



Changing patterns of daily rhythmicity across reproductive states in diurnal female Nile grass rats (*Arvicanthis niloticus*)

Jessica A. Schrader^a, Erin J. Walaszczyk^b, Laura Smale^{a,c,d,*}

^a Department of Zoology, Michigan State University, East Lansing, Michigan 48824, USA

^b Department of Fisheries and Wildlife, Michigan State University, East Lansing, Michigan 48824, USA

^c Department of Psychology, Michigan State University, East Lansing, Michigan 48824, USA

^d Neuroscience Program, Michigan State University, East Lansing, Michigan 48824, USA

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ABSTRACT

A suite of changes in circadian rhythms have been described in nocturnal rodents as females go through pregnancy and lactation, but there is no information on such patterns in diurnal species. As the challenges faced by these two groups of animals are somewhat different, we characterized changes in activity and core body temperature (T_b) in female diurnal Nile grass rats (*Arvicanthis niloticus*) as they went through a series of reproductive states: virgin, pregnant, pregnant and lactating, lactating only, and post-weaning. The phase of neither rhythm varied, but the amplitude did. Females increased their overall levels of daily activity from early to late pregnancy, regardless of whether they were also lactating. The pattern of activity was less rhythmic during early than mid-lactation, in both non-pregnant and pregnant females, as a consequence of a decrease in daytime relative to nighttime activity. The T_b rhythm amplitude dropped from mid-pregnancy through mid-lactation, and there were rises in T_b troughs during the mid-light and mid-dark phases of the day, though pregnancy and lactation affected T_b at these times in somewhat different ways. This study demonstrates that rhythms in diurnal grass rats change during pregnancy and lactation in different ways than those of nocturnal species that have been studied to date and that the effects of pregnancy and lactation are not additive in any simple way.

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1. Introduction

Circadian rhythms allow organisms to predict fluctuations in the environment and appropriately time behavioral and physiological events to anticipate those changes [1]. In mammals, the mechanisms governing these rhythms have been studied primarily in nocturnal rodents, particularly rats (*Rattus norvegicus*), hamsters (*Mesocricetus auratus*), and mice (*Mus musculus*), which exhibit behavioral and physiological rhythms that are roughly 12 h out of phase with those of diurnal species [2]. Circadian rhythms in females of these nocturnal species change across the estrous cycle [3] and throughout pregnancy and lactation [4,5]. The influences of ovarian hormones responsible for changes in non-pregnant females have been examined extensively [3,6–10], but little is known about how pregnancy and lactation change circadian rhythms despite the fact that wild adult female

mammals spend significantly more time in these states than they do undergoing estrous cycles.

To meet the demands of pregnancy and lactation, females must change many parameters of their behavior and physiology. Females of many species increase food intake or mobilize fat stores to sustain fetal and neonatal growth [11–13], and hyperthermia during lactation is almost universal [13]. In many species, activity levels are low throughout pregnancy and lactation [5,13–16], and sleep disruption is more common [13,17]. Although these general trends are well-established, relatively little is known about how circadian rhythms in these physiological and behavioral phenomena change [for an exception see 4].

Most studies of the effects of pregnancy and lactation on circadian rhythms have focused on locomotor activity and core body temperature (T_b) in nocturnal rodents and have found changes in the phase and/or the amplitude of these rhythms. Lab rats and various hamster species are less active, and that activity is less rhythmic, when they are pregnant or pseudopregnant than when they are non-reproductive [5,14–16]. The peak of the T_b rhythm in lab rats is phase-advanced by several hours during both pregnancy and lactation, and the amplitude of the rhythm decreases during gestation and increases during lactation [4]. We do not know whether any of these changes occur in diurnal rodents. One might expect them to be similar, as pregnancy

* Corresponding author. 108 Giltner Hall, Michigan State University, East Lansing, MI 48824, USA. Tel.: +1 517 432 1632; fax: +1 517 353 1652.

E-mail addresses: stjohnj4@msu.edu (J.A. Schrader), walaszcz@msu.edu (E.J. Walaszczyk), smale@msu.edu (L. Smale).

and lactation impose major metabolic challenges regardless of an animal's temporal niche [11,13]. However, animals that are active during the warmest time of the day may not be able to change their activity and T_b rhythms in the same manner as those that are most active during the coldest phase of the day–night cycle.

The current study used a diurnal species, the Nile grass rat (*Arvicanthis niloticus*), a murid rodent from sub-Saharan Africa [18], to examine two questions about how rhythms are modulated by reproductive state. The first was whether pregnancy and lactation affect circadian rhythms in activity and T_b in diurnal rodents in the same manner as they do in nocturnal ones. The second was whether the effects of pregnancy and lactation on circadian rhythms are additive or instead interact in more complex ways. To address these questions, we monitored T_b and activity of female grass rats as they progressed through a series of reproductive states: virgin, pregnant, pregnant and lactating, lactating only, and post-weaning.

2. Materials and methods

2.1. Animals

Animals were adult (>60 days) female and male Nile grass rats, *A. niloticus*, obtained from a breeding colony at Michigan State University [19]. All animals were housed in polycarbonate cages (48×28×16 cm) and given red transparent plastic huts (18×6×6 cm) for optional shelter. For singly housed animals and lactating females (see below), only one hut was provided, but mating couples were given two huts. Food (PMI Nutrition Prolab, RMH 2000, Brentwood, MD) and water were provided *ad libitum*. Animals were kept in a 12 h:12 h light/dark cycle (~250 lx during the light phase), with the lights on from Zeitgeber Time (ZT) 0 to ZT 12, and a red light (<5 lx) was on continuously to allow for visual observations and video recording during the dark phase. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care & Use Committee (04/03-053-00) and in accordance with the standard in the National Research Council Guide for Care and Use of Laboratory Animals (National Academy of Science).

2.2. Surgery

Calibrated temperature/activity transmitters (PDT ER-4000 G e-mitters, Minimitter, Bend, OR) were implanted intra-peritoneally into adult female grass rats under anesthesia (2–5% isoflurane), and females were given pre-surgery subcutaneous injections of the analgesic, buprenorphine hydrochloride (Buprenex, 0.06 mg/kg of body weight). A single 1 cm incision was made along the midline of the abdomen and muscle wall, and a transmitter was inserted into the peritoneal cavity. The muscle wall was sutured with nylon sutures, and the skin was sutured subcutaneously with dissolvable chromic gut sutures, which were reinforced with autoclips. Animals were then given sterile saline (2.0 cm³, 0.9% NaCl) subcutaneously, removed from anesthesia, and monitored until they regained normal motor capacity. They received injections of Buprenex (same dose as above) every 12 h for the next two days.

2.3. Experimental manipulations

Implanted females were housed singly in cages atop receiver bases (ER-4000, Minimitter, Bend, OR), which conveyed transmitter readings to a computer in an adjacent room that was equipped with Vitalview 4.0 software (Minimitter, Bend, OR). Gross motor activity and body temperature readings were collected and stored every 5 min, and the data file was exported once weekly, which required stopping the data collection for a five-minute period. Once all the females had healed from surgery, recordings were taken for two

weeks prior to splitting them into two treatment groups ($n=5$ per group): reproductive females and age-matched virgin controls. Each female in the reproductive group was paired with an adult virgin male grass rat, while the controls were kept singly housed throughout the experiment. Virgin females of this species do not exhibit a clear estrous cycle and appear to be in an anestrus state [20], but female grass rats do come into a reliable post-partum estrus [21]. Therefore, we could not assess the onset of pregnancy until the first litter was born. The mating couples were checked daily for the presence of their first litter, and the day that litter was discovered was designated day 0 of pregnancy and lactation (PL0, beginning at ZT 12 prior to birth) since a post-partum mating had occurred. Gestation in this species lasts approximately 25 days [21], and the day of conception of the first litter (pregnancy day 0 = P0) was calculated as 25 days prior to day PL0. At 21 days of age (PL21), the first litter was separated from the mother, and the male was also removed. The female's cage was checked daily for the birth of the second litter, and the day of birth was designated as day 0 of lactation (L0). The second litter was separated from the mother at day L21, and recordings were taken from the female for at least two more weeks after weaning. Readings were taken from all of the control females until recording from the last reproductive female was complete.

2.4. Activity and temperature data analysis

To determine how circadian rhythms in activity and body temperature varied among reproductive states, we sampled data from days 2, 10, and 19 of pregnancy (P), pregnancy plus lactation (PL), and lactation (L) from the reproductive females. These days were chosen because both pregnancy and lactation are dynamic states, and we wished to compare early, mid, and late days within these states without the confounds of other transitions, such as parturition (day P0), implantation (likely around P4 to P6), and weaning (L21). We also sampled on a pre-reproductive baseline day (pre: four days before pairing) and a post-reproductive day (post: 11 days after the second litter was weaned). Since the control females did not go through these reproductive states, we determined equivalent days for this group by sampling the data from each control female from the same calendar dates as we had sampled from a randomly pair-matched reproductive female so that five reproductive/control pairs of data were date-matched.

To examine how activity rhythms were altered by reproductive state, we generated actograms from the raw data in ClockLab v. 2.61 (Actimetrics, Wilmette, IL). For each day of interest, we used the program to determine both onset and offset of activity. Occasionally, the Clocklab algorithm designated the onset or offset to be at a time that was not consistent with visual inspection of the actogram. In these cases, the onset or offset was estimated instead by eye. To assess effects on the magnitude of general activity, we had to standardize the activity counts within each individual, since the activity outputs of the transmitters were not calibrated to one another. Therefore, to determine whether the total daily activity changed within individuals over the course of the study, we calculated the average daily activity for each reproductive day of interest (P2 through post or control equivalent) as the ratio of the average daily activity to the average daily activity of the pre-reproductive day. We also calculated the dark:light ratio of total activity for each day of interest, including the pre-reproductive day.

The temperature readings were calibrated for each transmitter by Minimitter (Bend, OR), and these readings could be directly compared between individuals. For each day of interest, T_b measurements were averaged for each half hour. The amplitude of the daily rhythm was calculated as the difference between the maximum and minimum half hour T_b . To examine how the phase of the rhythm changed, the start of the half hour with the minimum T_b was determined. This served as the best phase marker as the timing of the daily maximum was much more variable among and within individuals (data not shown). A

preliminary examination of the half-hour averages revealed that the time periods exhibiting the most noticeable and consistent changes were the midday (ZT 3 to 9) and midnight (ZT 15 to 21) periods. The average T_b for each of these two six-hour periods was therefore determined for each day of interest.

Since individual animals were randomly assigned to the two treatment groups and repeated measures were taken from each individual, a general mixed effects model (GMEM) was most appropriate to examine the influences of reproductive days on activity and T_b rhythms. For each dependent variable (rhythm parameter) described above, a GMEM was generated in SAS v. 9.1 (SAS Institute, Inc., Cary, NC) with reproductive day (or equivalent), treatment group, and the interaction between the two as fixed factors and reproductive day (or equivalent) as a repeated measure within the random effect of the individual within a group. In the GMEM, the covariance structure of the data had to be specified. To determine the best covariance structure for each dependent variable, the model was run with each of four different structures (compound symmetry, heterogeneous compound symmetry, autoregressive, and unstructured), and the Akaike's Information Criterion value for each model was used to determine which model

best fit the data. Post-hoc least significant difference tests were performed for any significant factors identified by the GMEM with the best covariance structure for pair-wise comparisons of means between groups within a reproductive day and between days within a group.

2.5. Video recordings and behavioral analysis of nesting females

In order to determine whether rhythms in nesting females might change across lactation and also to examine how core body temperatures might mediate nesting, we examined nesting and drinking rhythms in a subset of non-pregnant, lactating females ($n = 3$). Dams were videotaped with a low-light lens CCTV camera connected to a time-lapse video recorder that condensed a 24-hour period onto 2 h of videotape. For each female, days 2, 10, and 19 of lactation were scored by two different individuals. All individuals involved in video scoring performed preliminary test scoring to ensure 94% or higher agreement between all scorers. This agreement threshold was defined as having 94% of all time bins scored the same by all scorers. Each video was scored for off-nest and drinking behaviors. Off-nest behavior was defined as the female leaving contact with the pups; drinking, eating, and general

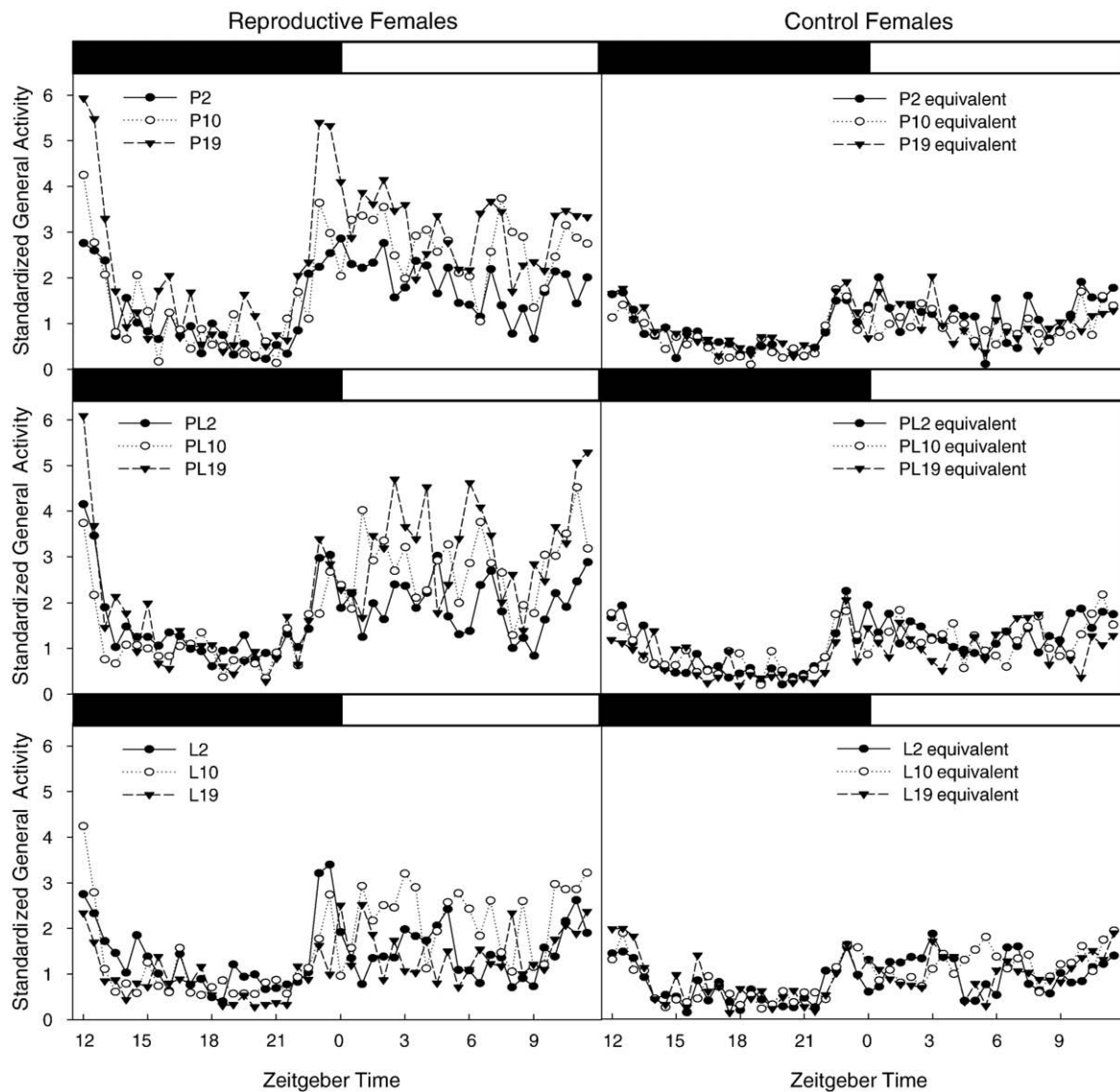


Fig. 1. Averaged general locomotor activity rhythms in the reproductive (left) and control (right) groups ($n = 5$ /group) under a 12 h:12 h light (open bars)/dark (black bars) cycle for pregnancy, pregnancy plus lactation, and lactation (or equivalents). For each individual, the average activity of each half hour was standardized by dividing the raw value for that half hour on the day of interest by the average total daily activity of that female on the pre-reproductive day. Plotted values are means of these standardized values for each treatment group for each half hour. Standard errors were removed for ease of comparing the rhythms.

locomotion occurred during this time. Drinking behavior was defined as the female placing her tongue or snout against the ball bearing in the water bottle spout. It usually occurred during off-nest bouts, but some females could rear up while still on the nest and reach the water bottles. The 24 h of each video were split into five-minute bins, and for each bin, each of the two scorers recorded in an all-or-none fashion which of the behaviors occurred. For each hour, the frequency for each behavior was calculated by dividing the number of bins scored positive for that behavior by both scorers by the total number of bins the scorers agreed upon within that hour (thus excluding any bins with discrepant scores).

For analysis of these frequencies, since all females were subjected to the same treatment, a two-way repeated measures ANOVA was performed for each behavior with the frequency for that behavior as the dependent variable and day of lactation (days 2, 10, or 19) and ZT (24 total hours) as fixed effects in SPSS v. 15 (SPSS, Inc., Chicago, IL). Least significant difference post-hoc tests were performed when significant effects were found. Differences were considered significant when $p < 0.05$. Data are given as means \pm SE unless otherwise stated.

3. Results

3.1. Reproductive output

For all reproductive females, the first litters were born within 27 to 32 days of pairing with males (mean 29.8 ± 1.2 days). These initial litters ranged in size from 4 to 7 pups (mean 5.8 ± 0.5 pups), and their sex ratios varied from twice as many males as females to the opposite ratio (mean 1.05 ± 0.25 males/females). Second litters were born 25 to 26 days after births of first litters (mean 25.8 ± 0.2 days) and ranged in size from 3 to 9 pups (mean 5.6 ± 1.1 pups). Their sex ratios varied from no males to 3 males for every 2 females (mean 1.23 ± 0.52 males/females).

3.2. Activity rhythms

As seen in previous laboratory work with this species [19,21], female grass rats in the current study exhibited diurnal activity rhythms with crepuscular tendencies (Fig. 1). This pattern is somewhat different from that seen in a natural setting where activity peaks in the middle of the day and there are no peaks around dawn or dusk [22]. Although females were diurnal in the current study, the amount of activity and its distribution across the day varied among reproductive states. We measured the effects of the reproductive days (pre through post or equivalent days for the control group to account for the effects of age) on the phases of activity onset and offset, the average daily activity (relative to the pre-reproductive day), and the

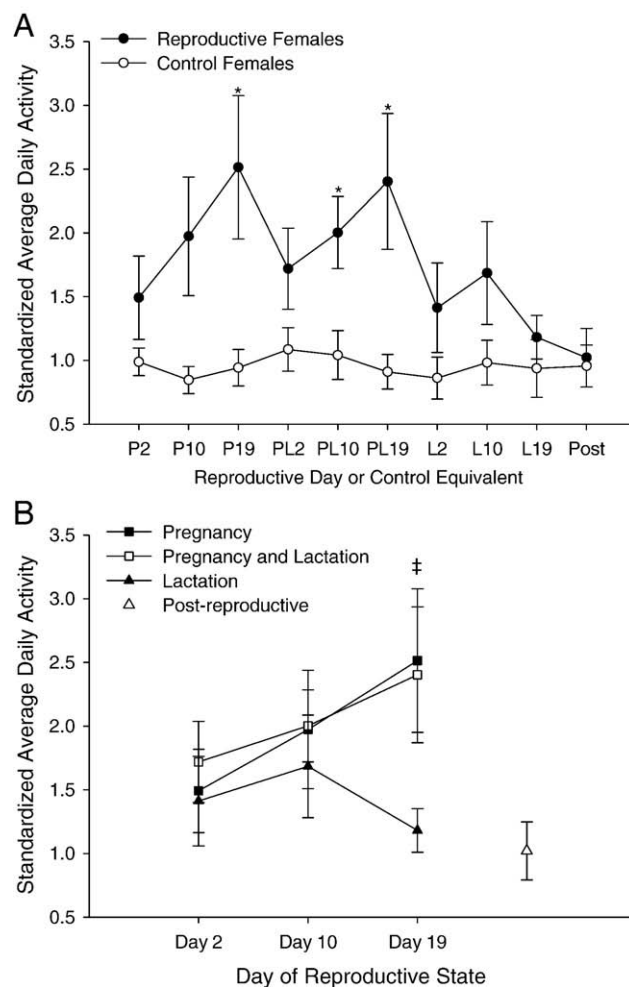


Fig. 2. (A) Average daily activity across reproductive days (or equivalents) in the two treatment groups ($n=5$ /group). The average daily activity was standardized by dividing the average activity of a female for each day of interest by the average activity of that female on the pre-reproductive (or equivalent) day. Values are means \pm SE. *Significant differences between the control and reproductive groups ($p < 0.05$; LSD test). The data for the reproductive group are re-plotted in (B) for comparisons of pregnancy and lactation to the combined state. ‡Significant differences between lactation and the combined (PL) state ($p < 0.05$; LSD test).

dark:light activity ratio. Table 1 contains a summary of the type III tests of fixed effects from the GMEM analyses for these variables. The phases of activity onset and offset were not significantly different

Table 1
GMEM results for type III tests of fixed effects for activity and temperature rhythms.

Rhythm parameter	Covariance structure ^a	Reproductive day (or equivalent) effect	Treatment group effect	Reproductive day \times group interaction
Phase of activity offset	CSH	$F(10, 26.6) = 1.25$ NS	$F(1, 7.94) = 0.76$ NS	$F(10, 26.6) = 1.24$ NS
Phase of activity onset	CSH	$F(10, 27.9) = 1.50$ NS	$F(1, 8.28) = 5.17$ NS	$F(10, 27.9) = 0.61$ NS
Average daily activity	CSH	$F(9, 27.6) = 3.51$ $p < 0.01$	$F(1, 8.01) = 5.11$ NS	$F(9, 27.6) = 2.74$ $p < 0.05$
Dark:light activity ratio	CSH	$F(10, 25.7) = 2.08$ NS	$F(1, 8.08) = 0.26$ NS	$F(10, 25.7) = 2.67$ $p < 0.05$
Amplitude of T_b rhythm	CS	$F(10, 80) = 10.60$ $p < 0.0001$	$F(1, 8) = 9.73$ $p < 0.05$	$F(10, 80) = 4.01$ $p < 0.0005$
Phase of minimum T_b	CSH	$F(10, 26.3) = 1.27$ NS	$F(1, 10.4) = 6.69$ $p < 0.05$	$F(10, 26.3) = 0.90$ NS
Average midday T_b	CS	$F(10, 80) = 12.90$ $p < 0.0001$	$F(1, 8) = 10.71$ $p < 0.05$	$F(10, 80) = 12.53$ $p < 0.0001$
Average midnight T_b	CS	$F(10, 80) = 14.36$ $p < 0.0001$	$F(1, 8) = 18.73$ $p < 0.005$	$F(10, 80) = 14.79$ $p < 0.0001$

$n = 5$ /group. NS = not significant ($p > 0.05$).

^a CSH = heterogeneous compound symmetry, CS = compound symmetry, see Materials and methods for model details.

across reproductive days (or equivalents) in either group, and they did not differ between groups (Table 1). Activity onsets occurred roughly 1.5 h before lights on (ZT 22.58 ± 0.10 h), and the offsets occurred around 1.75 h after lights off (ZT 13.76 ± 0.17 h).

Locomotor activity increased from early to late pregnancy, regardless of whether or not females were also lactating (Fig. 1). The average daily activity was significantly affected by the interaction between reproductive days (or equivalents) and treatment group and by the main effect of reproductive days (Table 1). In the reproductive group, the average daily activity was significantly higher than the post-reproductive day from day P19 through PL19 (Fig. 2A; $p < 0.05$; post-hoc pair-wise least significant difference (LSD) tests). Daily activity levels of the reproductive females appeared to return to pre-reproductive levels by the post-reproductive day (daily activity: 1.02 ± 0.23 , with a value of 1 indicating that activity levels were the same as on the pre-reproductive day). Therefore, females were also more active on days P19 through PL19 than during the pre-reproductive period. Additionally, reproductive females were more active on days P19, PL10, and PL19 than controls on the equivalent days (Fig. 2A; $p < 0.05$; LSD tests). Within pregnancy, activity levels significantly increased from day 2 to day 19 (Fig. 2B; $p < 0.05$; LSD test). A similar trend was apparent during the period of pregnancy plus lactation, but there were no significant differences across days in this state (Fig. 2B). During lactation this trend was not seen, and females on day L19 had significantly lower levels of activity than on PL19 (Fig. 2B; $p < 0.05$; LSD test). The average daily activity did not vary significantly among any of the days of interest in the control group (Fig. 2A; mean for all days: 0.955 ± 0.25 ; $F(9, 7.73) = 0.44$, $p > 0.5$; post-hoc GMEM).

The distribution of activity across the light/dark cycle was highly labile (Fig. 3A), but this distribution was affected by the interaction between reproductive days (or equivalents) and treatment group (Table 1). The reproductive females had a significantly lower dark:light activity ratio on PL19 (more diurnal) and a significantly higher ratio on L2 (more nocturnal) than controls ($p < 0.05$; LSD tests), but the ratio did not differ significantly from the pre- or post-reproductive day for any day examined in either group (Fig. 3A). Lactating females were more nocturnal during early than mid-lactation, regardless of whether or not they were pregnant, and the ratio was significantly higher on day PL2 than PL10 or PL19 and on day L2 than on L10 (Fig. 3B; $p < 0.05$; LSD tests). Within the control group, the ratio of activity in the night versus the day did not change across the days of interest, and females were more active in the light than the dark (Fig. 3A; dark:light ratio: 0.714 ± 0.070 ; post-hoc GMEM; $F(10, 9.14) = 0.53$, $p > 0.8$).

3.3. Temperature rhythms

Females were hyperthermic during much of pregnancy and lactation, with the most noticeable rises in T_b occurring during the mid-dark (major trough) and mid-light (minor trough) phases of the day, while rhythms in control females did not noticeably change over time (Fig. 4). Peaks occurred around the light/dark transitions. GMEM analysis of the amplitude of the T_b rhythms and midday and midnight T_b averages all revealed significant main effects of reproductive days (or equivalents) and treatment group and a significant interaction between the two (Table 1). However, reproductive state did not alter the phase of the rhythm as indicated by the half-hour interval with the lowest average T_b (Table 1). There was a significant effect of treatment group, however, with control females exhibiting a slightly later minimum T_b (ZT 18.19 ± 0.34) than reproductive females (ZT 17.09 ± 0.34) throughout the study, including prior to pairing the reproductive females with their mates.

The amplitude of the T_b rhythm (based on half-hour intervals) changed across time in both groups of animals but in different ways (Fig. 5A). Within the control group, the amplitude significantly increased toward the end of the study, from the PL19 and L2 equivalents to the post-reproductive equivalent (Fig. 5A; $p < 0.05$; LSD tests). In the reproductive group, the amplitude was also highest on the post-

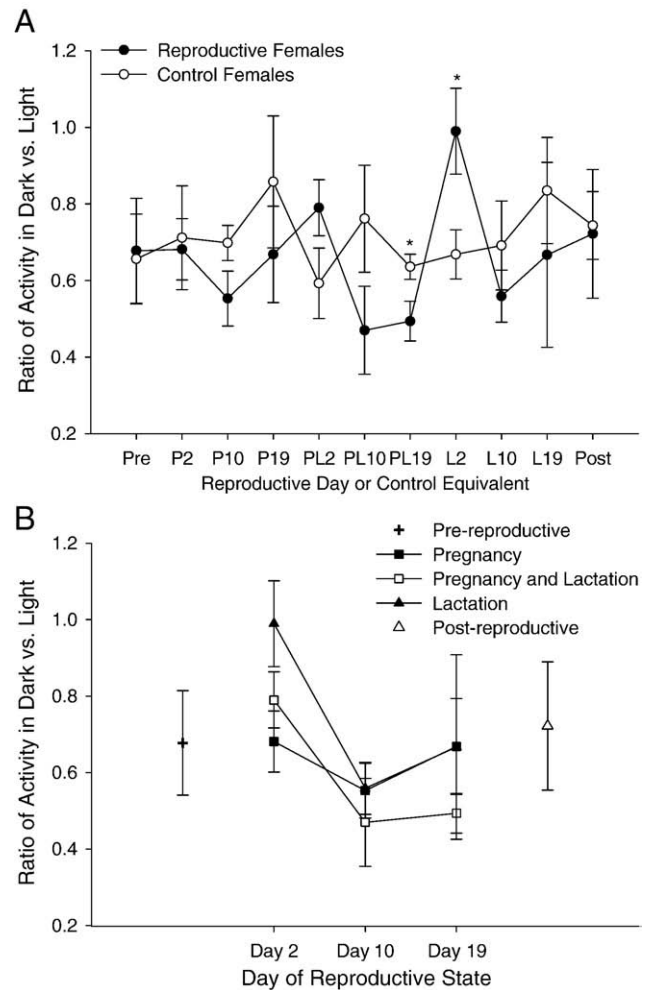


Fig. 3. (A) Ratio of activity in the dark versus light phase across reproductive days (or equivalents) in the two treatment groups ($n = 5/\text{group}$). Values are means \pm SE. Notation is the same as in Fig. 2A. The data for the reproductive group are re-plotted in (B) for comparisons of pregnancy and lactation to the combined state.

reproductive day, and it did not differ from that of controls at that point (Fig. 5A). These data indicate that amplitude increases with age. However, in the reproductive females, the amplitude of the T_b rhythm dropped from mid-pregnancy through mid-lactation and was significantly lower from days P10 through PL10 as well as on L2 and L10 than on the pre-reproductive day (Fig. 5A; $p < 0.05$; LSD tests). The amplitude from days P10 through PL10 was also significantly lower than in controls on the equivalent days (Fig. 5A; $p < 0.05$; LSD tests). Pregnancy and lactation influenced the rhythm amplitude differently during the combined state, with one masking the effects of the other on different days (Fig. 5B). The amplitude on PL10 was the same as on L10, but it was significantly higher than on P10 (Fig. 5B; $p < 0.01$; LSD test), suggesting that pregnancy has no effect on the rhythm amplitude in females that are 10 days into lactation. In contrast, the amplitude was the same on PL19 as on P19 but was lower on PL19 than on L19 (Fig. 5B; $p < 0.01$; LSD test), indicating that by 19 days, lactation no longer had an effect on the rhythm amplitude, regardless of whether or not females were pregnant.

Drops in rhythm amplitude appear to be due to rises in the average body temperatures of females during the midnight period in all three reproductive states relative to the pre-reproductive period and to controls. Midday temperatures rose in a similar fashion (Fig. 6A and B). During the midday period, T_b values did not change in the control group (Fig. 6A; $F(10, 40) = 0.83$, $p > 0.5$; post-hoc GMEM), and during the midnight, T_b actually dropped in controls from the P19 equivalent to the post-reproductive equivalent day (Fig. 6B; $p < 0.05$; LSD test), which is

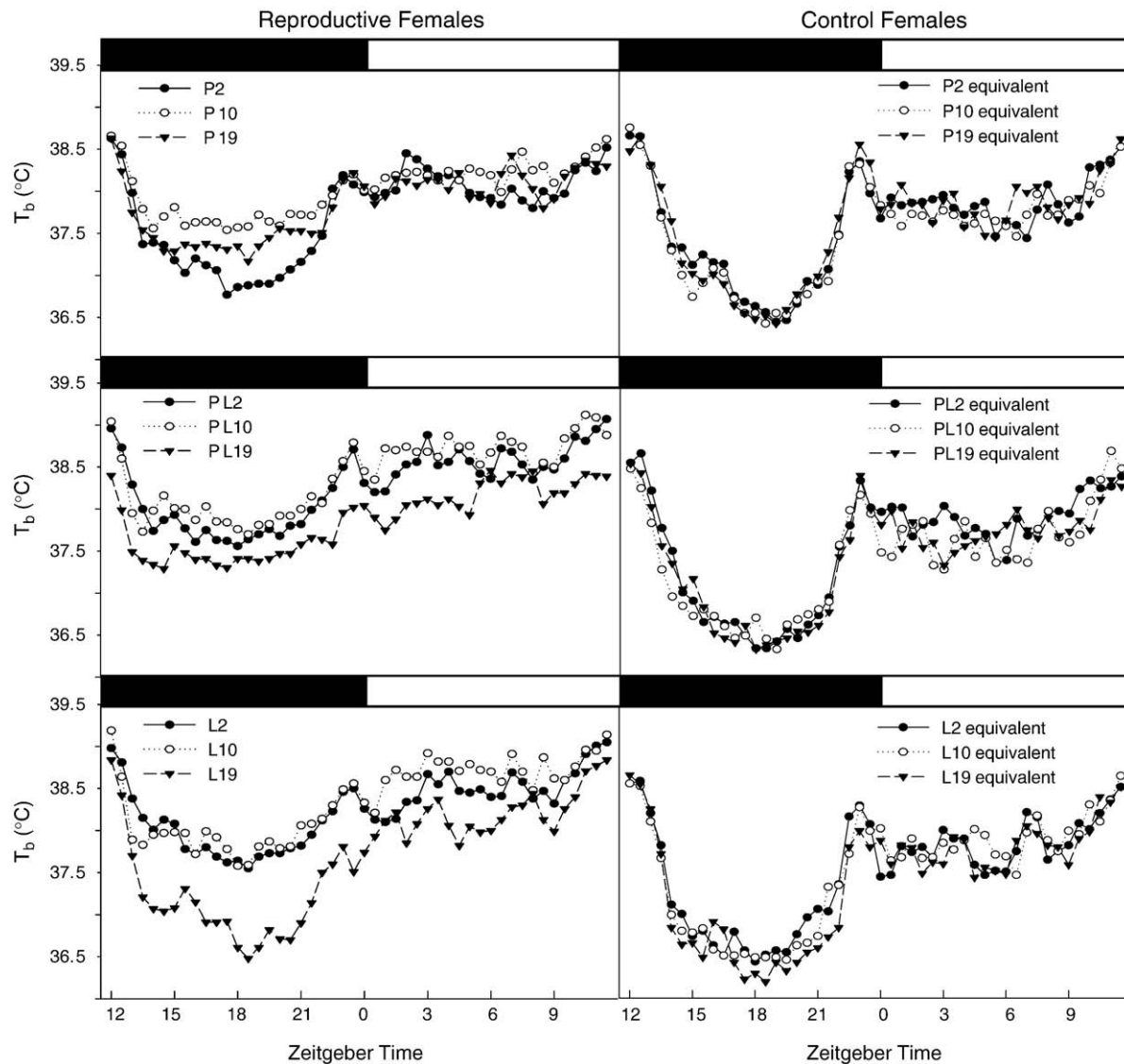


Fig. 4. Averaged T_b rhythms in the reproductive (left) and control (right) groups ($n = 5/\text{group}$) under a 12 h:12 h light (open bars)/dark (black bars) cycle for pregnancy, pregnancy plus lactation, and lactation (or equivalents). Values are means for each treatment group for each half hour. Standard errors were removed for ease of comparing the rhythms.

the probable cause of the rise in rhythm amplitude seen at this time (Fig. 5A). In the reproductive group, however, the midday T_b averages increased from the pre-reproductive day for all days of pregnancy and lactation and, except on P19, were also higher on these days than on the post-reproductive day and than controls on the equivalent days (Fig. 6A; $p < 0.05$; LSD tests). Similar deviations from the pre- and post-reproductive days were seen for the midnight T_b averages (Fig. 6B). The mean midnight T_b was significantly higher than on the pre-reproductive day from P2 through L10 and than on the post-reproductive day from P10 through L10; reproductive females had significantly higher midnight T_b means than controls on these days as well (Fig. 6B; $p < 0.05$; LSD tests).

Midday and midnight T_b values were highest in early to mid-lactation, whether or not females were also pregnant. During the midday period, the average T_b values during days 2 and 10 of pregnancy were significantly lower than the corresponding days of the combined pregnant/lactating state (Fig. 6C; $p < 0.01$; LSD tests), and midday T_b did not change throughout pregnancy (Fig. 6C). However, the midday T_b significantly dropped from days 10 to 19 in both lactation and the combined pregnant/lactating state (Fig. 6C; $p < 0.005$; LSD tests). Similar trends were evident in the midnight period with some differences (Fig. 6D). The midnight T_b average was significantly lower on P2 than on

PL2, and that on L19 was significantly lower than on PL19, again demonstrating differential effects of pregnancy and lactation during the combined state (Fig. 6D; $p < 0.05$; LSD tests). Midnight T_b means peaked on both P10 and PL10 and were significantly higher than on days 2 and 19 of the same states (Fig. 6D; $p < 0.05$; LSD tests). Similarly, the mean midnight T_b on L10 was significantly higher than on L19 ($p < 0.005$; LSD test) but did not differ from that on L2 (Fig. 6D).

3.4. Behavioral rhythms in nesting females

Three of the females from the reproductive group were videotaped on days 2, 10, and 19 of lactation, and the tapes were scored to assess the proportion of five-minute intervals each hour (beginning at the designated ZT) during which the lactating dam was off the nest and/or drinking. A two-way repeated measures ANOVA revealed no effects of day of lactation (off-nest: $F(2, 4) = 0.459$, $p > 0.5$; drinking: $F(2, 4) = 3.694$, $p > 0.1$) and no interaction between day of lactation and ZT for either behavior (off-nest: $F(46, 92) = 1.047$, $p > 0.1$; drinking: $F(46, 92) = 0.948$, $p > 0.5$). However, there were significant effects of time of day (ZT) on each behavior (off-nest: $F(23, 46) = 5.897$, $p < 0.001$; drinking: $F(23, 46) = 2.227$, $p < 0.05$), indicating a daily rhythm in nesting and drinking that did not change across lactation. These

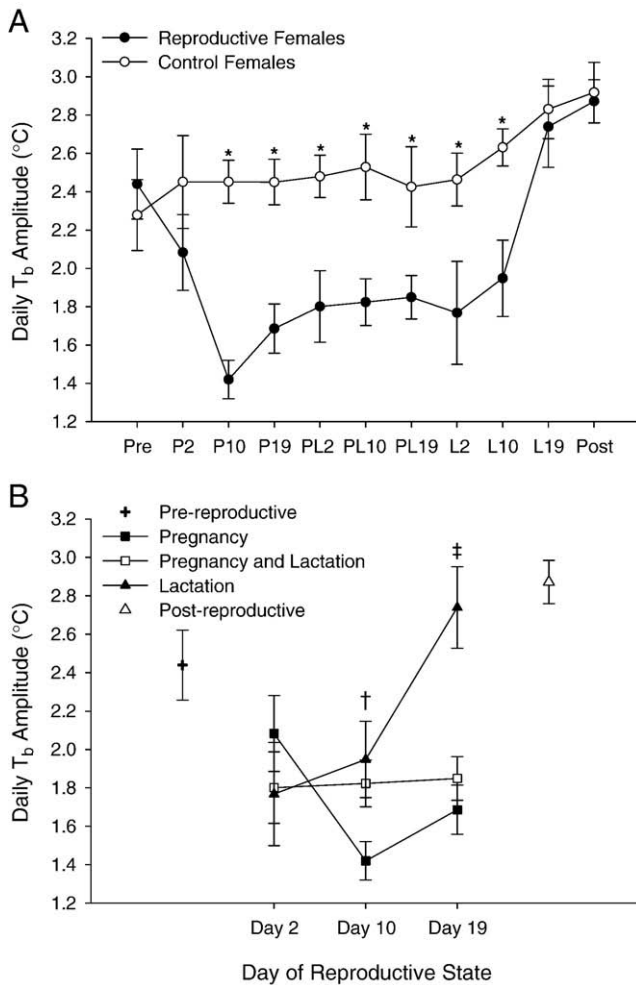


Fig. 5. (A) Daily amplitude of the T_b rhythm across reproductive days (or equivalents) in the two treatment groups ($n = 5/\text{group}$). Values are means \pm SE. Notation is the same as in Fig. 2A. The data for the reproductive group are re-plotted in (B) for comparisons of pregnancy and lactation to the combined state. †Significant differences between pregnancy and the combined (PL) state ($p < 0.05$; LSD test). ‡Significant differences between lactation and the combined (PL) state ($p < 0.05$; LSD test).

rhythms reflected the crepuscular nature of activity in this species in captivity. Off-nest activity peaked near the light/dark transitions (ZT 1 and 12), and troughs occurred in the mid-light and mid-dark phases (ZT 7 and 20; $p < 0.05$; LSD tests between peaks and troughs), though off-nest behavior was significantly less frequent at ZT 20 than at ZT 7 (Fig. 7A; $p < 0.01$; LSD test). Drinking was less frequent than off-nest behavior, but the rhythm looks similar in shape, though not in amplitude, to that behavior (Fig. 7B). There was not one distinct peak for this rhythm, although the highest frequencies of drinking were around the light/dark transitions (ZT 2 and 11; Fig. 7B). The significant troughs of the rhythm occurred a few hours before lights on (ZT 20 and 21) and again at lights on (ZT 0; Fig. 7B; $p < 0.05$; LSD tests between each trough and ZT 1 and 2).

4. Discussion

Many females spend most of their lives pregnant and/or lactating, which mandates major changes in various parameters of physiology and behavior that are rhythmic. In this study, we found that circadian strategies can be modulated during pregnancy and lactation in quite different ways in diurnal and nocturnal species. Additionally, we demonstrated that the effects of pregnancy and lactation are not simply additive during the combined state but rather that the effects

of pregnancy are evident in certain parameters of activity and temperature rhythms while the effects of lactation are evident in others. We have also found that rhythms in maternal nesting of grass rats do not change over the first 19 days of pup development.

One important issue to consider when interpreting these data is that males remained with their females during both the pregnant and the combined pregnant/lactating states but were absent during the lactation-only state and among control females. Therefore, differences seen within females in the P/PL states compared to the L condition, as well as differences between P/PL females and controls, could theoretically be due to the presence/absence of the male. We were unable to include P and PL conditions without males because grass rats do not undergo spontaneous estrous cycles, and the post-partum estrus is the only one that can be reliably predicted. Effects of reproductive state can be evaluated independently of effects that males might have had on T_b or activity when we compare P with PL states (both of which occurred when the male was present) and when we compare L females with non-pregnant controls (neither of which involved the presence of a male). Both sets of comparisons reveal clear effects of reproductive state. Furthermore, changes occurring within any given state (P, PL and L) are likely to begin with changes in the female and/or the pups and cannot be due to changes in the presence/absence of males. It should also be noted that all of the comparisons we have made are likely to reflect what occurs in nature, where adult male and female grass rats live together in communal burrows with one or two generations of their offspring [22–24] and where non-pregnant females (whether they are lactating or not) are unlikely to be living with males.

During pregnancy but not lactation, locomotor activity increased to roughly 2.5 times the level seen in virgin females (Figs. 1 and 2). However, there were no changes in the timing of the offset or onset of activity or in the relative amount of activity in the dark versus light portions of the day. This is entirely different from patterns observed in nocturnal rodent models, which become less rhythmic in their activity and show lower levels of both wheel-running and general activity [5,14–16]. In rats and hamsters, estradiol can consolidate, mildly phase-advance, and increase nighttime activity [3,7,8], and, in hamsters, progesterone counteracts these effects [7]. Takahashi and Menaker [7] argued that this might explain why pregnant rats, which have high levels of circulating progesterone relative to estrogens, are much less active and less rhythmic in their activity. Although we do not know the effects of these hormones on activity in grass rats, the patterns we see here suggest that responses to these hormones may be very different in this species compared to lab rats and hamsters.

The most dramatic effects of reproductive state on rhythms stemmed from rises in T_b that occurred during pregnancy, lactation, and when the two states were combined (Fig. 4). The increases were largest during the midday and midnight phases of the rhythm rather than at the light/dark transitions. This may reflect a physiological constraint limiting increases at those transitions, when T_b was high even in females that were not reproductive. The increases were also larger at night than during the day (Fig. 6), which resulted in a reduction in rhythm amplitude (Fig. 4). During pregnancy, the rises in T_b could have been partially driven by changes in activity, which, although not necessary for them, has modulatory effects on rhythms in T_b [25,26]. However, it is unlikely that activity was solely responsible for the changes since it increased from early to late pregnancy. An increase in the midnight T_b did occur from day 2 to day 10 of pregnancy, but if increased activity was inducing hyperthermia, this would have also been evident in the midday, which it was not. Various other factors probably contributed to increasing T_b and changes in its temporal pattern during pregnancy. First, the increased secretion of certain hormones may have driven hyperthermia. Progesterone and prolactin increase T_b in rodents [27,28], as do birth control pills, which contain progestagens, in humans [29–31]. During the first half of pregnancy there is an endogenous circadian rhythm in prolactin secretion in many

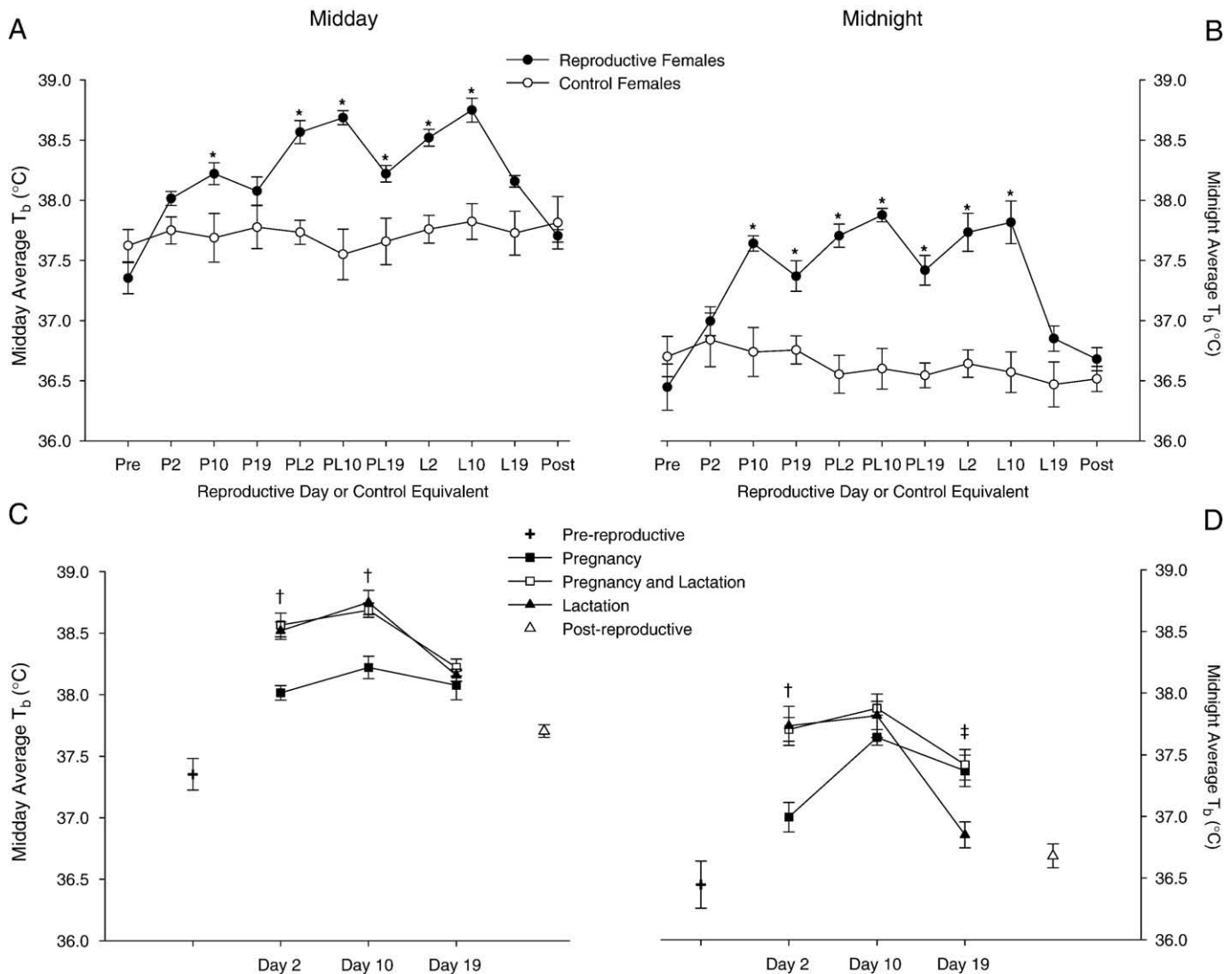


Fig. 6. (A and B) Average T_b during the midday (ZT 3 to 9, A) and midnight (ZT 15 to 21, B) periods across reproductive days (or equivalents) in the two treatment groups ($n = 5/\text{group}$). Values are means \pm SE. Notation is the same as in Fig. 2A. The data for the reproductive group are re-plotted in (C) and (D) for comparisons of pregnancy and lactation to the combined state during the midday (C) and midnight (D) periods. Notation is the same as in Fig. 5B.

rodents [32–35]. The rhythmic release of this hormone might therefore influence the rhythmic rises in T_b . Other physiological processes associated with pregnancy could also contribute to rises in T_b and changes in its temporal patterning. Since this is an energetically expensive state [11,13], an increase in feeding or metabolic turnover, as has been seen in other rodent species [36–39], could account for the time-dependent changes in hyperthermia seen in grass rats.

Hyperthermia was even more pronounced in lactation than in pregnancy, especially during the energetically expensive early to mid-lactational period (Fig. 6). There are several possible explanations for this. Circulating hormones related to lactation and maternal care could contribute directly to increased T_b [27,28], as may be the case with pregnancy. However, an increase in metabolic turnover causing hyperthermia is more consistent with our data. Hyperthermia during the mid-dark phase disappeared from mid to late lactation, when pups began to incorporate solid food into their diet, reducing energetic demands of the pups on their mother. Also, the fact that hyperthermia was more pronounced during lactation than pregnancy, which is less energetically expensive [11,13], lends support to this hypothesis. An alternative explanation was proposed by Crookery et al. [40] suggesting that contact with pups in the nest induces maternal hyperthermia and serves as a constraint on the amount of

time a dam may spend on her nest. This hypothesis has received much attention, and evidence both for and against it has been gathered from various species [41–43], but see [44–46]. Our data indicate that contact with pups did not correlate with peaks in T_b in lactating grass rats. The behavioral data show that females were off the nest least from ZT 15 to 22, when core body temperature had not yet peaked. If females were overheating on the nest, then they should have been warmest at these times. However, significant increases in T_b did occur during this mid-dark phase of the day, and maternal-pup thermal transfer may thus have contributed to some of the increase in T_b , though it certainly did not appear to constrain maternal care in grass rats. Kittrell and Satinoff [4] argued that their data from lab rats were also inconsistent with the hypothesis of Crookery et al. [40]. Their females exhibited the highest core body temperatures when they were most active off of the nest, not after long bouts on the nest. However, in grass rats, activity also could not account for lactational hyperthermia, as females were no more active than controls or than they themselves were before or after the period of reproduction. This would indicate that metabolic and/or hormonal processes were the most important factors driving hyperthermia in lactating grass rats.

Patterns of change in the phase of daily rhythms in T_b across reproductive states were different in grass rats from those seen in

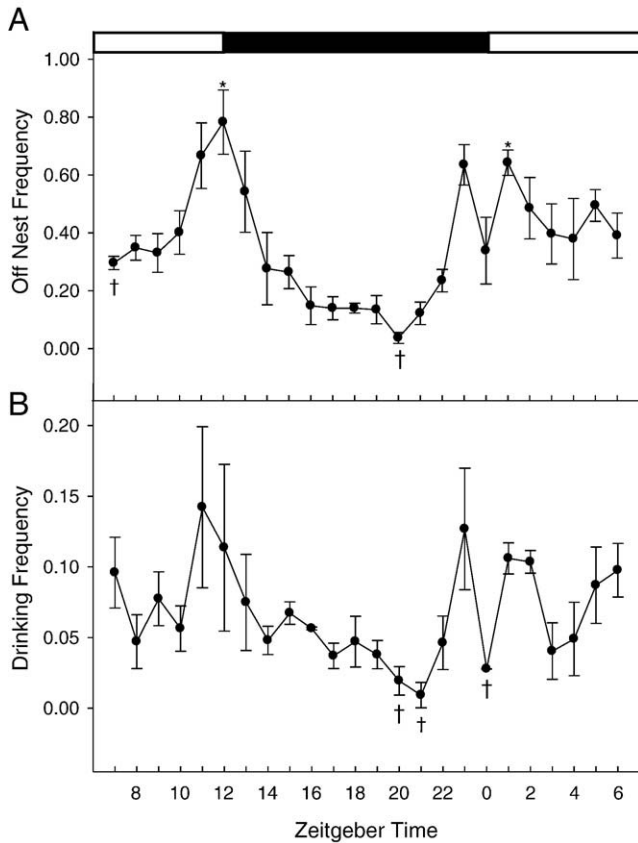


Fig. 7. Frequencies of off-nest (A) and drinking (B) behaviors for each hour of the light (open bars, top): dark (black bar, top) cycle during lactation in a subset of the reproductive group ($n = 3$). Values are pooled means \pm SE for days 2, 10, and 19 of lactation in 3 lactating dams. *Peaks and †troughs in each rhythm as explained in the Results section.

nocturnal lab rats. Specifically, whereas in lab rats, the phase of the peak of the T_b rhythm advances during both pregnancy and lactation [4], no changes in phase occurred in the rhythms of grass rats. This difference between the species may reflect their evolutionary histories in environments with different ecological constraints during the active phase. For instance, it may be that for a nocturnal animal, it would be more energetically expensive to increase T_b further during the cold night, when the T_b peak usually occurs, and in order to do so, the phase must be shifted so that it occurs under warmer ambient conditions. Another possibility is that the species difference is directly related to activity rhythms. When grass rats became pregnant, the amplitude of their activity rhythms remained high, and their phase did not change (Fig. 1). However, in pregnant lab rats, activity rhythms break down in constant darkness [16], and in hamsters, activity becomes less rhythmic under a light/dark cycle [15]. Therefore, a lack of a stable activity rhythm in these nocturnal species may allow for a change in phase of the T_b rhythm whereas the presence of a stable activity rhythm may prevent such a change in grass rats.

When we focus directly on the rhythms and ask how they were affected by reproductive state, clear time-of-day effects on the nature of the interaction between pregnancy and lactation emerge. One example is apparent when considering reproductive females on day 10. Here, the midday T_b was identical in the two lactating conditions and significantly lower when females were just pregnant (Fig. 6C), but midnight T_b was indistinguishable among the three states (Fig. 6D). Thus, effects of lactation were considerably higher than those of pregnancy during the day, but not during the night. Another example is evident when considering day 19, at which point midday T_b was the same in the three reproductive states (Fig. 6C), but the midnight T_b

was considerably lower when females were lactating but not pregnant (Fig. 6D). This pattern suggests that the return to patterns characteristic of the non-reproductive state, (evident at day 19 of lactation in non-pregnant females) occurs more rapidly for the nighttime than the daytime phase of the rhythm. In neither of these examples, at either time of day, were effects of pregnancy and lactation on rhythms simply additive. This could be due to time-of-day effects on some of the processes noted above. For example, in some cases (e.g. day 10) the non-additive effects could reflect a rhythm in a physiological ceiling on the maximum T_b (compare Fig. 6C and 6d). Although pregnancy and lactation are both energetically demanding, they are nonetheless physiologically different states. Perhaps it should not be surprising, therefore, that their effects are non-additive, but the intriguing part of the patterns are that they can be additive at some times of day but not others.

5. Conclusions and implications

The current data reveal clear patterns of change in daily rhythms of grass rats as they transition from one reproductive state to another and that these are not identical to the changes seen in nocturnal rodents. They also show interacting effects of lactation and pregnancy that could not have been predicted from either state alone and that these interactions themselves change as a function of time of day. The patterns observed here highlight the importance of considering the circadian timekeeping system as a dynamic one whose influence on behavioral and physiological processes can change in systematic ways across different life history stages. These data raise the question of what neural and physiological processes might produce changes in temporal organization across these reproductive states. One possibility is that they are driven by modulation of the mechanisms generating circadian rhythms and transmitting temporal information from them to other regulatory systems. Alternatively, these basic circadian mechanisms could be buffered against changes in reproductive state, and the emergence of new patterns of rhythmicity could instead be brought about by more direct influences of reproductive processes on systems regulating T_b and activity. The most likely possibility, however, is that the modulation of rhythms from one reproductive state to another is driven by changes both within and beyond the circadian timekeeping system.

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