Melatonin secretion in a strictly subterranean mammal, the Damaraland mole-rat (*Cryptomys damarensis*)

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Abstract

Subterranean mammals inhabit an environment that is normally devoid of light and are therefore deprived of photoperiodic information that can be used to time important life-history events. An assessment was made of whether melatonin secretion in a strictly subterranean rodent, the Damaraland mole-rat *Cryptomys damarensis*, can be modified by photoperiod. In experiment 1, a clear diurnal rhythm of melatonin secretion in animals housed under a neutral photoperiod (12L:12D) was observed, with significantly higher melatonin concentrations in the dark compared to the light phase. The same diurnal melatonin rhythm was found 1 day after animals were transferred to either continuous light or continuous dark, suggesting that a circadian rhythm was maintained under acute exposure to light and dark. In experiment 2, melatonin secretion was monitored in a long (14L:10D) and short day (10L:14D) photoperiod and was found to be modified by the photoperiodic change. We therefore suggest that the Damaraland mole-rat possesses a circadian melatonin rhythm that can be physiologically modulated in response to photoperiod.

Key words: subterranean, mole-rat, melatonin, photoperiod, *Cryptomys damarensis*

INTRODUCTION

Subterranean mammals inhabit a challenging environment that is characterized by a high $CO_2:O_2$ ratio, low nutrient availability and poor opportunity for dispersal (Bennett & Faulkes, 2000; Roper, Bennett *et al*., 2001). Consequently, subterranean mammals have evolved numerous remarkable adaptations, including physiological (e.g. progression of non-visual senses such as olfaction: Heth *et al*., 1992; and hearing: Nevo, Heth & Pratt, 1991; Credner, Burda & Ludescher, 1997; Klauer, Burda & Nevo, 1997; Narins *et al*., 1997) and sociological (e.g. complex social structures: Jarvis *et al*., 1994; Clarke & Faulkes, 1997; Faulkes *et al*., 1997). The most striking characteristic of subterranean environments is that they are devoid of light, which has led to regression of ocular structures that subserve vision in many subterranean species (Cooper *et al*., 1993*a,b*). Furthermore, the absence of light means that subterranean animals are deprived of the most ubiquitous environmental cue (or *zeitgeber*), namely photoperiod, that is available to time life-history events.

Photoperiod determines the pattern of secretion of a number of endogenous substances that exhibit diurnal variation. For instance, in almost all mammals, secretion of the hormone melatonin is far greater at night, when animals are in the dark, than during the day, when animals are exposed to light (Reiter, 1991; Malpaux, Thiery & Chemineau, 1999). Melatonin secretion thus has a diurnal rhythm, the phase of which is determined by the length of day (Reiter, 1988). As annual changes in day length are predictable, the phase of the melatonin rhythm can be used as a neurochemical index of season, facilitating the use of photoperiod as a *zeitgeber* (Reiter, 1991, 1993; Malpaux, Viguie *et al*., 1997; Chemineau & Malpaux, 1998; Malpaux *et al*., 1999). To effectively reflect different day lengths, the phase of the melatonin rhythm needs to be regulated at a resolution that is equal to, or greater than, the average magnitude of change in photoperiod (Reiter, 1987*a,b*; Malpaux, Thiery *et al*., 1998, 1999).

Despite being widely distributed in seasonal habitats, most strictly subterranean mammals do not restrict reproduction to a particular time but are reproductively active throughout the year (Shanas *et al*., 1995; Bennett, Faulkes & Molteno, 1996; Spinks, Van der Horst & Bennett, 1997), although seasonal fluctuations in some reproductive parameters have been reported in some species (e.g. sperm count in male common mole-rats *Cryptomys hottentotus*; Spinks *et al*., 1997). One explanation for the absence of seasonal breeding in subterranean mammals is that they cannot interpret and

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respond to seasonal changes in day length because there is not an appropriate photoperiodic signal below ground. Diurnal rhythms of melatonin secretion that resemble those of other mammals have been described in some fossorial (Reiter, Hurlburt *et al.*, 1983; Reiter, Steinlechner *et al.*, 1983) and subterranean (Reiter, Reiter *et al.*, 1994) species. However, the effects of photoperiod on reproductive parameters in subterranean mammals are unknown. Consequently, whether the phase of melatonin secretion can be modulated in response to changes in photoperiod in subterranean mammals (i.e. whether or not photoperiod is a *zeitgeber*) is not known.

Melatonin secretion was assessed in a strictly subterranean rodent, the Damaraland mole-rat *C. damarensis*, initially to determine whether there is a diurnal pattern of melatonin secretion in this species. Subsequently, an assessment was made of whether a change in photoperiod caused a change in the melatonin rhythm, to test the hypothesis that the pattern of melatonin secretion in this subterranean mammal can be regulated at a resolution that allows a distinction between different photoperiods. There are several reasons why *C. damarensis* is an excellent model species in which to test this hypothesis. First, *C. damarensis* inhabits extensive, sealed tunnel systems that are located > 25 cm underground (Bennett, Jarvis & Davies, 1988; Jarvis *et al.*, 1994) and which no light penetrates (T. A. Richter & N. C. Bennett, pers. obs.). Damaraland mole-rats feed on roots and tubers located beneath the surface (Jarvis & Bennett, 1990) and have never been observed above the ground in > 4000 h of field observation (N. C. Bennett & A. J. Molteno, pers. obs.). Consequently, *C. damarensis* is rarely, if ever, exposed to external light. Second, the structures that subserve vision in *C. damarensis* are significantly reduced, although light can still be perceived (Negroni, 1998).

MATERIALS AND METHODS

Experimental model

Adult *C. damarensis* were captured in the field at Dorbabis, Namibia (22°58′S, 17°41′E) and transferred to artificial holding facilities in light- (~ 200 μW/cm^2 during the light phase, < 0.01 μW/cm^2 during the dark phase) and temperature- (25 ± 1 °C) controlled rooms. The mole-rats were housed in their intact colony in plastic crates (49.5×28 cm) under red light for 1 month before the start of the trials (habituation period and experimentation). A small plastic box served as a nest in which the animals slept. Wood shavings and paper towelling were provided as nesting material. The mole-rats were fed chopped sweet potato, pumpkin, carrots and apples daily, but drank no free water. They were allowed 2–3 months to habituate under the specific lighting regime being tested before starting experimentation. As colonies of *C. damarensis* comprise large (mean mass: males $= 165$ g; females $= 191$ g), reproductively active, and smaller (mean mass: males $= 124$ g; females $= 116$ g), suppressed individuals (Bennett, Jarvis *et al.*,

1994; Bennett, Faulkes *et al.*, 1996), experimental groups were matched for reproductive status and sex.

Blood sampling

Blood samples were collected from the brachial vein following venepuncture. Not more than 450 μl of blood was collected from an individual on each sampling occasion. Owing to the extended period over which experiment 2 was carried out, samples were taken from individual animals not more than twice every 10 days. To ensure minimal exposure of the animals to light when sampling at night, individuals were captured with the aid of a weak (< 0.05 μW/cm^2) red-filtered headlamp. As an additional precaution, hand-restrained animals were immediately blindfolded with a light-impenetrable hood. Blood sampling was usually completed within 5 min. All experimental procedures were carried out under UP Ethics Committee guidelines (permit no. 960426–006).

Melatonin assays

Plasma was assayed in duplicate 100 μl aliquots using a previously described, double antibody radio-immunoassay technique (Malpaux, Daveau *et al.*, 1983). Briefly, antiserum (described in Tillet *et al.*, 1996) was diluted 1:80 000 and the labelled ligand was 2–^{125}I melatonin. The standard curve and buffer controls were made from ovine plasma from pinealectomized animals. Specificity of the antibody was checked by the inhibition test of melatonin binding with 17 structurally related endogenous molecules. Cross-reaction was $< 0.01\%$ for all tested compounds except 2% for 6-OH-melatonin and 0.1% for *N*-acetyl-serotonin (Tillet *et al.*, 1996). Parallelism was demonstrated between a standard curve in ovine plasma by double diluting 2 pools of mole-rat plasma from an animal maintained in the dark. In addition, a pool of mole-rat plasma with 0, 16, 32, 64 and 128 pg/ml of melatonin added (slope $= 0.99$, least-squares $r^2 = 0.99$; data not shown) revealed recovery between 85% and 100%. Samples for each experiment were assayed separately. The inter- and intra-assay coefficient of variation for the combined assays was 10.0% and 7.7%, respectively. Sensitivity averaged 4 pg/ml.

Experiment 1 (Fig. 1)

The aim of this experiment was to determine whether a circadian rhythm of melatonin secretion exists in *C. damarensis* and whether this rhythm is maintained under acute exposure to continuous light or continuous dark. Laboratory-housed animals ($n = 24$) were habituated to a standard photoperiod (12L:12D) for 3 months. The experiment was then conducted on 2 consecutive days. On day 1, blood samples were collected midway through the dark (< 0.01 μW/cm^2) and light (~ 200 μW/cm^2) phase (at T0 and T12, respectively) to monitor melatonin
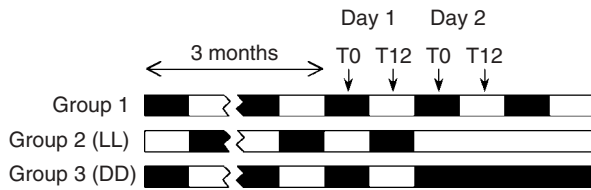
Fig. 1. Design of experiment 1. All *Cryptomys damarensis* ($n = 24$) were held in a neutral photoperiod (12L:12D) for 3 months before experimentation. Note that group 2 was 12 h out of phase with groups 1 and 3 (see Materials and Methods for details). To assess melatonin secretion blood samples were collected on day 1 of the experiment at T0 and T12, which corresponded, respectively, to the middle of the dark and light phase in the control group (group 1). Subsequently (on day 2), while control animals (group 1, upper panel) were kept in the neutral photoperiod, animals in group 2 (middle panel) were transferred to continuous light (LL) and group 3 (lower panel) was transferred to continuous dark (DD). Melatonin concentrations were assessed again by obtaining blood samples on day 2 at the same times as on day 1 (T0 & T12).

Fig. 2. Design of experiment 2. Melatonin secretion in a short day (SD, upper panel) and long day (LD, lower panel) photoperiod was assessed in blood samples from *Cryptomys damarensis* collected at times (T4, 6, 8, 16, 18 & 20) that spanned the transition from dark to light and vice versa (lights on/off at 07:00/17:00 in SD; lights on/off at 05:00/19:00 in LD). A single group of animals ($n = 24$) was used to assess melatonin secretion in SD and LD to minimize variability. The mole-rats were held in the prevailing photoperiod for 2–3 months before blood sampling. See Materials and Methods for further details.

secretion. Animals were then transferred to 1 of the following treatment groups. Group 1 ($n = 8$) was held in the same (neutral) photoperiod (12L:12D) to act as a control. Group 2 ($n = 8$) was held in continuous light (LL, $\sim 200 \ \mu W/cm^2$; 24L:0D), while group 3 ($n = 8$) was held in continuous dark (DD, $< 0.01 \ \mu W/cm^2$; 0L:24D). On day 2, blood samples were again taken at T0 and T12.

If all 3 groups were in phase with each other, the blood sampling in group 2 would have had to be delayed by 12 h, relative to groups 1 and 3, to compensate for the transfer from the standard photoperiod to continuous light. As this may have invalidated a direct comparison between the groups, group 2 was maintained exactly 12 h out of phase with groups 1 and 3. Thus, blood samples were taken at the same chronological time in all groups, and the same time relative to the change in photoperiod, although in different phases (see Fig. 1 for details).

Data for statistical analysis were the log-transformed values of melatonin concentration, since the raw data were not normally distributed. For one individual in group 3 (DD), a single sample was lost (day 1, T12), resulting in complete sample sizes of 8, 8 and 7 for groups 1, 2 and 3, respectively. A repeated-measures analysis of variance (RM-ANOVA, Statistica) was carried out to determine the effect of time of sampling on melatonin levels for day 1 and day 2 separately. This enabled analysis of melatonin concentrations under the neutral photoperiod on day 1 and the manipulated photoperiod on day 2. For this analysis, T0 and T12 samples for group 2 (LL) were switched to examine the effect of light on melatonin, rather than time of day. Subsequently, RM-ANOVA analyses were carried out for each group to assess the effects of the different light regimes, using day (day 1 and day 2) and time (T0 and T12) as repeated factors. Post-hoc analysis was carried out by Tukey's honest significant difference test (Tukey's HSD).
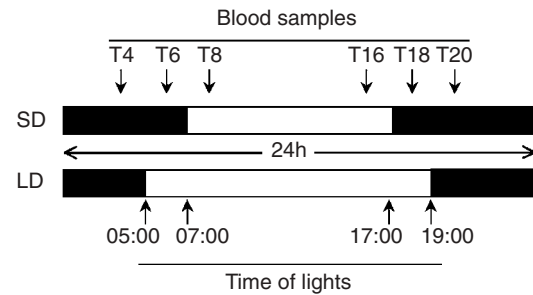
Experiment 2 (Fig. 2)

The aim of this experiment was to determine whether the phase of the melatonin rhythm in *C. damarensis* changes in response to photoperiods of different duration. A single group of mole-rats ($n = 24$) balanced for sex and reproductive status was used. Animals were housed in a long day (LD) photoperiod (14L:10D, lights on at 05:00 and off at 19:00) for 3 months before experimentation. To monitor melatonin secretion, blood samples were taken at 6 time-points (T4, 6, 8, 16, 18, 20). Note that these time-points spanned 'lights on/off' (i.e. the switch from light to dark and vice versa). Once blood samples had been collected from each animal at each time-point (which took *c.* 3 months), all animals were transferred to a short day (SD) photoperiod (10L:14D, lights on at 07:00 and off at 17:00) and allowed 2 months to become habituated. The same blood sampling regime that was used in LD was then used in SD. Note that, as for LD, time-points at which blood samples were collected were over a period that spanned lights on/off (see above).

Data for statistical analysis were the log-transformed values of melatonin concentration since the raw data were not normally distributed. Analysis of plasma melatonin levels was carried out by 4-way RM-ANOVA using sex and reproductive status as independent factors and day length as factors. The analysis was repeated with day length and time of day as factors. Post-hoc analysis was carried out by Tukey's HSD test. Values are presented throughout as the means ± 1 SE.

RESULTS

Experiment 1 (Fig. 3)

There is a significant circadian rhythm in melatonin secretion. For all groups, melatonin concentrations were
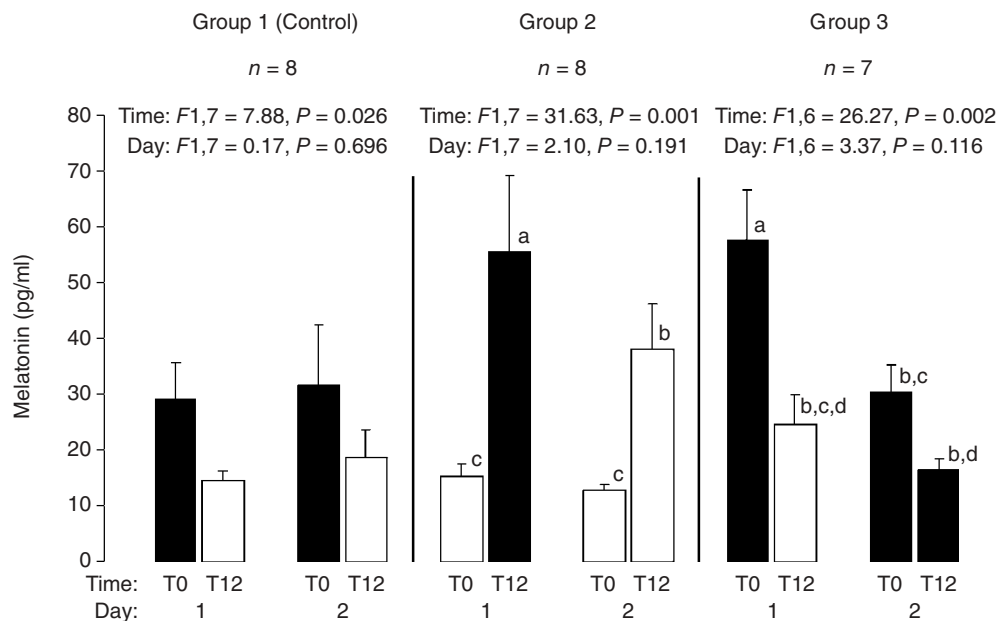
Fig. 3. Mean ± SE melatonin concentrations (pg/ml) in *Cryptomys damarensis* observed in experiment 1. On day 1 of the experiment, all mole-rats were held in a neutral (12L:12D) photoperiod. On day 2, group 2 was exposed to constant light (LL) and group 3 was exposed to constant darkness (DD). Group 1 (control) was kept in the neutral photoperiod. Blood samples were collected at T0 and T12 on each day. See legend of Fig. 1 and Materials and Methods for further details. Solid bars, samples taken during dark phase; open bars, samples taken during light phase. Letters link groups that were significantly different from each other at $P < 0.05$ (RM-ANOVA followed by Tukey's HSD). Note that the light and dark phase of group 2 was 12 h out of phase with group 1 and 3 (see Materials and Methods for explanation).

significantly higher in the dark compared to the light phase under the neutral (12L:12D) photoperiod on day 1 ($F_{1,20} = 55.52$, $P < 0.0001$). Melatonin during the dark phase (47.6 ± 6.2 pg/ml) was 2.7 times higher than during the light phase (17.9 ± 2.0 pg/ml). A significant effect of treatment on melatonin levels on day 1, when all groups are subject to a neutral photoperiod (treatment: $F_{2,20} = 3.92$, $P = 0.037$), reflects the variability between individuals, with a greater difference in melatonin concentration between the dark and light phases for groups 2 and 3 than in the control group.

By contrast with day 1 results, under the manipulated photoperiods on day 2, there was no significant effect of treatment ($F_{2,20} = 0.02$, $P = 0.980$), while the circadian rhythm continued to have an effect on melatonin concentrations (time: $F_{1,20} = 18.53$, $P < 0.001$). This was also evident for repeated measures analysis of data for each group, there being a significant effect of time of sampling, but no significant difference in melatonin concentrations between day 1 and day 2 (Fig. 3). In groups 2 and 3, the circadian rhythm evident on day 1 was still evident on day 2, despite the change to continuous light or continuous dark, respectively. This suggests that the diurnal rhythm of melatonin secretion observed was not readily inhibited by exposure to light (~ 200 μW/cm^2) at night (group 2) and was circadian (group 3).

Experiment 2 (Fig. 4)

Four-way RM-ANOVA revealed a significant effect of time of day on plasma melatonin levels ($F_{5,100} = 24.89$,
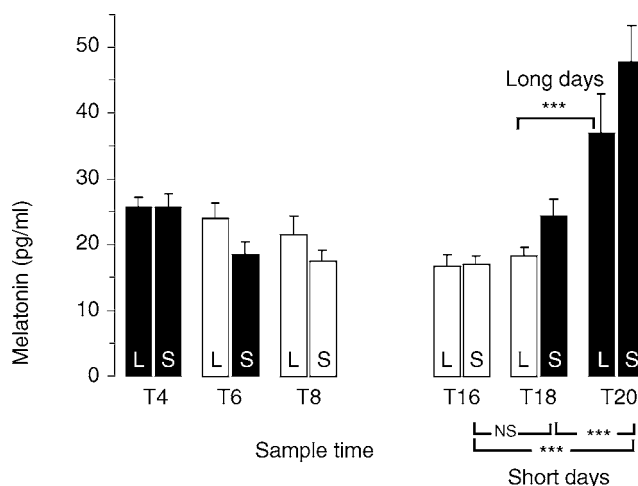

Fig. 4. Mean ± SE melatonin concentrations (pg/ml) in *Cryptomys damarensis* observed in experiment 2. See Fig. 2 for details of experimental design. Left and right columns are for blood samples collected at the same times for long (L) and short (S) days. Solid bars, samples taken during dark phase; open bars, samples taken during light phase. At no sampling time was there a significant difference in melatonin levels between the long- and short-day-length treatments (RM-ANOVA followed by Tukey's HSD). For long days, melatonin concentration for T20 samples was significantly higher than for T18. For short days, melatonin concentration was significantly higher for T20 than either T16 or T18 (NS different). NS, not significant; ***, $P < 0.001$.

$P < 0.001$), while sex, reproductive status and day length treatment were not significant factors each on their own. Reproductive females showed significantly lower melatonin concentrations than the other groups, being 19.68 (2.18 pg/ml compared with 26.46 (3.13* pg/ml for non-reproductive females and $26.94 \pm 2.70*$ and 25.30 ± 1.61 pg/ml for reproductive and non-reproductive males respectively ($n = 6$ for each group; *, significantly different to reproductive females at $P < 0.05$, Tukey HSD).

When the analysis was repeated with day length and time of day as the only factors, there was no significant effect of day length ($F_{1,23} = 0.216$, $P = 0.646$), a significant effect of time of day ($F_{5,115} = 21.712$, $P < 0.0001$) and a significant interaction between time of day and day length ($F_{5,115} = 3.872$, $P = 0.0028$). At no time point were the differences between long and short-day treatments significant (Tukey's HSD). The significant interaction between time and day length reflects that while short days have only marginally higher melatonin concentrations at the times sampled (25.95 ± 1.45 compared with 23.95 ± 1.36 pg/ml on long days), the largest difference between the treatments was observed after lights-out. For both treatments the significant change in melatonin secretion was between T18 and T20, 1 and 3 h after lights-out for the long and short-day photoperiods, respectively.

DISCUSSION

Despite being widely distributed in habitats that are subject to seasonal variations in environmental factors, most strictly subterranean mammals are reproductively active throughout the year (see Bennett & Faulkes, 2000, for review). The absence of light underground suggests that subterranean mammals are normally deprived of the most ubiquitous *zeitgeber* to time life-history events, namely photoperiod. However, it has been suggested that brief exposure to light can entrain melatonin secretion in subterranean rodents (Rado *et al*., 1993; Reiter, Reiter *et al*., 1994), as it does in some other species (Reiter, 1993; Shanahan *et al*., 1997). In this study, melatonin secretion in a strictly subterranean rodent *C. damarensis* was found to be both diurnal and circadian, but relatively insensitive to acute exposure to light and dark.

The diurnal pattern of melatonin secretion in *C. damarensis* was similar to other mammalian species in which a 24-h rhythm of melatonin secretion has been described (Bartness & Goldman, 1997). The existence of a diurnal rhythm of melatonin secretion in a subterranean animal is not unprecedented, as ocular regression that is associated with adaptation to a subterranean habitat is not necessarily accompanied by a loss of circadian melatonin secretion. In fact, diurnal melatonin rhythms have been described previously in blind (Pevet *et al*., 1984; Green & Romero, 1997) and anopthalmic (Bartness & Goldman, 1989; Jagota *et al*., 1999) animals. In addition to being diurnal, melatonin secretion in *C. damarensis* seems to be circadian, as evidenced by the similar pattern of diurnal melatonin secretion in mole-rats transferred from a neutral photoperiod to continuous darkness in experiment 1. Furthermore, melatonin secretion was relatively insensitive to exposure to light at night, as evidenced by the uninhibited nocturnal rise in melatonin secretion observed in animals transferred to continuous light. This characteristic resembles melatonin secretion in most diurnally active rodents, where melatonin secretion does not seem to be easily suppressed by exposure to light at night (Reiter & Peters, 1984).

In addition to the existence of a circadian rhythm of melatonin secretion in *C. damarensis*, the alteration of this rhythm when animals are changed from a long (14L:10D) to a short (10L:14D) day photoperiod (indicating that a phase change can occur in response to the change in photoperiod) suggests these animals detect day length. The results of the present study therefore support the hypothesis that the pattern of melatonin secretion in *C. damarensis* can be physiologically regulated to allow different photoperiods to be distinguished. The period of melatonin secretion is required to interpret day length (and thereby season), the presence of a response to a change in photoperiod in *C. damarensis* suggests that photoperiod has the potential to function as a *zeitgeber*, despite these mole-rats being strictly subterranean.

In a completely dark, subterranean environment, there may be a lack of selective pressure for the maintenance of a functional melatonin system, if there are no clear fitness advantages associated with its maintenance, as has been suggested for the lack of photoperiodism in some species of cave fish (Green & Romero, 1997). In fact, selection may promote visual sensory atrophy if there are metabolic economies to be gained by reducing visual structures (Cooper *et al*., 1993*a*,*b*). *Crypotmys damarensis* may therefore still be sensitive to photoperiod as a relict trait that has survived from a potential evolutionary above-ground ancestry.

We found that reproductive females showed lower melatonin concentrations than other individuals. Note that other studies have found no significant effect of gender (e.g. Santana *et al*., 1991; Reiter, Reiter *et al*., 1994) or reproductive status (Reiter & Richardson, 1992; Zarazaga, Malpaux & Chemoneau, 1996, 1997). An explanation for this unusual finding warrants further investigation, particularly in light of the extensive reproductive skew in this eusocial mammal.

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