

# Diel changes in plasma melatonin and corticosterone concentrations in tropical Nazca boobies (*Sula granti*) in relation to moon phase and age

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## Abstract

We investigated the effects of moon phases and age on diel rhythms of plasma melatonin and corticosterone in free-living Nazca boobies (*Sula granti*) on the Galápagos Islands, Ecuador. Melatonin and corticosterone secretion are regulated by the circadian system and the two hormones play a role in the control of locomotor activity and foraging, which can be influenced by moon phases. These seabirds have a long life span and in many vertebrates circadian function deteriorates with age. The functioning of the circadian system under different environmental conditions and changes related to age are poorly understood and hardly studied in wild birds. Nazca boobies had generally low plasma melatonin concentrations but showed a diel variation with higher concentrations at 00:00 and 16:00 h. The diel variations in melatonin concentrations disappeared during full moon, suggesting that natural light levels at night can suppress melatonin secretion in Nazca boobies. Maximal melatonin concentrations tended to decline in older birds (10–19 years). Birds showed a clear diel variation in basal plasma corticosterone with a peak in the early morning, before the active period begins, and low concentrations throughout the day. As with melatonin, there were no diel variations in corticosterone at full moon, which may be due to different activity patterns in response to food availability or changes in the circadian system. While other studies have found a relationship between corticosterone and melatonin, we found no such correlation in Nazca boobies. The lunar cycle appears to affect the hormone titers of Nazca boobies both directly and indirectly. First, melatonin rhythms can be directly affected by the light intensity associated with full moon. Second, prey availability may change foraging patterns and can therefore indirectly alter corticosterone secretion in Nazca boobies.

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## 1. Introduction

Many behaviors, including sleep–awake cycles, activity or foraging patterns are controlled by the circadian system. Circadian rhythms are endogenously generated and synchronized to the environment by light–dark cycles (Aschoff, 1981; Cassone et al., 1993; Saunders, 1977; Underwood, 1984). To investigate the properties of circadian systems and how they are regulated, many studies have been conducted in the labora-

tory under controlled conditions. Only very few studies have been conducted in the wild under natural conditions where the functioning of circadian systems and their responses to the environment can be witnessed (e.g., Cockrem, 1991a; Daan and Koene, 1981; DeCoursey et al., 2000; Miché et al., 1991; Palmer, 1991; Reierth et al., 1999; Wikelski and Hau, 1995). The circadian system is affected by a multitude of stimuli, including light–dark cycles, food availability, and social cues (Aschoff, 1981; Mistlberger, 1994; Pittendrigh, 1981; Regal and Connolly, 1980). Only studies of circadian rhythms in the wild are likely to involve all relevant natural stimuli.

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Nazca boobies (*Sula granti*) are tropical seabirds of the eastern tropical Pacific (Pitman and Jehl, 1998; Friesen et al., 2002). They are an ideal system for studying circadian functions in the wild because they live in a natural, undisturbed environment without light pollution. Additionally, they accept close observation and capture without fear or disturbance. The photoperiod at the equator is nearly constant, but lunar effects can be strong at this latitude (Erkert et al., 1986). Indeed, lunar cycles have been shown to affect the behaviors of many organisms (Alonso et al., 1985; Kavaliers, 1982; Morrison, 1978; Naylor, 1982; Watanuki, 1986; Ydenberg et al., 1984). In the Galápagos, for example, lunar phases affect prey availability and foraging efficiency in Galápagos fur seals (Horning and Trillmich, 1999). Nazca boobies may also adjust their foraging patterns to lunar phases.

Hormone rhythms are one output of the circadian system that can easily be quantified. Here we investigate melatonin rhythms, a crucial component in the circadian system of birds. Most vertebrates and many invertebrates exhibit a daily melatonin rhythm with low concentrations during the day and high concentrations at night, corresponding to the light–dark cycle (Binkley, 1993; Cassone, 1990; Reiter, 1993). In general, light exposure during the dark phase suppresses melatonin production (Binkley et al., 1980; Bojkowski et al., 1987; Honma et al., 1992; Ralph et al., 1975) and suppression or elimination of the melatonin rhythm impairs circadian function in birds (e.g., Gwinner and Hau, 2000). Over recent years, studies of captive vertebrates have shown how night light of varying intensities affects melatonin rhythms (Cockrem, 1991b; Kumar et al., 2000; Lynch et al., 1981; Meyer and Millam, 1991; Wever, 1980). However, recent studies on natural populations of birds living in polar regions under continuous daylight have detected a persistent melatonin rhythm in some species (Cockrem, 1991a; Hau et al., 2002; Reierth et al., 1999). With the robust difference of light intensity in the Galápagos between the full and new moon phases, we predicted that plasma melatonin concentrations will vary between these moon phases in Nazca boobies.

As a second output of the circadian system we investigated the diel pattern of corticosterone (CORT) secretion in free-living Nazca boobies. CORT rhythms are often associated with activity and foraging patterns and birds typically show a CORT rhythm with high concentrations during the inactive period and lower concentrations during the active period (Breuner et al., 1999; Dufty and Belthoff, 1997; Romero and Remage-Healey, 2000). CORT may also be linked to metabolism (Widmaier, 1992) and foraging rhythms (Astheimer et al., 1992).

The amplitude of circadian rhythms, especially in melatonin secretion, has been shown to decrease with

age in vertebrates, such as rats (Pang et al., 1984), hamsters (Reiter et al., 1982) and humans (Sack et al., 1986) and it has been argued that low melatonin concentrations play a role in senescence (for review see Reiter, 1992). Nazca boobies have a long life span as they can live for more than 20 years, making them an appropriate system for aging studies in the wild. By sampling banded animals of known ages we attempted to determine if melatonin declines with age in these seabirds.

## 2. Methods

We studied Nazca boobies at Punta Cevallos, Isla Española (89° 37'W, 1° 23'S) on the Galápagos Islands during the January and February 2000 breeding season. Total colony size at our study site is about 10,000 individuals. Ninety-one individuals were sampled for hormone analysis: 44 on the days surrounding the full moon (January 19–23; full moon on January 21) and 47 on the days surrounding the new moon (February 3–7; new moon on February 5). Each individual was sampled only once. In all cases, individuals were captured and carefully removed from their nest site. Only awake (eyes open) birds were captured for all daytime sampling (08:00, 12:00, 16:00, and 20:00 h). Only non-breeding birds were used for CORT analysis because CORT concentrations can change during different phases of the breeding cycle in Nazca boobies (Tarlow, unpublished data); this only required us to eliminate three CORT samples from analysis. Not all individuals had both CORT and melatonin samples, however for determination of the 'relationship between melatonin and CORT' only individuals that had both a CORT and melatonin samples were used. Band numbers were recorded for later determination of sex and age (long term data collected by David Anderson). Ages are determined by a conservative estimate; birds are at least as old as the age they are assigned for analysis (some previously banded birds were banded as 'adults,' without knowing their exact age; this was only the case for 25% of the birds). Blood samples were obtained by puncturing the superficial brachial wing vein with a 26-gauge needle. Blood (300–600 µl) was collected in heparinized microcapillaries. All blood samples were taken within 4 min (except for two samples, one taken within 5 min and one taken within 5.30 min); only samples taken within 3 min were used for CORT analysis. Each individual was also weighed in a bag with a spring scale and then released. Plasma was separated from blood cells by centrifugation (at  $588 \times 10^2$  m/s for 4 min) within one hour after collection, removed, and treated with 10 µl of a 0.2% β-propiolactone solution to destroy viruses, according to US import regulations for avian blood. Five hundred microliter of

ethanol was added to each sample and samples were shaken. Within three weeks of collection, samples were first stored at the Charles Darwin station in Galápagos and then transported to the University of Illinois and stored at  $-20^{\circ}\text{C}$  until analysis, later done at Princeton University. The use of ethanol as a method of steroid hormone preservation had been validated prior to our investigations (Hau et al., in preparation; Müllner et al., submitted; Tarlow et al., 2003). The measured levels of hormone samples of Nazca boobies preserved in ethanol had not changed significantly after 4 weeks of storage at ambient temperatures compared to samples stored at  $-20^{\circ}\text{C}$  immediately after collection in the field (Wikelski, unpublished data).

Research conducted during this project was permitted under the regulations of the University of Illinois, Urbana-Champaign Office of Laboratory Animal Research and adheres to NIH standards for the use of animals in research.

### 2.1. Hormone analysis

Plasma concentrations of CORT and melatonin were determined separately by indirect radioimmunoassay (RIA; e.g., for CORT see Tarlow et al., 2001; Wingfield and Farner, 1975; for melatonin see Fraser et al., 1983; Hau et al., 2002; Van't Hof and Gwinner, 1996; but for modifications see below). All samples were analyzed in one single CORT and one single melatonin assay. In short, ethanol samples were mixed with water to an equal volume as were blanks and standards that consisted of equally mixed volumes of water and ethanol. For estimation of recovery 2000 dpm of tritiated label was used for CORT or 3000 dpm of tritiated label for melatonin. Samples equilibrated overnight at  $4^{\circ}\text{C}$ . Samples were extracted one time with 4 ml dichloromethane for CORT. For melatonin the first extraction was done by adding 120  $\mu\text{l}$  NaOH and 4 ml chloroform and the second by adding only 2 ml chloroform. Samples were dried in a  $40^{\circ}\text{C}$  water bath under nitrogen gas and redissolved in 550  $\mu\text{l}$  PBSG buffer for CORT or 300  $\mu\text{l}$  PBSG buffer for melatonin. Samples equilibrated with buffer overnight at  $4^{\circ}\text{C}$ . Fractions of 200  $\mu\text{l}$  were taken for duplicates used in the RIA and fractions of 100  $\mu\text{l}$  were directly counted for the determination of recovery for CORT (CORT antibody, B3-163 Endocrine Sciences, Calabasas, CA 91301); for melatonin fractions of 100  $\mu\text{l}$  were taken for duplicates and fractions of 50  $\mu\text{l}$  were used for recovery (melatonin antibody, Stockgrand, Surrey, UK). Blanks were below detection limit, and intra-assay variation for CORT and melatonin (for a total of 9 standards and 11 standards, respectively) was 8% for CORT and 11% for melatonin and lower detection limit was at 2.0 ng/ml for CORT and 0.015 ng/ml for melatonin.

### 2.2. Statistical analysis

Data were analyzed with SPSS 10.0 for Windows (SPSS, Chicago). Values below the lower detection limit were set at that limit as a conservative estimate for statistical analysis. For analysis of diel rhythms in hormones we used analysis of variance (ANOVA) followed by post hoc comparisons with Tukey post hoc tests. Body mass, sex and age were included as co-variables to test their potential effects on hormone concentrations; no such effects were detected. To determine whether a consistent relationship existed between the basal plasma concentrations of melatonin and CORT, we conducted a Pearson correlation analysis. ANOVA was used when comparing the deviation from the mean between new and full moon. The overall difference of melatonin and CORT concentrations between the two moon phases was tested with Mann-Whitney  $U$  tests. Effect of age on melatonin concentrations was determined for individuals sampled during full moon in the following way: the mean was determined for the total of all full moon samples and then the deviation from this mean was taken for each individual; for new moon, the deviation was taken from the mean of the time period the individual was a part (i.e., if an individual was sampled at 12:00 h, the mean would be determined for all samples taken at 12:00 h and then the deviation from this mean would be taken for that individual). Significance of tests was accepted at the  $\alpha = 0.05$  level. A natural logarithmic transformation was done on all CORT data before analysis to satisfy the normality assumption of the ANOVA; no transformation was necessary on the melatonin data.

## 3. Results

### 3.1. Melatonin

Nazca boobies showed a significant diel variation in melatonin when data from both moon phases were combined ( $F_{(5,72)} = 2.455$ ,  $P = 0.041$ ; Fig. 1). However, Tukey post hoc tests failed to show which time periods differed significantly from each other. When we analyzed data from the two moon phases separately, we found no significant diel variation during the full moon ( $F_{(5,32)} = 0.723$ ,  $P = 0.611$ ), only during the new moon ( $F_{(5,34)} = 2.970$ ,  $P = 0.025$ ; again Tukey post hoc tests failed to show which time periods differed significantly from each other). There was no overall difference between the two moon phases (Mann-Whitney  $U = 634$ ,  $n = 40$  new moon, 38 full moon,  $P = 0.208$ ).

### 3.2. Corticosterone

When data from both moon phases were combined, Nazca boobies showed a clear diel variation of plasma

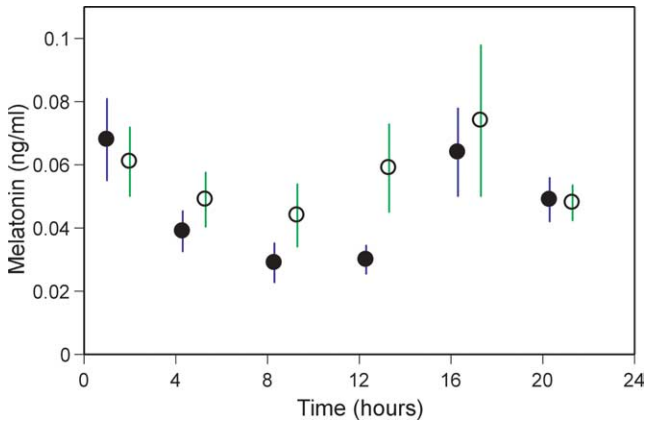


Fig. 1. Comparison of plasma melatonin concentrations (means  $\pm$  95% CI) during new moon (full circles) and full moon (open circles) by time of day. The horizontal bar above each graph represents photoperiod as obtained by sunrise/sunset charts: open bars represent daytime and black bars represent nighttime. Sample sizes: new moon 00:00 h = 7, 04:00 h = 6, 08:00 h = 6, 12:00 h = 5, 16:00 h = 6, 20:00 h = 8; full moon 00:00 h = 5, 04:00 h = 8, 08:00 h = 7, 12:00 h = 7, 16:00 h = 4, 20:00 h = 7.

CORT ( $F_{(5,74)} = 4.990$ ,  $P = 0.001$ ; sign. Differences: 04:00 > 08:00 h, 04:00 > 16:00 h, 04:00 > 20:00 h; Fig. 2). During the new moon birds showed a diel variation in CORT with peak concentrations at midnight and 4 am and decreasing concentrations throughout the active period ( $F_{(5,39)} = 3.868$ ,  $P = 0.006$ ; sign. Differences: 00:00 > 16:00 h, 04:00 > 16:00 h, 04:00 > 20:00 h). However, as with melatonin, plasma CORT concentrations did not vary significantly during the full moon ( $F_{(5,29)} = 2.284$ ,  $P = 0.072$ ). There was, again, no overall difference between the two moon phases (Mann–

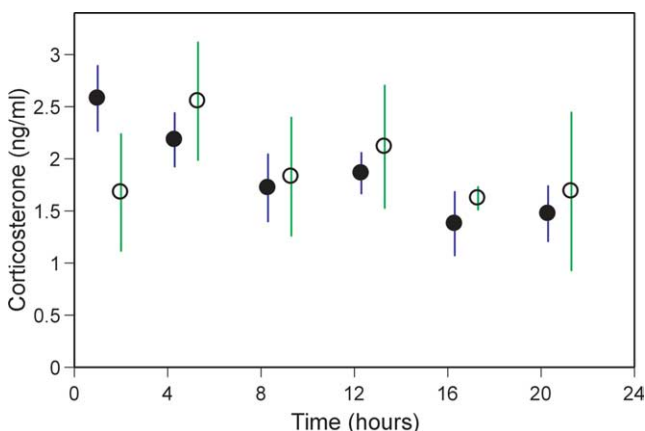


Fig. 2. Comparison of plasma corticosterone concentrations (means  $\pm$  95% CI) during new moon (full circles) and full moon (open circles) by time of day. The horizontal bar above each graph represents photoperiod as obtained by sunrise/sunset charts: open bars represent daytime and black bars represent nighttime. Sample sizes: new moon 00:00 h = 7, 04:00 h = 6, 08:00 h = 8, 12:00 h = 8, 16:00 h = 7, 20:00 h = 8; full moon 00:00 h = 6, 04:00 h = 8, 08:00 h = 4, 12:00 h = 7, 16:00 h = 2, 20:00 h = 8.

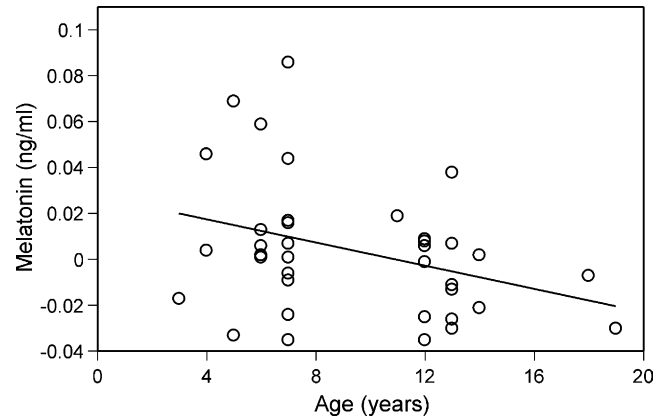


Fig. 3. Scatterplot of the deviation of plasma melatonin concentrations from the mean of full moon by age. Sample sizes: age 3 = 1, age 4 = 2, age 5 = 3, age 6 = 6, age 7 = 11, age 11 = 1, age 12 = 6, age 13 = 6, age 14 = 2, age 18 = 1, age 19 = 1.

Whitney  $U = 748$ ,  $n = 45$  new moon, 35 full moon,  $P = 0.666$ ).

### 3.3. Relationship melatonin—corticosterone

Overall, the concentrations of CORT did not fluctuate in synchrony with the concentrations of melatonin (Pearson's correlation  $r = -0.175$ ,  $P = 0.164$ ). When the Pearson correlation analysis was done separately for new moon and full moon, there was a trend for melatonin and corticosterone concentrations to fluctuate in synchrony during new moon ( $P = 0.063$ ) but not during full moon ( $P = 0.688$ ).

### 3.4. Age

We found a significant difference in melatonin concentrations of birds of different ages ( $F_{(1,36)} = 5.028$ ,  $P = 0.031$ ; Fig. 3) during full moon. However, if we removed the two oldest individuals the result only shows a trend ( $F_{(1,34)} = 3.348$ ,  $P = 0.076$ ). We did not find a difference in melatonin concentrations of birds of different ages at new moon ( $F_{(1,37)} = 0.238$ ,  $P = 0.628$ ). However this is expected because at new moon we did not have birds of all ages represented in the sample. CORT concentrations of birds of different ages did not differ in either moon phase.

## 4. Discussion

This is the first study to examine the diel variations of both plasma melatonin and CORT concentrations in a free-living seabird. Nazca boobies living at the equator showed significant diel variations in both melatonin and CORT during new moon, but not during full moon. The general pattern of these rhythms was similar to that of

other day active birds, although melatonin concentrations overall were very low. Plasma melatonin concentrations decreased with age during full moon, when birds of all ages were represented in the sample.

#### 4.1. Melatonin

Three aspects of the diel melatonin differences of Nazca boobies merit attention: (i) the low night-time melatonin concentrations, (ii) the apparent increase in melatonin concentrations during the day, and (iii) the lack of a diel pattern during full moon.

*Low nocturnal melatonin concentrations.* Nocturnal plasma melatonin concentrations of Nazca boobies during both lunar phases were almost one order of magnitude lower than in many other diurnal birds under the same photoperiod, either when held under artificial illumination (Gwinner et al., 1993; Kumar et al., 2000; Meyer and Millam, 1991; Van't Hof and Gwinner, 1996), or under natural light conditions (Brandstatter et al., 2001; Guchhait and Haldar, 1999; Hau et al., 2002). However, Nazca boobies resemble three high latitude residents, the emperor penguin (*Aptenodytes forsteri*), the Adelie penguin (*Pygoscelis adeliae*), and the Svalbard ptarmigan (*Lagopus mutus hyperboreus*), which also have low night-time melatonin concentrations under a similar photoperiod and natural light conditions (Cockrem, 1991a,b; Miché et al., 1991; Reierth et al., 1999). In future studies on captive and free-living birds, it would be interesting to elucidate mechanistic and functional aspects of low-amplitude melatonin rhythms. It has been shown that the lack of a melatonin rhythm facilitates resynchronization of the circadian system to changing environmental cues (Abraham et al., 2000; Hau and Gwinner, 1994, 1995), which might be beneficial for animals living in a changing environment (Gwinner and Brandstätter, 2001; Gwinner et al., 1997).

*Melatonin increases in the afternoon.* Most vertebrates show a diel melatonin pattern with high concentrations during the dark phase and low concentrations during the light phase (Binkley, 1988; Cassone, 1990; Underwood, 1984). In our study however, melatonin secretion appears to be elevated at 16:00 h. The daytime increase in Nazca booby melatonin concentrations is unusual because melatonin concentrations should be suppressed by daylight. Light intensities on the arid, equatorial Galápagos islands are typically very high and there was no heavy cloud cover or rains during any of the day time sampling periods. Previous findings in the Indian spotted owl (*Athene brama*) also show a daytime peak in melatonin at 14:00 h (Guchhait and Haldar, 1999). Guchhait and Haldar (1999) suggest that this peak may be due to the birds hiding in dark burrows at that time of the day. Nazca boobies, however, are exposed to light at all times of the day. The fact that the boobies had increased melatonin concentrations at 16:00 h at both

moon phases suggests that factors other than light may influence melatonin secretion in Nazca boobies. Kumar et al. (2000) found that European starlings (*Sturnus vulgaris*) generally display a melatonin rhythm with high concentrations at night, when locomotor and feeding activity are low. However, one starling showed a melatonin peak during the light phase which appeared more strongly correlated with its feeding and locomotor activity than with the light–dark cycle (Kumar et al., 2000). Although this hypothesis is currently speculative, it can be tested in wild Nazca boobies using feeding experiments.

John et al. (1993) found that after daytime flight, homing pigeons had significantly elevated melatonin concentrations. They suggest that this increase in melatonin during flight may have many benefits including thermoregulation, energy metabolism, and pain relief (John et al., 1993). Nazca boobies have been shown to return from foraging excursions between approximately 14:15–15:20 h (Anderson and Ricklefs, 1987) and return rates are high until after 20:00 h (Anderson and Ricklefs, 1992). It is possible that the afternoon increase in melatonin in Nazca boobies is associated with flights back to the colony.

*Moon effects on melatonin rhythms.* Nazca boobies showed a significant diel variation in plasma concentrations of melatonin during new moon, but not during full moon. It is possible that Nazca boobies forage more during the day during the full moon period, which would explain the overall higher daytime concentrations during that period and result in a lack of a melatonin rhythm at full moon. During new moon Nazca boobies may forage less intensively or shift their foraging pattern towards early morning, thereby forestalling the afternoon peak observed during full moon. Future studies under captive conditions are necessary to uncover the mechanistic explanation for the lack of a melatonin rhythm at full moon.

#### 4.2. Corticosterone

During new moon, Nazca boobies had a clear diel variation in basal plasma CORT concentrations. Nazca booby plasma CORT concentrations were low throughout the active period and peaked in the second part of the night. This pattern is consistent with the 'pre-active peak' observed in other diurnal bird species (Breuner et al., 1999; Romero and Ramage-Healey, 2000; Westerhof et al., 1994). CORT is one of the hormones intimately involved in the regulation of energy utilization (see Dallman et al., 1993) and the presumed function of the 'pre-active peak' is to prepare the body for the energy-demanding active period (e.g., Breuner et al., 1999).

CORT patterns of Nazca boobies showed a clear diel variation during new moon, but not during full moon. Thus, during full moon both hormones did not

fluctuate, suggesting that either the circadian system has stopped cycling or has become arrhythmic, or both rhythms are ‘masked’ and directly influenced by activity levels.

High activity levels can increase plasma concentrations of CORT (Belthoff and Dufty, 1998; Heath, 1997; Jessop et al., 2002). Thus, we hypothesize that the lunar differences in CORT profiles—like those in melatonin—may be affected by activity patterns (i.e., foraging patterns). Many birds have been shown to vary their behavior with lunar cycles (Alonso et al., 1985; Mills, 1986; Milsom et al., 1990; Watanuki, 1986; Ydenberg et al., 1984).

Data show that food availability can synchronize behavioral and hormonal circadian rhythms in birds (Hau and Gwinner, 1992; Reiherth and Stokkan, 1998), mammals (for review see Mistlberger, 1994; see also Stephan and Davidson, 1998), reptiles (Wikelski and Hau, 1995), fish (Davis and Bardach, 1965; Naruse and Oishi, 1994; Sanchez-Vazquez et al., 1995), and invertebrates (e.g., Fernandez de and Arechiga, 1994). Indeed, periodic food availability can also affect and synchronize hormonal rhythms in mammals, especially CORT (Honma et al., 1984; Krieger, 1974). Thus, the different CORT patterns during the two lunar phases in Nazca boobies could result from shifted activity and foraging patterns of the birds.

Consistent with this theory, a previous behavioral study on radio-tagged Nazca boobies around new moon showed that birds first leave the colony for foraging trips at approximately 05:25 h (Anderson and Ricklefs, 1987). Although we currently lack behavioral data from earlier in the morning (we began recording activity at 04:50 h; Anderson and Ricklefs, 1987), these behavioral observations fit with our hormonal profiles. It is conceivable that at new moon some individuals start foraging earlier in the morning, resulting in high CORT concentrations at 00:00 h. Such a temporal change in activity pattern is likely associated with food availability. The lunar cycle has been shown to influence food availability and foraging efficiency in Galápagos fur seals. Horning and Trillmich (1999) found that fur seal foraging activity is affected by deep scattering layer organisms migrating to different water depths at different light intensities. We hypothesize that Nazca boobies may take advantage of a similar change in prey density at new moon and begin foraging earlier in the morning when prey is easier to capture, more close to the surface, or boobies are less likely to be detected by the prey. The majority of the diet for Nazca boobies includes sardines (*Clupeids*) and flying fish (*Exocoetids*; Anderson, 1989), which spend almost all of their time within the top few meters of the water column.

#### 4.3. Age

We found an effect of age on melatonin concentrations, with melatonin titers decreasing in older birds.

However, this effect was not very strong, possibly because our sample size for old individuals was small and plasma melatonin concentrations overall were generally low. A very low proportion of the banded Nazca booby population are older birds (26% of birds are over 10 years old and only 8% are over 15 years old; Anderson, unpublished data). Nisbet et al. (1999) found a decline with age in androgen concentrations of male common terns (*Sterna hirundo*). In these seabirds androgen concentrations appear to drop off in 16–21 year old individuals even though they are still reproductively active (Nisbet et al., 1999). Nisbet et al. (1999) suggests that the lower androgen concentrations may indicate the early stages of reproductive senescence in the common tern. Accordingly, melatonin found at lower concentrations in older Nazca boobies may indicate senescence as suggested for other animals (Reiter, 1992). Old rodents, for example, possess much lower melatonin concentrations than younger individuals (Miguez et al., 1998). A decrease in melatonin can have severe consequences including immunodeficiency (Maestroni, 1993), tumor growth (Blasko, 1984; Relkin, 1983), and decreased longevity (for overall review see Reiter, 1992). We suggest that known-age, long-lived sea birds can be used as a system to study potential physiological effects of hormonal changes throughout ontogeny. The results from our study also suggest that valuable information on the adaptive function of circadian hormone release can be gained by studying alternative, new systems in the wild.

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