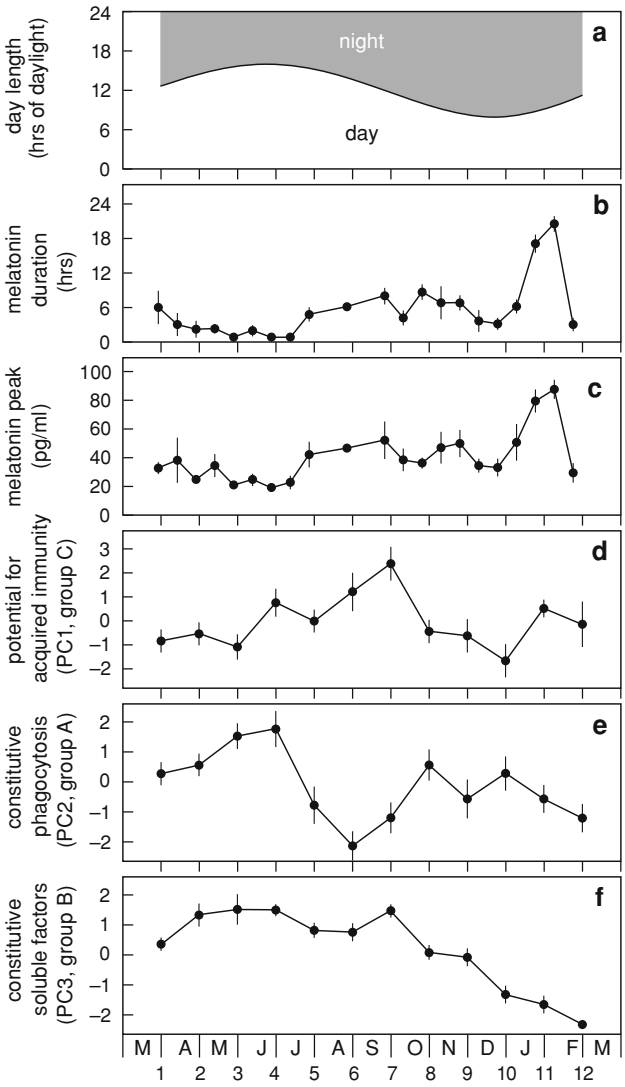

Fig. 2 Patterns of melatonin and immune function over the annual cycle of red knots. **a** Day length at 50°N, compiled from the information in Fig. 5 in (Piersma and Davidson 1992), **b** melatonin duration, **b** melatonin amplitude, **d** immune indices associated with the potential for acquired immunity (PC1, lymphocytes and monocytes, Group C in Buehler et al. 2008a), **e** immune indices associated with phagocytosis and inflammation (PC2, *S. aureus* and *C. albicans* killing, heterophils and lysis, Group A in Buehler et al. 2008a), and **f** immune indices associated with soluble factors of constitutive immunity (PC3, *E. coli* killing and agglutination, Group B in Buehler et al. 2008a). For **b** and **c** statistics were performed on log 10 transformed values, but the raw data are shown for ease of interpretation. Melatonin sampling was not performed during August and early September, thus in **b** and **c** the point in late August (without error bars) is an interpolation. Measures of microbial killing were not available for late March and early April thus principal component scores for month 1 in **e** were calculated using heterophil data only. *Symbols* show means and *error bars* show one SE

Discussion

In this study, we examine whether the winter immunoenhancement hypothesis holds in an avian species, the red knot. This discussion addresses two predictions: first, exposure to melatonin peaks during the shortest days of the year, and second immune function also peaks during short days and is associated with melatonin exposure.

The winter immunoenhancement hypothesis is based on the premise that the short days of winter increase the duration of melatonin secretion which in turn could bolster immune function (Nelson 2004). We found that both melatonin duration and amplitude were highest in winter; however, not during the winter solstice when days were shortest. Melatonin peaked during late January and early February when day lengths were increasing (Fig. 2a–c). This result is surprising, but not unprecedented. In Svalbard ptarmigans *Lagopus mutus hyperboreus,* melatonin secretion

was highest from January to April and night time plasma melatonin concentration peaked in March (Reierth et al. 1999). Furthermore, in house sparrows *Passer domesticus*, although melatonin duration followed day length, melatonin amplitude peaked in March and April (Brandstätter et al. 2001). Finally, in garden warblers *Sylvia borin* and blackcaps *Sylvia atricapilla* melatonin concentrations were lowest, not during the summer solstice, but during migratory restlessness (Gwinner et al. 1993; Fusani and Gwinner 2005). Thus, our findings add to evidence that peaks and troughs in plasma melatonin concentration do not necessarily coincide with summer and winter solstices.

We do not know why melatonin duration and amplitude peaked in late January and early February in knots; however, we are confident that methodological artifacts do not underlie this pattern. Our sampling and assay methods were constant throughout the year and the birds experienced no light contamination during this period or any other period during the experiment. Furthermore, sampling melatonin five to six times over a 24-h period is common (Gwinner et al. 1993; Reierth et al. 1999; Hau et al. 2002) and although this sampling schedule may have resulted in the underestimation of melatonin duration throughout our study (due to the 4-h intervals between samplings), we were still able to detect significant fluctuations in melatonin duration.

The red knots in this study had low melatonin amplitudes ranging from 17 to 116 pg/ml, comparable to nocturnal and polar birds (range 16–110 pg/ml; Miché et al. 1991; Taniguchi et al. 1993; Van't Hof and Gwinner 1998; Reierth et al. 1999; Hau et al. 2002 wild sample in Alaska), but lower than diurnal birds (range 200–900 pg/ml; Miché et al. 1991; Taniguchi et al. 1993; Van't Hof and Gwinner 1998; Reierth et al. 1999; Hau et al. 2002 captive sample in Seattle). Red knots are tidal foragers and maintain foraging activity in darkness (van Gils and Piersma 1999; van Gils et al. 2006). Low levels of melatonin in species active at night suggest that avian melatonin rhythms may be fitted to lifestyle with a flexible link to day length cues. This flexibility may be related to the complexity of the avian circadian system which consists of several interacting oscillators (Gwinner and Brandstätter 2001; Kumar 2001). Unlike in mammals, the role of melatonin in the annual cycle of birds is unclear (Dawson et al. 2001; Kumar 2001).

A second premise of the winter immunoenhancement hypothesis is that immune function should be bolstered during winter when days are short, because winter is the most stressful period of the annual cycle (Nelson 2004). However, plots of principal component scores for immune function (Fig. 2d–f), show that fluctuations in immune function did not correspond to changes in day length or to variation in melatonin. In knots the most difficult periods of the year are predicted not during winter, but during spring migration and arrival on the breeding grounds (reviewed in Buehler and Piersma 2008). During migration, strenuous flights necessitate high energetic demands while dense feeding flocks increase the chances of disease transmission and travel through many environments increases the chances of encountering novel pathogens. Then birds arrive on Arctic breeding grounds and face very limited food resources until the insect bloom. Thus, if knots do bolster immune function in anticipation of higher disease risk and lower resource availability, this enhancement would be predicted during spring and early summer. Certain immune indices, especially those associated with phagocytosis, are elevated during this period (Fig. 2e; Buehler et al. 2008a). This suggests that the idea of immunoenhancement in anticipation of predictable fluctuations in disease risk and resource availability during the annual cycle has merit for birds. However, since we found no association (positive or negative) between melatonin and immune function (Fig. 2), we suggest that melatonin is an unlikely mechanism for this immunoenhancement.

Assessment of immunoenhancement due to seasonal increases in melatonin exposure is complicated by the fact that immune function is not a monolithic entity (Nelson et al. 2002; Adamo 2004; Matson et al. 2006). The immune system can divided along two axes: the first concerning degree of specificity from *non-specific* to *specific* and the second concerning temporal dynamics from *constitutive* to *induced* (Schmid-Hempel and Ebert 2003). In mammals there appears to be a trend towards increased aspects of induced specific immunity (lymphocyte proliferation and the cytolytic killer cell capacity; (Yellon et al. 1999; Bilbo et al. 2002a; Mann et al. 2000; Nelson et al. 2002) with exposure to short days. In contrast, aspects of constitutive non-specific immunity (phagocytosis and oxidative burst) and aspects of the induced non-specific sickness responses decrease with exposure to short days (Yellon et al. 1999; Bilbo et al. 2002b). In birds no similar trends have emerged. This study indicates that measures of constitutive immunity show fluctuations that do not coincide with changes in day length. Studies of induced specific immunity (cell-mediated and humoral) also indicate no clear association with day length with some studies finding increases while others find decreases during short days (reviewed in Hasselquist 2007). Finally, a study examining the induced non-specific sickness response found decreased sickness duration during short days, but only in males (Owen-Ashley et al. 2006). This variation in the relationship between immune function and day length in birds suggests that immunoenhancement is context dependent and implies that melatonin is not the proximate mechanism for annual variation in immune function in birds.

In summary, our study does not provide support for winter immunoenhancement in red knots. Increased exposure to melatonin was not associated with the winter solstice or

with enhanced immune function. Furthermore, this study questions whether immunoenhancement need necessarily be tied to a particular time of the year (winter), and suggests that immune function may be linked to the severity of environmental conditions rather than time of the year per se. We suggest future studies examining species with a range of ecologies (i.e. migratory and tropical species); as well as studies that examine alternative proximate mechanisms for annual variation in immune function in birds.

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