



## Photic phase-response curves for cycling female mice<sup>☆</sup>

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### ABSTRACT

The photic entrainment system is critical for the internal circadian clock to be synchronized by external time cues. In nocturnal rodents, exposure to light during the early subjective night causes a phase delay, whereas it causes a phase advance during the late subjective night. This is represented by a phase-response curve (PRC). The PRC of females has not been well studied due to their estrous cycles. Our aim in this study was to understand the characteristics of photic entrainment in female cycling rodents and identify differences in photic entrainment among the stages of the estrous cycle. To establish two types of PRC, immediate PRC (iPRC) and steady state PRC (ssPRC), in each stage of the estrous cycle, we recorded circadian rhythms of wheel running activity, applying a 15-min light pulse to cycling female mice in constant darkness. In the iPRC, which was evaluated on the next day of the light pulse, the amount of phase shift in the diestrus was larger than that in the metestrus stage at circadian time (CT) 2. Similarly, the amount of phase shift in metestrus was larger than that in proestrus at CT 10. In the ssPRC, which was evaluated after completion of a new steady state, no significant estrous variations in the amount of photic phase shifts were detected for any CTs. Although these results indicate that the intrinsic photic entrainment system is not influenced by the estrous cycle, it may affect photoreception and cause sudden behavioral changes.

### 1. Introduction

Circadian rhythms are oscillations with daily periodicities in physiological and behavioral functions of organisms (see review in [Kriegsfeld and Silver, 2006](#)). Characteristic properties of circadian rhythms in mammals have long been investigated by researchers, beginning around the early 20th century ([Richter, 1922](#)). Among the defining properties of circadian rhythms, the photic entrainment system is important and necessary for the internal circadian clock to be synchronized by external time cues. The amount of photic response differs depending on the phase in the cycle at which light is presented ([DeCoursey, 1960](#); [Pittendrigh, 1960](#)). For instance, in nocturnal rodents, exposure to light during the early part of the dark phase (subjective night) generally results in a phase delay, whereas exposure to light during the late part of the subjective night generally results in a phase advance. This difference in response can be represented by a phase-response curve (PRC). The PRC can predict the manner in which an organism will entrain not only to shifts in the light-dark (LD) cycles but also to unusual light cycles, such as non-24-hour cycles or different

LD ratios ([Daan and Pittendrigh, 1976](#)).

Although the characteristics of photic entrainment in males have been well studied, little has been reported in females owing to complications that might result from their estrous cycles. Female rodents show alteration in circadian rhythms of locomotor activity during their estrous cycle ([Morin, 1980](#); [Albers et al., 1981](#); [Wollnik and Turek, 1988](#); [Morin et al., 1977](#)). For instance, female rats display phase advances in locomotor activity and higher total activity during the proestrus and estrus stages relative to the diestrus ([Wollnik and Turek, 1988](#)). In fact, physiologically high levels of estrogen alter circadian rhythms, as implantations of estrogen shorten the period of locomotor activity in female rats and hamsters, while increasing both the amplitude and activity bout length ([Morin et al., 1977](#); [Morin, 1980](#); [Albers et al., 1981](#)). Interestingly, these estrogenic effects are weakened in the presence of progesterone ([Takahashi and Menaker, 1980](#)), suggesting that complex regulation of the hormonal environment during the estrous cycle is necessary for the observed behavioral effects. Recently, we have developed a new algorithm to detect alterations in the circadian rhythms of female mice during their cycle. It allows us to easily

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predict which estrous stage the mouse is in by only observing wheel running activity (Takasu et al., 2015).

The aim of the present study was to better understand photic entrainment in cycling female rodents and determine the differences in photic entrainment among the estrous cycle stages. To establish PRCs in each stage of the estrous cycle, we recorded circadian rhythms of wheel running activity and applied 15-min light pulse to cycling female mice housed in constant darkness (DD).

## 2. Material and methods

### 2.1. Animals and housing

Nine-week-old female C57BL6/J mice were purchased from Japan SLC (Shizuoka, Japan) and maintained in a 12:12 h light-dark cycle under controlled air conditions (room temperature,  $23 \pm 1$  °C; humidity,  $50 \pm 10\%$ ) with food (Certified Diet MF, Oriental Yeast Co., LTD, Tokyo, Japan) and water available *ad libitum*. All animal housing and experimental procedures were in accordance with the guidelines of the Japanese Physiological Society and approved by the Institutional Animal Care and Use Committee at School of Agriculture Meiji University (permission #: IACUC16-0012).

### 2.2. Measuring wheel running activity

For experiments assessing wheel-running activity, each mouse was singly housed in a cage ( $183 \times 340 \times 148$  mm; CL-0135, CLEA Japan, Tokyo Japan) with a running wheel (12-cm diameter, SANKO, Osaka, Japan). The cages were placed in light-tight, ventilated boxes in which the light intensity at the bottom of the cage was 200–300 lx (light source: white LED light bulb, 230 lm, LDA2N-G AG5, Ohm electric Inc., Tokyo, Japan). The number of wheel revolutions was counted by a magnet-sensor-activated signal between a button magnet on the running wheel and a magnet relay (59070-010, Littelfuse, Inc., Chicago, IL, USA) fixed on a side wall of the cage and was fed into a computer every minute. A chronobiology kit (Stanford Software Systems, Naalehu, HI, USA) and ClockLab software (Actimetrics, Wilmette, IL, USA) were used to collect and display the activity data.

### 2.3. Determination of the estrous stage

The number of daily wheel revolutions was analyzed by an algorithm that we recently developed to detect the estrous cycle stage in individual mice (Takasu et al., 2015). To determine the significance of regularity in estrous cycles, activity data of at least 14 days were analyzed using a periodogram. To focus on the estrous cycle, periodicity ranging from 3.5 to 5.5 cycles was investigated. The significance of periodicity was examined by the permutation test (Odell Jr. et al., 1975; Ptitsyn et al., 2006), in which the strength of the periodicity detected in the original data was ranked among those of 105 sets of randomly shuffled data. If the *p* value obtained by the ranking was  $< 5\%$ , the periodicity was considered significant. Because the day showing the peak of daily activity during one estrous cycle matches to the day of proestrus confirmed by vaginal smear characterized by nucleated cells (Fig. 1), the day with increased activity was set to proestrus. For the 4-day estrous cycle, the next cycle was determined as estrus, 2 cycles later as metestrus, and 3 cycles later as diestrus. For the 5-day estrous cycle, the next cycle was basically determined as estrus, 2 cycles later as metestrus, and 3 and 4 cycles later as diestrus. Animals that did not show clear or significant peak activity during the estrous cycle were eliminated from the experiment.

### 2.4. Light pulse and constructing PRCs

Nine-week-old animals were kept under a 12:12 h light-dark schedule for 2 weeks and then transferred to DD. When animals were kept in

DD, they were randomly and remotely applied a 15-min light pulse (light intensity: 200–300 lx at the bottom of the cage; light source: White LED light bulb, 230 lm, LDA2N-G AG5, Ohm electric Inc.) every 2 weeks until their estrous cycle disappeared or they reached 10 month of age. Effects of the light pulse were evaluated on the next day of pulse treatment (immediate PRC; iPRC) as well as after achieving a steady state phase shift (steady state PRC; ssPRC). The amount of the iPRC was calculated by subtracting the time of activity onset actually observed on the next day of the pulse treatment from the pre-pulse onset time. Similarly, the amount of the ssPRC was determined by subtracting the post-pulse onset time from the pre-pulse onset time. The post-pulse time was estimated by backward extrapolation to the day of the pulse treatment along the regression line fitted to the onset of activity for 5 days, following the first 5 days since the light pulse.

Circadian rhythm phases were expressed in h of circadian time (CT). The PRC was defined as a PRC in which the activity onset was designated as CT 12 and used as a reference for phase shifts in the rhythm. The lights-on time of the light pulse was taken as the pulse reference. Advance phase shifts were plotted above the midline with a positive sign, and below the line were delay shifts with a negative sign (Honma et al., 1985).

### 2.5. Statistics

To compare the extent of phase shift among the estrous cycle stages, the 4-h binning data (total 6 time points: CT 0 to 4 as CT 2; CT 4 to 8 as CT 6; CT 8 to 12 as CT 10; CT 12 to 16 as CT 14; CT 16 to 20 as CT 18; CT 20 to 24 as CT 22) were used in one-way ANOVA with Tukey post hoc test. In addition, eta squared for one-way ANOVA and Cohen's *d* for post hoc test were carried out to present these effect size estimates. All results are presented as the mean  $\pm$  standard deviation (SD) and were considered significant at  $p < 0.05$ .

## 3. Results

We randomly applied a 15-min light pulse to 4–5 days cycling mice every 2 weeks (Supplemental Fig. 1). Fig. 2A and B illustrate the iPRC for all estrous stages in the cycling mice. Each point in both figures represents the phase shift resulting from a light pulse and that in Fig. 2B represents a mean with SD, which was obtained by averaging data over 2-h bins of circadian time. The phase delays were observed from the late subjective day to early subjective night with the maximum ( $-2.65$  h) at CT 15. The phase advances occurred from late subjective night to early subjective day with the maximum (0.55 h) at CT 5. To determine whether the estrous cycle affected the iPRC, we divided the PRC into each estrous stage (Fig. 2C) and obtained the averaged values of each stage in 4-h bins for CT 2, 6, 10, 14, 18, and 22 (Fig. 2D). One-way ANOVA (Supplemental Table 1) revealed the estrous variations in the immediate photic phase shift values at CT 2 and 10 (CT 2:  $F(3, 7) = 3.566$ ;  $p = 0.036$ , eta squared = 0.386; CT 10:  $F(3, 14) = 3.683$ ;  $p = 0.033$ , eta squared = 0.394). In addition, the amount of phase shift in diestrus was larger than that in metestrus at CT 2 ( $p = 0.023$ , Tukey test; Cohen's  $d = -1.93$ ). Similarly, the amount of phase shift in metestrus was larger than that in proestrus at CT 10 ( $p = 0.036$ , Tukey test; Cohen's  $d = -1.50$ ).

Fig. 3A and B illustrate the ssPRC for all estrous stages in the cycling mice. Each point in both figures represents a phase shift resulting from the light pulse. Phase delays were detected from the late subjective day to early subjective night with the maximum phase shift ( $-3.10$  h) at CT 15. Phase advances were observed from late subjective night to early subjective day with the maximum phase shift (0.59 h) at CT 21. We also divided the PRC of each estrous stage (Fig. 3C) and obtained the averaged values of each stage in 4-h bins for CT 2, 6, 10, 14, 18, and 22 (Fig. 3D). A one-way ANOVA revealed no significant estrous variations in the amounts of steady state photic phase shifts for any CTs (Supplemental Table 2).

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