# The estrous cycle coordinates the circadian rhythm of eating behavior in mice

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# The estrous cycle coordinates the circadian rhythm of eating behavior in mice

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**Abstract**

The estrous cycle regulates rhythms of locomotor activity, body temperature, and circadian gene expression. In female mice, activity increases on the night of proestrus, when elevated estrogens cause ovulation. Exogenous estradiol regulates eating behavior rhythms in female mice fed high-fat diet, but it is unknown whether endogenous estrogens regulate eating rhythms. In this study, we investigated whether diurnal and circadian eating behavior rhythms change systematically across the estrous cycle. We first studied diurnal eating behavior rhythms in female C57BL/6J mice in 12L:12D. Estrous cycle stages were determined by vaginal cytology while eating behavior and wheel revolutions were continuously measured. The mice had regular 4- to 5-day estrous cycles. Consistent with prior studies, the greatest number of wheel revolutions occurred on the night of proestrus into estrus when systemic levels of estrogens peak. The amplitude, or robustness, of the eating behavior rhythm also fluctuated with 4- to 5-day cycles and peaked primarily during proestrus or estrus. The phases of eating behavior rhythms fluctuated, but not at 4- or 5-day intervals, and phases did not correlate with estrous cycle stages. After ovariectomy, the eating behavior rhythm amplitude fluctuated at irregular intervals. In constant darkness, the amplitude of the circadian eating behavior rhythm peaked every 4 or 5 days and coincided with the circadian day with the greatest wheel revolutions, a marker of proestrus. These data suggest that fluctuations of ovarian hormones across the estrous cycle temporally organize the robustness of circadian eating behavior rhythms so that it peaks during ovulation and sexual receptivity.

## Introduction

Circadian rhythms are approximately 24-hour cycles of physiology and behavior that entrain to environmental cycles, but are endogenous, and thus persist in constant environmental conditions (Halberg, 1959; Pittendrigh and Daan, 1976). Because circadian rhythms are endogenous, they allow animals to anticipate environmental cycles to improve survival and reproductive success (Hurd and Ralph, 1998; Penev et al., 1998; DeCoursey et al., 2000; Woelfle et al., 2004; Davidson et al., 2006; Hozer et al., 2020; Jabbur et al., 2024). The circadian system may improve fitness by coordinating reproductive behaviors and physiology, as disruption of the circadian system impairs reproductive success in mammals (Loudon et al., 1994; Lucas et al., 1999; Miller et al., 2004; Sen and Sellix, 2016; Kobayashi et al., 2018; Swamy et al., 2018; Fernandez et al., 2020). Recent studies found that mistimed eating (i.e., during the inactive phase) impaired the timing of ovulation and mating behaviors and reduced reproductive success (Swamy et al., 2018; Kukino et al., 2022). Thus, the daily rhythm of eating is intricately connected to reproduction in mice.

The estrous cycle in mice is a 4- to 5-day cycle of ovarian follicle development that culminates in the luteinizing hormone (LH) surge and ovulation (Barry, 1979; Bronson and Vom Saal, 1979). As the follicle develops, systemic estrogen levels rise and peak on the afternoon of proestrus (Bronson and Vom Saal, 1979). The main circadian clock in the suprachiasmatic nucleus (SCN) sends a daily permissive signal that allows for the pulsatile release of GnRH (Gu and Simerly, 1997; Christian and Moenter, 2008; Williams and Kriegsfeld, 2012). This daily signal, when coupled with high levels of estrogens, leads to the LH surge on the afternoon of proestrus (Christian et al., 2005; Williams and Kriegsfeld, 2012). The LH surge stimulates ovulation, which occurs about 12 hours later (Bingel and Schwartz, 1969; Miller and Takahashi, 2013). Thus, the circadian system coordinates the timing of ovulation so that it coincides with the time of peak sexual receptivity on the night of estrus.

Prior studies have shown that fluctuations in estrogens across the estrous cycle regulate daily and circadian rhythms of wheel-running activity in rodents. In hamsters, the onset of nighttime activity is earliest on proestrus and estrus, when estrogen levels peak, compared to other days of the estrous cycle, called “scalloping” (Morin et al., 1977). Scalloping occurs because estradiol shortens the period of the activity rhythm (Morin et al., 1977; Albers et al., 1981). Rats display scalloping and a lengthening of activity duration (alpha) during the dark phase (or subjective dark) on the night of proestrus (Albers et al., 1981; Wollnik and Turek, 1988). C57BL/6J mice do not have scalloped activity onsets, but they do have longer wheel-running activity durations on the night of proestrus into estrus (Takasu et al., 2015). The temporal alignment of ovulation and increased activity during heightened sexual receptivity could increase reproductive success.

The estrous cycle also regulates food consumption in rodents. Rats eat less food on the day of estrus compared to other stages of the estrous cycle (Tarttelin and Gorski, 1971; Eckel et al., 2000). This may reflect a trade-off so that more time can be spent seeking a mate, instead of eating, during estrus when the female has ovulated and is sexually receptive. There are mixed findings in mice regarding food consumption during the estrous cycle. Some studies find that females eat less during proestrus and estrus, while others do not (Kopp et al., 2006; Basterfield et al., 2009; Smarr et al., 2019). Thus, regulation of food consumption by endogenous, cycling estrogens in mice is weak at best. However, no study has investigated whether the daily rhythm of eating is affected by the estrous cycle in mice. Previous studies from our lab showed that exogenous estradiol increased the amplitude of the eating behavior rhythm in female mice fed high-fat diet (Omotola et al., 2019). Therefore, in this study we sought to determine whether endogenous, cycling estrogens regulate daily and circadian rhythms of eating behavior in female mice.

## Methods

### Animals

Female C57BL/6J mice were used for experiments. Mice were generated from breeding pairs of heterozygous PERIOD2::LUCIFERASE mice (originally obtained from Dr. Joseph Takahashi and backcrossed to C57BL/6J mice from The Jackson Laboratory for 32 to 35 generations) crossed with wild-type C57BL/6J mice purchased from The Jackson Laboratory. All mice used in these experiments, except for one, were wild-type females (that did not carry the PER2::LUC transgene). Pups were weaned at 3 weeks old, and group housed (2 to 5 mice per cage) until 12 weeks old. Mice were bred and housed in 12L:12D and given standard chow diet (Teklad 2918, 18% protein diet) and water ad libitum. For all experiments, 12-week-old female mice were single-housed in cages (33x17x14 cm) with running wheels (11 cm diameter) in light-tight boxes with white LEDs (intensity 80 to 90 lux) and fed standard chow diet. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (protocol numbers 2015-2211 and 2021-3842).

### Experimental protocols

#### Experiment 1: Measuring daily eating behavior rhythms and estrous cycles in the light-dark cycle

Mice were single housed in 12L:12D and acclimated to running wheels for 3 to 5 weeks before data collection. Mice that had regular 4-to-5-day cycles of total wheel revolutions in their nocturnal activity were used for experiments (Kopp et al., 2006; Takasu et al., 2015; Nakamura et al., 2023). Mice that did not have clear 4- or 5-day cycles in the duration of their activity were not used for experiments because we used infradian wheel cycles as a proxy for regular estrous cycling (8 of 28 mice were excluded across all experiments, all mice shown in Fig. S1). Eating behavior rhythms and wheel revolutions were measured for 3 to 5 weeks before vaginal lavage and then for 15 to 16 days with daily vaginal lavage at Zeitgeber time (ZT) 2 to 3, where ZT0 is

lights on and ZT12 is lights off. Mice were then ovariectomized, allowed to recover for 2 weeks after surgery, and then eating behavior and wheel running rhythms were measured for about 15 days. Vaginal lavage was performed daily to confirm estrous cycles had ceased after ovariectomy.

**Experiment 2: Measuring circadian eating behavior rhythms and estrous cycles in constant darkness**

Mice were single housed in 12L:12D and acclimated to running wheels for 3 to 5 weeks. In the first cohort of mice, eating rhythms and wheel running activity were measured for 1 week and then mice were transferred to constant darkness for 5 to 6 weeks. Cage changes were performed in constant darkness using an infrared viewer (FJW Optical Systems Inc, FIND-R-SCOPE). Mice were then ovariectomized in 12L:12D and kept in LD for 3 weeks to confirm complete wound healing. The ovariectomized mice were then released into constant darkness and eating behavior and wheel running were measured for 15 to 21 days.

We also performed vaginal lavage in constant dim red light (LED safelight, 620 nm, NDT consultants, light intensity 5 to 15 lux) to measure estrous cyclicity in constant conditions that were as similar to constant darkness as possible. This method was chosen as it was technically difficult to lavage in constant darkness. Mice were single housed in 12h light (80 to 90 lux white light):12h dim red light, where the dim red light was on constantly, and the white LEDs were also on during the 12h light phase. After 2 weeks in this condition, the mice were released into constant dim red light. Vaginal lavage was performed daily for 3 to 4 weeks, at approximately Circadian Time (CT) 2 to 3, where the onset of wheel running activity was designated as CT12 (CT2 was defined as 14h after activity onset).

**Vaginal cytology for estrous cycle staging**

Vaginal lavage was performed daily in 12L:12D at ZT 2 to 3 and in dim red light at CT2 to 3. Vaginal samples were collected by aspirating 175µl of sterile saline near the vaginal opening. Samples were placed in a 96-well plate and cell morphology was examined under a light microscope and viewed at 40X magnification to assign estrous cycle stage. Briefly, samples comprised primarily of leukocytes were defined as diestrus, samples with nucleated epithelial cells were proestrus, and samples with cornified epithelial cells were estrus. Metestrus samples had all 3 cell types. The stage observed in the morning sample collected at ZT2 to 3 (or CT2 to 3) was assigned to the prior day since transitions between estrous cycle stages typically occur in the middle of the light phase (~ZT6) (Takasu et al., 2015).

**Ovariectomy surgery**

Female mice were anesthetized with inhaled isoflurane (3 to 5% for induction) and continuous isoflurane (1 to 2%) was administered throughout the procedure. Ketofen or meloxicam (5-10 mg/kg) was given subcutaneously prior to surgery. After removing the hair on the back in a 1 cm x 1cm square, a midline dorsal incision was made followed by 2 lateral incisions on the peritoneal wall. With the ovary clamped, polyglycolic acid ligatures were tied around the oviducts proximal to the ovaries. The ovaries were removed, and the peritoneal incisions were closed with 1 to 2 simple interrupted stitches. The skin was closed using metal wound clips. After surgery, mice were single housed. Ketofen or meloxicam (5-10 mg/kg) was administered subcutaneously 24 hours post-surgery and wound clips were removed 1 week after surgery. Successful removal of circulating estrogens was verified by vaginal lavage that showed no vaginal cells or no change in cytology across days.

**Wheel-running activity**

Wheel revolutions were measured using a wheel counter interfaced to ClockLab Acquisition Software (Actimetrics, Wilmette, IL). Actograms were plotted using the normalized setting in

Clocklab Analysis software in 6-minute bins. The peak of wheel-running activity across multiple days was defined as previously described (Takasu et al., 2015; Nakamura et al., 2023). Briefly, the peak day was defined as the day with 2 preceding days and 2 succeeding days with lower wheel counts. If there were 2 successive days of nearly identical high counts, then the second day was defined as the peak.

**Eating behavior rhythms**

An infrared camera (HD 48Led 940nm Outdoor CMOS 800TVL IR-Cut Dome camera waterproof IF CCTV) connected to MPX HD 1080p Security System DVR (Lorex player) was positioned to record mouse behavior at the feeder. An eating event was defined as (i) the mouse took food from the feeder, (ii) the mouse took food and ate food away from the feeder, or (iii) the mouse moved food with mouth or paws for more than 3 seconds as previously described (Pendergast et al., 2013). Individual days of eating behavior were analyzed using Oriana and plotted in 10-minute bins to create circular histograms (Oriana 4.02; Kovach Computing Services, Anglesey, UK). To determine if a daily rhythm was present, a Rayleigh test was performed ($p$<0.05 indicated a rhythm was present). We defined the length of the vector as the amplitude of the rhythm and the direction of the vector as the phase of the rhythm.

The peak amplitude of the eating behavior rhythm across multiple days was defined as the day with 2 preceding days and 2 succeeding days with lower amplitudes. If there were 2 successive days of nearly identical high amplitudes, then the second day was defined as the peak. When there was no clear infradian cycle of eating behavior rhythm amplitude after ovariectomy, the peak day was defined as the day with greater than 25% increase in the amplitude of the eating behavior rhythm compared to the lowest value in the preceding days. The peak phase was defined as the day where phase was the most advanced (earliest) compared to the 2 preceding and 2 succeeding days.

## Statistics

Three infradian cycles (15 to 16 days) of wheel-running activity and eating behavior were analyzed for each mouse in 12L:12D and constant darkness. For each mouse, the ratio of wheel revolution and eating behavior rhythm peaks during high estrogen (proestrus and estrus) and low estrogen (metestrus and diestrus) signaling stages was determined and these data were compared with ratio paired *t*-tests. Pearson's chi-squared tests were used to compare infradian cycles of wheel-running activity rhythms and eating behavior rhythm amplitudes and phases between intact and ovariectomized mice. Daily and circadian periods in 12L:12D and constant darkness, respectively, were analyzed using wavelet analysis in ClockLab Analysis software and compared across estrous cycle stages using one-way ANOVA with repeated measures. Cosinor analysis (ClockLab Analysis software) was used to measure the amplitudes of infradian cycles of eating behavior rhythm amplitudes with periods set to 96h or 120h for mice that had 4- or 5-day cycles, respectively. An unpaired t-test was then used to compare infradian cycle amplitudes between gonadally-intact mice housed in 12L:12D and constant darkness. One-way ANOVA with repeated measures was used to compare the ratio of daytime or subjective daytime eating events across stages of the estrous cycle in 12L:12D and constant darkness, respectively. Results were considered significant when $p<0.05$.



## Results

### Wheel-running activity is regulated by ovarian hormones that fluctuate across the estrous cycle

The number of daily wheel revolutions fluctuated with 4- or 5-day cycles (Fig. 1A, 1B, all mice shown in Fig. S2) in female mice. Consistent with prior studies, we found the greatest number of daily wheel revolutions usually occurred during proestrus or estrus (Fig. 1B, 1C, 1D: ratio paired *t*-test $p<0.0001$) and at 4- to 5-day intervals (Fig. 1G). The phases of wheel-running activity onsets did not correlate with stages of the estrous cycle (Fig. 1A, Fig. S3). The periods

of wheel-running activity daily rhythms did not differ across the estrous cycle in 12L:12D (Fig. S4A). Ovariectomy, which removes virtually all circulating ovarian hormones, reduced the occurrence of infradian 4- to 5-day cycles of daily wheel revolutions (Fig. 1E, 1F, 1G, all mice shown in Fig. S2). Ovariectomy reduced the likelihood that peaks of daily wheel-running activity occurred at 4- to 5-day intervals (Fig. 1G, Pearson's chi-squared test $p$=0.03).

**The amplitude of the eating behavior rhythm is coordinated by ovarian hormones that fluctuate across the estrous cycle**

The amplitudes and phases of eating behavior rhythms and the stages of the estrous cycle were measured daily in female mice (Fig. 2, 3). In gonadally-intact mice, eating behavior rhythm amplitudes fluctuated and peaked every 4 to 5 days (Fig. 3A; all mice shown in Fig. S5, S6). Peak amplitudes most often occurred during proestrus and estrus, when estrogen signaling is greatest during the estrous cycle (Fig. 3B, 3C: ratio paired $t$-test, $p$=0.008). Gonadally-intact females had more 4-to 5-day infradian cycles of eating behavior rhythm amplitudes compared to ovariectomized females, whose eating rhythm amplitudes rarely peaked at 4-or 5-day intervals (Fig. 3D, Pearson's chi-squared, $p$=0.02, all ovariectomized mice shown in Fig. S7). Daily eating events (minutes when the mice were eating) did not significantly vary across estrous cycle stages (Fig. S8, one-way ANOVA with repeated measures $p$=0.23). These data suggest that changes in ovarian hormones across the estrous cycle regulate the amplitude of the eating rhythm.

In contrast to the amplitude of the eating rhythm, there was no clear relationship between the phases of eating behavior rhythms and stages of the estrous cycle (Fig. 3E, 3F, 3G: ratio paired $t$-test $p$=0.68, all mice shown in Fig. S5). The phases of the eating rhythms fluctuated slightly each day but did not consistently occur at 4- or 5-day intervals in gonadally-intact females (Fig. 3E, 3H). Earliest phases did not coincide with a specific stage(s) of the estrous cycle (Fig. 3F, 3G). Moreover, removal of circulating estrogens by ovariectomy did not

affect the proportion of earliest phases that occurred at 4- or 5-day intervals (Fig. 3H, all ovariectomized mice in Fig. S7, Pearson's chi-squared, $p$=0.71). These data suggest that the estrous cycle does not regulate the phase of the eating rhythm in female mice.

**Estrous cycle regulation of wheel-running activity persists in constant conditions**

Gonadally-intact female mice in constant darkness had clear 4- to 5-day infradian cycles of wheel revolutions (Fig. 4A, 4B, all mice shown in Fig. S9). The periods of wheel-running activity circadian rhythms were shorter in estrus compared to diestrus and proestrus (Fig. S4B, one-way ANOVA with repeated measures, $p$=0.02; post-hoc LSD post-hoc: $p$<0.05). Ovariectomy abolished infradian cycles of wheel-running activity (Fig. 4C, 4D, all mice shown in Fig. S9). Ovariectomized mice had fewer 4- and 5-day cycles of wheel revolutions than gonadally-intact females (Fig. 4E, Pearson's chi-squared test, $p$<0.001).

**The amplitude of the eating behavior rhythm is regulated by the estrous cycle in constant conditions**

Gonadally-intact female mice in constant darkness maintained 4- to 5-day cycles of eating behavior rhythm amplitudes (Fig. 5A, 5B, 5C, 5D all mice shown in Fig. S10). Peak eating rhythm amplitudes coincided more often with days ~~with~~ having the greatest wheel revolutions (Fig. 5B, 5C: ratio paired $t$-test $p$=0.008). Based on our results in 12L:12D and in constant dim red light, the greatest number of wheel revolutions occurred on proestrus and estrus (Fig. S11). Removal of circulating estrogens by ovariectomy reduced the likelihood of 4- to 5-day cycles of eating behavior rhythm amplitude (Fig. 5D, all mice in Fig. S12, Pearson's chi-squared test, $p$=0.037). The phases of eating behavior rhythms did not fluctuate systematically with wheel revolution cycles or with predicted stages of the estrous cycle (Fig. 5E, 5F, 5G: ratio of paired $t$-test $p$=0.83, all mice in Fig. S12). There was no difference in the

likelihood of peak phase to occur at 4-to 5-day cycles between gonadally-intact and ovariectomized females (Fig. 5H, Pearson's chi-squared test, $p$=0.56).

## Discussion

~~The circadian system regulates the timing of behaviors and physiological processes, including those related to reproduction. For example, in female rodents circulating estrogens regulate the locomotor activity rhythm and the timing of the LH surge (Morin et al., 1977; Albers et al., 1981; Miller et al., 2004). Meal timing is another rhythmic behavior that regulates reproductive behavior and physiology (Swamy et al., 2018; Kukino et al., 2022).~~ The goal of this study was to determine whether ~~the estrous cycle coordinates the eating rhythm in mice. We previously found that female mice treated with physiological levels of estradiol and fed high-fat diet had high-amplitude rhythms of eating behavior compared to ovariectomized females with virtually no circulating estrogens (Palmisano et al., 2017; Omotola et al., 2019). These data led to our hypothesis that~~ endogenous ovarian hormones, whose levels systematically change across the estrous cycle, regulate daily and circadian rhythms of eating behavior. We found that amplitudes, but not phases, of eating behavior rhythms~~,~~ peaked during proestrus and estrus, which are high estrogen signaling stages, in both the light-dark cycle and constant darkness. Thus, the circadian rhythm of eating is coordinated by the estrous cycle in female mice.

The relationship between the estrous cycle and eating rhythm amplitude was abolished in ovariectomized mice, demonstrating that ovarian hormones are necessary for the coordination of eating rhythms. While we cannot rule out a role for progesterone, the current study, combined with our prior results showing~~, shows~~ that treatment with exogenous estradiol regulates the eating rhythm in female mice, suggest~~s~~ing that cycles of endogenous estrogens regulate the eating rhythm amplitude (Omotola et al., 2019). E~~Prior studies showed that e~~strogens bind to estrogen response elements (ERE) to regulate the expression of core

circadian genes in rodents (Gery et al., 2007; Rossetti et al., 2012). Other studies have observed this relationship through changes in clock gene expression across the estrous cycle or with exogenous estradiol treatments (Nakamura et al., 2005; Perrin et al., 2006; He et al., 2007; Nakamura et al., 2008). Thus, estrogens may regulate the amplitude of the eating rhythm by directly modifying the expression of circadian genes in brain regions that control eating rhythms. However, it is difficult to study the mechanisms of estrogenic regulation of the eating rhythm because the neural circuit that controls the eating rhythm is unknown.

In this study, we did not find a relationship between the phase of the eating behavior rhythm and the estrous cycle in female mice. This is consistent with prior studies in mice that showed the phase of the wheel-running activity rhythm was not regulated by estradiol nor the estrous cycle (Kopp et al., 2006; Takasu et al., 2015; Joye and Evans, 2022). Scalloping occurs in hamsters and rats because elevated estrogen signaling during proestrus and estrus transiently shortens the period of wheel-running activity, resulting in an earlier phase of activity onset (Morin et al., 1977; Albers et al., 1981). While mice do not show fluctuations in the phase of activity onset across the estrous cycle, they do have increases in amplitude of activity that coincide with the shortest period (Leise and Harrington, 2011). Our results are consistent with these prior studies; we found the greatest amplitude (most wheel revolutions) during proestrus and estrus, but no systematic fluctuations in the phase of the wheel-running activity rhythm.

It is possible that estrous cycle coordination of wheel-running activity and eating rhythms contributes to reproductive success. Others have speculated that greater locomotor activity on the night of estrus, when the female ovulates and is sexually receptive, represents mate-seeking behavior and increases the likelihood of mating after ovulation. We hypothesize that regulation of the eating rhythm amplitude may increase reproductive success. High amplitude eating rhythms consolidate eating to a smaller time window, thus allotting more time for mate seeking behaviors on the night of proestrus into estrus. It is also possible that the increase in

wheel-running activity secondarily leads to increases in eating behavior rhythm amplitude, as there is more demand for increased energy intake on the night of the greatest wheel--running activity. Further studies in mice housed without running wheels will determine whether the coordination of the amplitude of the eating rhythm with the estrous cycle persists in the absence of increased energy demand.

This study also further supports the interplay between estrogens and eating behavior rhythms. We previously showed that estradiol regulation of the eating behavior rhythm is critical for regulation of energy balance and metabolism during a nutritional challenge (Palmisano et al., 2017; Omotola et al., 2019). This study extends the model of estrogen regulation of meal timing and shows that endogenous estrogens regulate the eating behavior rhythm in gonadally-intact females fed low-fat diet. Thus, this study, together with prior studies, suggest that estrous cycle regulation of eating rhythms may be important for reproductive success.

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**Declaration of Conflicting Interests**

The authors declare that there is no conflict of interest.

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## Figure Legends

**Figure 1. Wheel-running activity is regulated by the estrous cycle.** Representative actograms of wheel-running activity of gonadally-intact (A) and ovariectomized (D) female mice housed in 12L:12D. The stage of the estrous cycle was determined by vaginal lavage (B,C,D,G) and plotted relative to wheel revolutions per day (B,E) and peak wheel revolutions (C,F) in gonadally-intact (B,C) and ovariectomized (E,F) female mice. The ratios of wheel-running activity peaks that occurred in metestrus (M) and diestrus (D) compared to proestrus (P) and estrus (E) were analyzed across 3 estrous cycles in gonadally intact mice (D, ratio paired t-test $*p<0.0001$). Wheel-running activity peaks were more likely to occur every 4 to 5 days than at other intervals in gonadally-intact compared to ovariectomized females (G, n=9/group across 15 to 16 days per mouse, $*p=0.03$, Pearson's chi-squared test).

**Figure 2. The amplitude of the eating behavior rhythm fluctuates every 4- to 5-days in female mice.** Estrous cycle stages were determined from cytology of vaginal lavage samples collected daily at ZT2 to 3 (P: proestrus, E: estrus, D: diestrus, M: metestrus). Eating behavior was monitored using infrared video cameras and amplitudes and phases of eating behavior rhythms were determined from the lengths and directions, respectively, of vectors from Rayleigh statistics (top panel).

**Figure 3. The amplitude of the eating behavior rhythm is coordinated with the estrous cycle.** Representative graphs of estrous stage and amplitudes (A) and phases (ED) of eating behavior rhythms in gonadally-intact female mice. Peak amplitudes (B, C: ratio paired *t*-test $**p=0.008$) and earliest phases (F, G: ratio paired *t*-test $p=0.68$) across the stages of the estrous cycle of all mice. The proportion of 4- to 5-day cycles of amplitudes (D, Pearson's chi-squared

test *$p$=0.02) and phases (H, Pearson's chi-squared test $p$=0.71) in intact compared to ovariectomized mice (n=9/group, 3 infradian cycles per mouse).

**Figure 4. Circadian wheel-running activity rhythms are regulated by the estrous cycle in constant darkness.** Representative actograms (A,C) and daily wheel revolutions (B,D) from a gonadally- intact (A,B) and ovariectomized (C,D) mouse in constant darkness. The proportion of 4-and 5-day cycles of wheel running activity in intact (n=9) and ovariectomized (n=7) mice (E, Pearson's chi-squared test, *$p$<0.001).

**Figure 5. The amplitude of the eating behavior rhythm coincides with peak wheel revolutions in constant darkness.** Representative graphs of eating behavior rhythm amplitudes (A) and phases (E) plotted with wheel revolutions in gonadally-intact females in constant darkness. Peak amplitudes (B, C: ratio paired *t*-test $p$=0.008) and earliest phases (F, G: ratio paired *t*-test $p$=0.83) across the stages of the estrous cycle of all gonadally-intact mice. The proportion of 4- to 5-day cycles of amplitudes (D, Pearson's chi-squared test *$p$=0.037) and phases (H, Pearson's chi-squared test $p$=0.56) in intact and ovariectomized mice (n=7-9/group, 3 infradian cycles per mouse).

# The estrous cycle coordinates the circadian rhythm of eating behavior in mice

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**Abstract**

The estrous cycle regulates rhythms of locomotor activity, body temperature, and circadian gene expression. In female mice, activity increases on the night of proestrus, when elevated estrogens cause ovulation. Exogenous estradiol regulates eating behavior rhythms in female mice fed high-fat diet, but it is unknown whether endogenous estrogens regulate eating rhythms. In this study, we investigated whether diurnal and circadian eating behavior rhythms change systematically across the estrous cycle. We first studied diurnal eating behavior rhythms in female C57BL/6J mice in 12L:12D. Estrous cycle stages were determined by vaginal cytology while eating behavior and wheel revolutions were continuously measured. The mice had regular 4- to 5-day estrous cycles. Consistent with prior studies, the greatest number of wheel revolutions occurred on the night of proestrus into estrus when systemic levels of estrogens peak. The amplitude, or robustness, of the eating behavior rhythm also fluctuated with 4- to 5-day cycles and peaked primarily during proestrus or estrus. The phases of eating behavior rhythms fluctuated, but not at 4- or 5-day intervals, and phases did not correlate with estrous cycle stages. After ovariectomy, the eating behavior rhythm amplitude fluctuated at irregular intervals. In constant darkness, the amplitude of the circadian eating behavior rhythm peaked every 4 or 5 days and coincided with the circadian day with the greatest wheel revolutions, a marker of proestrus. These data suggest that fluctuations of ovarian hormones across the estrous cycle temporally organize the robustness of circadian eating behavior rhythms so that it peaks during ovulation and sexual receptivity.

## Introduction

Circadian rhythms are approximately 24-hour cycles of physiology and behavior that entrain to environmental cycles, but are endogenous, and thus persist in constant environmental conditions (Halberg, 1959; Pittendrigh and Daan, 1976). Because circadian rhythms are endogenous, they allow animals to anticipate environmental cycles to improve survival and reproductive success (Hurd and Ralph, 1998; Penev et al., 1998; DeCoursey et al., 2000; Woelfle et al., 2004; Davidson et al., 2006; Hozer et al., 2020; Jabbur et al., 2024). The circadian system may improve fitness by coordinating reproductive behaviors and physiology, as disruption of the circadian system impairs reproductive success in mammals (Loudon et al., 1994; Lucas et al., 1999; Miller et al., 2004; Sen and Sellix, 2016; Kobayashi et al., 2018; Swamy et al., 2018; Fernandez et al., 2020). Recent studies found that mistimed eating (i.e., during the inactive phase) impaired the timing of ovulation and mating behaviors and reduced reproductive success (Swamy et al., 2018; Kukino et al., 2022). Thus, the daily rhythm of eating is intricately connected to reproduction in mice.

The estrous cycle in mice is a 4- to 5-day cycle of ovarian follicle development that culminates in the luteinizing hormone (LH) surge and ovulation (Barry, 1979; Bronson and Vom Saal, 1979). As the follicle develops, systemic estrogen levels rise and peak on the afternoon of proestrus (Bronson and Vom Saal, 1979). The main circadian clock in the suprachiasmatic nucleus (SCN) sends a daily permissive signal that allows for the pulsatile release of GnRH (Gu and Simerly, 1997; Christian and Moenter, 2008; Williams and Kriegsfeld, 2012). This daily signal, when coupled with high levels of estrogens, leads to the LH surge on the afternoon of proestrus (Christian et al., 2005; Williams and Kriegsfeld, 2012). The LH surge stimulates ovulation, which occurs about 12 hours later (Bingel and Schwartz, 1969; Miller and Takahashi, 2013). Thus, the circadian system coordinates the timing of ovulation so that it coincides with the time of peak sexual receptivity on the night of estrus.

Prior studies have shown that fluctuations in estrogens across the estrous cycle regulate daily and circadian rhythms of wheel-running activity in rodents. In hamsters, the onset of nighttime activity is earliest on proestrus and estrus, when estrogen levels peak, compared to other days of the estrous cycle, called “scalloping” (Morin et al., 1977). Scalloping occurs because estradiol shortens the period of the activity rhythm (Morin et al., 1977; Albers et al., 1981). Rats display scalloping and a lengthening of activity duration (alpha) during the dark phase (or subjective dark) on the night of proestrus (Albers et al., 1981; Wollnik and Turek, 1988). C57BL/6J mice do not have scalloped activity onsets, but they do have longer wheel-running activity durations on the night of proestrus into estrus (Takasu et al., 2015). The temporal alignment of ovulation and increased activity during heightened sexual receptivity could increase reproductive success.

The estrous cycle also regulates food consumption in rodents. Rats eat less food on the day of estrus compared to other stages of the estrous cycle (Tarttelin and Gorski, 1971; Eckel et al., 2000). This may reflect a trade-off so that more time can be spent seeking a mate, instead of eating, during estrus when the female has ovulated and is sexually receptive. There are mixed findings in mice regarding food consumption during the estrous cycle. Some studies find that females eat less during proestrus and estrus, while others do not (Kopp et al., 2006; Basterfield et al., 2009; Smarr et al., 2019). Thus, regulation of food consumption by endogenous, cycling estrogens in mice is weak at best. However, no study has investigated whether the daily rhythm of eating is affected by the estrous cycle in mice. Previous studies from our lab showed that exogenous estradiol increased the amplitude of the eating behavior rhythm in female mice fed high-fat diet (Omotola et al., 2019). Therefore, in this study we sought to determine whether endogenous, cycling estrogens regulate daily and circadian rhythms of eating behavior in female mice.

## Methods

### Animals

Female C57BL/6J mice were used for experiments. Mice were generated from breeding pairs of heterozygous PERIOD2::LUCIFERASE mice (originally obtained from Dr. Joseph Takahashi and backcrossed to C57BL/6J mice from The Jackson Laboratory for 32 to 35 generations) crossed with wild-type C57BL/6J mice purchased from The Jackson Laboratory. All mice used in these experiments, except for two, were wild-type females (that did not carry the PER2::LUC transgene). The two heterozygous PER2::LUC mice were used in the light-dark cycle experiment. Pups were weaned at 3 weeks old, and group housed (2 to 5 mice per cage) until 12 weeks old. Mice were bred and housed in 12L:12D and given standard chow diet (Teklad 2918, 18% protein diet) and water ad libitum. For all experiments, 12-week-old female mice were single-housed in cages (33x17x14 cm) with running wheels (11 cm diameter) in light-tight boxes with white LEDs (intensity 80 to 90 lux) and fed standard chow diet. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (protocol numbers 2015-2211 and 2021-3842).

### Experimental protocols

#### Experiment 1: Measuring daily eating behavior rhythms and estrous cycles in the light-dark cycle

Mice were single housed in 12L:12D and acclimated to running wheels for 3 to 5 weeks before data collection. Mice that had regular 4-to-5-day cycles of total wheel revolutions in their nocturnal activity were used for experiments (Kopp et al., 2006; Takasu et al., 2015; Nakamura et al., 2023). Mice that did not have clear 4- or 5-day cycles in the duration of their activity were not used for experiments because we used infradian wheel cycles as a proxy for regular estrous cycling (8 of 28 mice were excluded across all experiments, all mice shown in Fig. S1). Eating behavior rhythms and wheel revolutions were measured for 3 to 5 weeks before vaginal lavage

and then for 15 to 16 days with daily vaginal lavage at Zeitgeber time (ZT) 2 to 3, where ZT0 is lights on and ZT12 is lights off. Mice were then ovariectomized, allowed to recover for 2 weeks after surgery, and then eating behavior and wheel running rhythms were measured for about 15 days. Vaginal lavage was performed daily to confirm estrous cycles had ceased after ovariectomy.

**Experiment 2: Measuring circadian eating behavior rhythms and estrous cycles in constant darkness**

Mice were single housed in 12L:12D and acclimated to running wheels for 3 to 5 weeks. In the first cohort of mice, eating rhythms and wheel running activity were measured for 1 week and then mice were transferred to constant darkness for 5 to 6 weeks. Cage changes were performed in constant darkness using an infrared viewer (FJW Optical Systems Inc, FIND-R-SCOPE). Mice were then ovariectomized in 12L:12D and kept in LD for 3 weeks to confirm complete wound healing. The ovariectomized mice were then released into constant darkness and eating behavior and wheel running were measured for 15 to 21 days.

We also performed vaginal lavage in constant dim red light (LED safelight, 620 nm, NDT consultants, light intensity 5 to 15 lux) to measure estrous cyclicity in constant conditions that were as similar to constant darkness as possible. This method was chosen as it was technically difficult to lavage in constant darkness. Mice were single housed in 12h light (80 to 90 lux white light):12h dim red light, where the dim red light was on constantly, and the white LEDs were also on during the 12h light phase. After 2 weeks in this condition, the mice were released into constant dim red light. Vaginal lavage was performed daily for 3 to 4 weeks, at approximately Circadian Time (CT) 2 to 3, where the onset of wheel running activity was designated as CT12 (CT2 was defined as 14h after activity onset).

**Vaginal cytology for estrous cycle staging**

Vaginal lavage was performed daily in 12L:12D at ZT 2 to 3 and in dim red light at CT2 to 3. Vaginal samples were collected by aspirating 175µl of sterile saline near the vaginal opening. Samples were placed in a 96-well plate and cell morphology was examined under a light microscope and viewed at 40X magnification to assign estrous cycle stage. Briefly, samples comprised primarily of leukocytes were defined as diestrus, samples with nucleated epithelial cells were proestrus, and samples with cornified epithelial cells were estrus. Metestrus samples had all 3 cell types. The stage observed in the morning sample collected at ZT2 to 3 (or CT2 to 3) was assigned to the prior day since transitions between estrous cycle stages typically occur in the middle of the light phase (~ZT6) (Takasu et al., 2015).

**Ovariectomy surgery**

Female mice were anesthetized with inhaled isoflurane (3 to 5% for induction) and continuous isoflurane (1 to 2%) was administered throughout the procedure. Ketofen or meloxicam (5-10 mg/kg) was given subcutaneously prior to surgery. After removing the hair on the back in a 1 cm x 1cm square, a midline dorsal incision was made followed by 2 lateral incisions on the peritoneal wall. With the ovary clamped, polyglycolic acid ligatures were tied around the oviducts proximal to the ovaries. The ovaries were removed, and the peritoneal incisions were closed with 1 to 2 simple interrupted stitches. The skin was closed using metal wound clips. After surgery, mice were single housed. Ketofen or meloxicam (5-10 mg/kg) was administered subcutaneously 24 hours post-surgery and wound clips were removed 1 week after surgery. Successful removal of circulating estrogens was verified by vaginal lavage that showed no vaginal cells or no change in cytology across days.

**Wheel-running activity**

Wheel revolutions were measured using a wheel counter interfaced to ClockLab Acquisition Software (Actimetrics, Wilmette, IL). Actograms were plotted using the normalized setting in Clocklab Analysis software in 6-minute bins. The peak of wheel-running activity across multiple days was defined as previously described (Takasu et al., 2015; Nakamura et al., 2023). Briefly, the peak day was defined as the day with 2 preceding days and 2 succeeding days with lower wheel counts. If there were 2 successive days of nearly identical high counts, then the second day was defined as the peak.

**Eating behavior rhythms**

An infrared camera (HD 48Led 940nm Outdoor CMOS 800TVL IR-Cut Dome camera waterproof IF CCTV) connected to MPX HD 1080p Security System DVR (Lorex player) was positioned to record mouse behavior at the feeder. An eating event was defined as (i) the mouse took food from the feeder, (ii) the mouse took food and ate food away from the feeder, or (iii) the mouse moved food with mouth or paws for more than 3 seconds as previously described (Pendergast et al., 2013). Individual days of eating behavior were analyzed using Oriana and plotted in 10-minute bins to create circular histograms (Oriana 4.02; Kovach Computing Services, Anglesey, UK). To determine if a daily rhythm was present, a Rayleigh test was performed ($p$<0.05 indicated a rhythm was present). We defined the length of the vector as the amplitude of the rhythm and the direction of the vector as the phase of the rhythm.

The peak amplitude of the eating behavior rhythm across multiple days was defined as the day with 2 preceding days and 2 succeeding days with lower amplitudes. If there were 2 successive days of nearly identical high amplitudes, then the second day was defined as the peak. When there was no clear infradian cycle of eating behavior rhythm amplitude after ovariectomy, the peak day was defined as the day with greater than 25% increase in the

amplitude of the eating behavior rhythm compared to the lowest value in the preceding days. The peak phase was defined as the day where phase was the most advanced (earliest) compared to the 2 preceding and 2 succeeding days.

**Statistics**

Three infradian cycles (15 to 16 days) of wheel-running activity and eating behavior were analyzed for each mouse in 12L:12D and constant darkness. For each mouse, the ratio of wheel revolution and eating behavior rhythm peaks during high estrogen (proestrus and estrus) and low estrogen (metestrus and diestrus) signaling stages was determined and these data were compared with ratio paired *t*-tests. Pearson's chi-squared tests were used to compare infradian cycles of wheel-running activity rhythms and eating behavior rhythm amplitudes and phases between intact and ovariectomized mice. Daily and circadian periods in 12L:12D and constant darkness, respectively, were analyzed using wavelet analysis in ClockLab Analysis software and compared across estrous cycle stages using one-way ANOVA with repeated measures. Cosinor analysis (ClockLab Analysis software) was used to measure the amplitudes of infradian cycles of eating behavior rhythm amplitudes with periods set to 96h or 120h for mice that had 4- or 5-day cycles, respectively. An unpaired t-test was then used to compare infradian cycle amplitudes between gonadally-intact mice housed in 12L:12D and constant darkness. One-way ANOVA with repeated measures was used to compare the ratio of daytime or subjective daytime eating events across stages of the estrous cycle in 12L:12D and constant darkness, respectively. Results were considered significant when $p<0.05$.

**Results**

**Wheel-running activity is regulated by ovarian hormones that fluctuate across the estrous cycle**

The number of daily wheel revolutions fluctuated with 4- or 5-day cycles (Fig. 1A, 1B, all mice shown in Fig. S2) in female mice. Consistent with prior studies, we found the greatest number of daily wheel revolutions usually occurred during proestrus or estrus (Fig. 1B, 1C, 1D: ratio paired *t*-test $p<0.0001$) and at 4- to 5-day intervals (Fig. 1G). The phases of wheel-running activity onsets did not correlate with stages of the estrous cycle (Fig. 1A, Fig. S3). The periods of wheel-running activity daily rhythms did not differ across the estrous cycle in 12L:12D (Fig. S4A). Ovariectomy, which removes virtually all circulating ovarian hormones, reduced the occurrence of infradian 4- to 5-day cycles of daily wheel revolutions (Fig. 1E, 1F, 1G, all mice shown in Fig. S2). Ovariectomy reduced the likelihood that peaks of daily wheel-running activity occurred at 4- to 5-day intervals (Fig. 1G, Pearson's chi-squared test $p=0.03$).

**The amplitude of the eating behavior rhythm is coordinated by ovarian hormones that fluctuate across the estrous cycle**

The amplitudes and phases of eating behavior rhythms and the stages of the estrous cycle were measured daily in female mice (Fig. 2, 3). In gonadally-intact mice, eating behavior rhythm amplitudes fluctuated and peaked every 4 to 5 days (Fig. 3A; all mice shown in Fig. S5, S6). Peak amplitudes most often occurred during proestrus and estrus, when estrogen signaling is greatest during the estrous cycle (Fig. 3B, 3C: ratio paired *t*-test, $p=0.008$). Gonadally-intact females had more 4-to 5-day infradian cycles of eating behavior rhythm amplitudes compared to ovariectomized females, whose eating rhythm amplitudes rarely peaked at 4-or 5-day intervals (Fig. 3D, Pearson's chi-squared, $p=0.02$, all ovariectomized mice shown in Fig. S7). Daily eating events (minutes when the mice were eating) did not significantly vary across estrous cycle stages (Fig. S8, one-way ANOVA with repeated measures $p=0.23$). These data suggest that changes in ovarian hormones across the estrous cycle regulate the amplitude of the eating rhythm.

In contrast to the amplitude of the eating rhythm, there was no clear relationship between the phases of eating behavior rhythms and stages of the estrous cycle (Fig. 3E, 3F, 3G: ratio paired *t*-test $p$=0.68, all mice shown in Fig. S5). The phases of the eating rhythms fluctuated slightly each day but did not consistently occur at 4- or 5-day intervals in gonadally-intact females (Fig. 3E, 3H). Earliest phases did not coincide with a specific stage(s) of the estrous cycle (Fig. 3F, 3G). Moreover, removal of circulating estrogens by ovariectomy did not affect the proportion of earliest phases that occurred at 4- or 5-day intervals (Fig. 3H, all ovariectomized mice in Fig. S7, Pearson's chi-squared, $p$=0.71). These data suggest that the estrous cycle does not regulate the phase of the eating rhythm in female mice.

**Estrous cycle regulation of wheel-running activity persists in constant conditions**

Gonadally-intact female mice in constant darkness had clear 4- to 5-day infradian cycles of wheel revolutions (Fig. 4A, 4B, all mice shown in Fig. S9). The periods of wheel-running activity circadian rhythms were shorter in estrus compared to diestrus and proestrus (Fig. S4B, one-way ANOVA with repeated measures, $p$=0.02; post-hoc LSD post-hoc: $p$<0.05). Ovariectomy abolished infradian cycles of wheel-running activity (Fig. 4C, 4D, all mice shown in Fig. S9). Ovariectomized mice had fewer 4- and 5-day cycles of wheel revolutions than gonadally-intact females (Fig. 4E, Pearson's chi-squared test, $p$<0.001).

**The amplitude of the eating behavior rhythm is regulated by the estrous cycle in constant conditions**

Gonadally-intact female mice in constant darkness maintained 4- to 5-day cycles of eating behavior rhythm amplitudes (Fig. 5A, 5B, 5C, 5D all mice shown in Fig. S10). Peak eating rhythm amplitudes coincided more often with days having the greatest wheel revolutions (Fig. 5B, 5C: ratio paired *t*-test $p$=0.008). Based on our results in 12L:12D and in constant dim

red light, the greatest number of wheel revolutions occurred on proestrus and estrus (Fig. S11). Removal of circulating estrogens by ovariectomy reduced the likelihood of 4- to 5-day cycles of eating behavior rhythm amplitude (Fig. 5D, all mice in Fig. S12, Pearson's chi-squared test, $p$=0.037). The phases of eating behavior rhythms did not fluctuate systematically with wheel revolution cycles or with predicted stages of the estrous cycle (Fig. 5E, 5F, 5G: ratio of paired $t$-test $p$=0.83, all mice in Fig. S12). There was no difference in the likelihood of peak phase to occur at 4-to 5-day cycles between gonadally-intact and ovariectomized females (Fig. 5H, Pearson's chi-squared test, $p$=0.56).

## Discussion

The goal of this study was to determine whether endogenous ovarian hormones, whose levels systematically change across the estrous cycle, regulate daily and circadian rhythms of eating behavior. We found that amplitudes, but not phases, of eating behavior rhythms peaked during proestrus and estrus, which are high estrogen signaling stages, in both the light-dark cycle and constant darkness. Thus, the circadian rhythm of eating is coordinated by the estrous cycle in female mice.

The relationship between the estrous cycle and eating rhythm amplitude was abolished in ovariectomized mice, demonstrating that ovarian hormones are necessary for the coordination of eating rhythms. While we cannot rule out a role for progesterone, the current study, combined with our prior results showing that treatment with exogenous estradiol regulates the eating rhythm in female mice, suggests that cycles of endogenous estrogens regulate the eating rhythm amplitude (Omotola et al., 2019). Estrogens bind to estrogen response elements (ERE) to regulate the expression of core circadian genes in rodents (Gery et al., 2007; Rossetti et al., 2012). Other studies have observed this relationship through changes in clock gene expression across the estrous cycle or with exogenous estradiol treatments

(Nakamura et al., 2005; Perrin et al., 2006; He et al., 2007; Nakamura et al., 2008). Thus, estrogens may regulate the amplitude of the eating rhythm by directly modifying the expression of circadian genes in brain regions that control eating rhythms. However, it is difficult to study the mechanisms of estrogenic regulation of the eating rhythm because the neural circuit that controls the eating rhythm is unknown.

In this study, we did not find a relationship between the phase of the eating behavior rhythm and the estrous cycle in female mice. This is consistent with prior studies in mice that showed the phase of the wheel-running activity rhythm was not regulated by estradiol nor the estrous cycle (Kopp et al., 2006; Takasu et al., 2015; Joye and Evans, 2022). Scalloping occurs in hamsters and rats because elevated estrogen signaling during proestrus and estrus transiently shortens the period of wheel-running activity, resulting in an earlier phase of activity onset (Morin et al., 1977; Albers et al., 1981). While mice do not show fluctuations in the phase of activity onset across the estrous cycle, they do have increases in amplitude of activity that coincide with the shortest period (Leise and Harrington, 2011). Our results are consistent with these prior studies; we found the greatest amplitude (most wheel revolutions) during proestrus and estrus, but no systematic fluctuations in the phase of the wheel-running activity rhythm.

It is possible that estrous cycle coordination of wheel-running activity and eating rhythms contributes to reproductive success. Others have speculated that greater locomotor activity on the night of estrus, when the female ovulates and is sexually receptive, represents mate-seeking behavior and increases the likelihood of mating after ovulation. We hypothesize that regulation of the eating rhythm amplitude may increase reproductive success. High amplitude eating rhythms consolidate eating to a smaller time window, thus allotting more time for mate seeking behaviors on the night of proestrus into estrus. It is also possible that the increase in wheel-running activity secondarily leads to increases in eating behavior rhythm amplitude, as there is more demand for increased energy intake on the night of the greatest wheel-running

activity. Further studies in mice housed without running wheels will determine whether the coordination of the amplitude of the eating rhythm with the estrous cycle persists in the absence of increased energy demand.

This study also further supports the interplay between estrogens and eating behavior rhythms. We previously showed that estradiol regulation of the eating behavior rhythm is critical for regulation of energy balance and metabolism during a nutritional challenge (Palmisano et al., 2017; Omotola et al., 2019). This study extends the model of estrogen regulation of meal timing and shows that endogenous estrogens regulate the eating behavior rhythm in gonadally-intact females fed low-fat diet. Thus, this study, together with prior studies, suggest that estrous cycle regulation of eating rhythms may be important for reproductive success.

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**Declaration of Conflicting Interests**

The authors declare that there is no conflict of interest.

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## Figure Legends

**Figure 1. Wheel-running activity is regulated by the estrous cycle.** Representative actograms of wheel-running activity of gonadally-intact (A) and ovariectomized (D) female mice housed in 12L:12D. The stage of the estrous cycle was determined by vaginal lavage (B,C,D,G) and plotted relative to wheel revolutions per day (B) and peak wheel revolutions (C,F) in gonadally-intact (B,C) and ovariectomized (E,F) female mice. The ratios of wheel-running activity peaks that occurred in metestrus (M) and diestrus (D) compared to proestrus (P) and estrus (E) were analyzed across 3 estrous cycles in gonadally intact mice (D, ratio paired t-test *$p$<0.0001). Wheel-running activity peaks were more likely to occur every 4 to 5 days than at other intervals in gonadally-intact compared to ovariectomized females (G, n=9/group across 15 to 16 days per mouse, *$p$=0.03, Pearson's chi-squared test).

**Figure 2. The amplitude of the eating behavior rhythm fluctuates every 4- to 5-days in female mice.** Estrous cycle stages were determined from cytology of vaginal lavage samples collected daily at ZT2 to 3 (P: proestrus, E: estrus, D: diestrus, M: metestrus). Eating behavior was monitored using infrared video cameras and amplitudes and phases of eating behavior rhythms were determined from the lengths and directions, respectively, of vectors from Rayleigh statistics (top panel).

**Figure 3. The amplitude of the eating behavior rhythm is coordinated with the estrous cycle.** Representative graphs of estrous stage and amplitudes (A) and phases (E) of eating behavior rhythms in gonadally-intact female mice. Peak amplitudes (B, C: ratio paired *t*-test ***p*=0.008) and earliest phases (F, G: ratio paired *t*-test *p*=0.68) across the stages of the estrous cycle of all mice. The proportion of 4- to 5-day cycles of amplitudes (D, Pearson's chi-squared test **p*=0.02) and phases (H, Pearson's chi-squared test *p*=0.71) in intact compared to ovariectomized mice (n=9/group, 3 infradian cycles per mouse).

**Figure 4. Circadian wheel-running activity rhythms are regulated by the estrous cycle in constant darkness.** Representative actograms (A,C) and daily wheel revolutions (B,D) from a gonadally- intact (A,B) and ovariectomized (C,D) mouse in constant darkness. The proportion of 4-and 5-day cycles of wheel running activity in intact (n=9) and ovariectomized (n=7) mice (E, Pearson's chi-squared test, **p*<0.001).

**Figure 5. The amplitude of the eating behavior rhythm coincides with peak wheel revolutions in constant darkness.** Representative graphs of eating behavior rhythm amplitudes (A) and phases (E) plotted with wheel revolutions in gonadally-intact females in constant darkness. Peak amplitudes (B,C: ratio paired *t*-test *p*=0.008) and earliest phases (F, G: ratio paired *t*-test *p*=0.83) across the stages of the estrous cycle of all gonadally-intact mice. The proportion of 4- to 5-day cycles of amplitudes (D, Pearson's chi-squared test **p*=0.037) and phases (H, Pearson's chi-squared test *p*=0.56) in intact and ovariectomized mice (n=7-9/group, 3 infradian cycles per mouse).

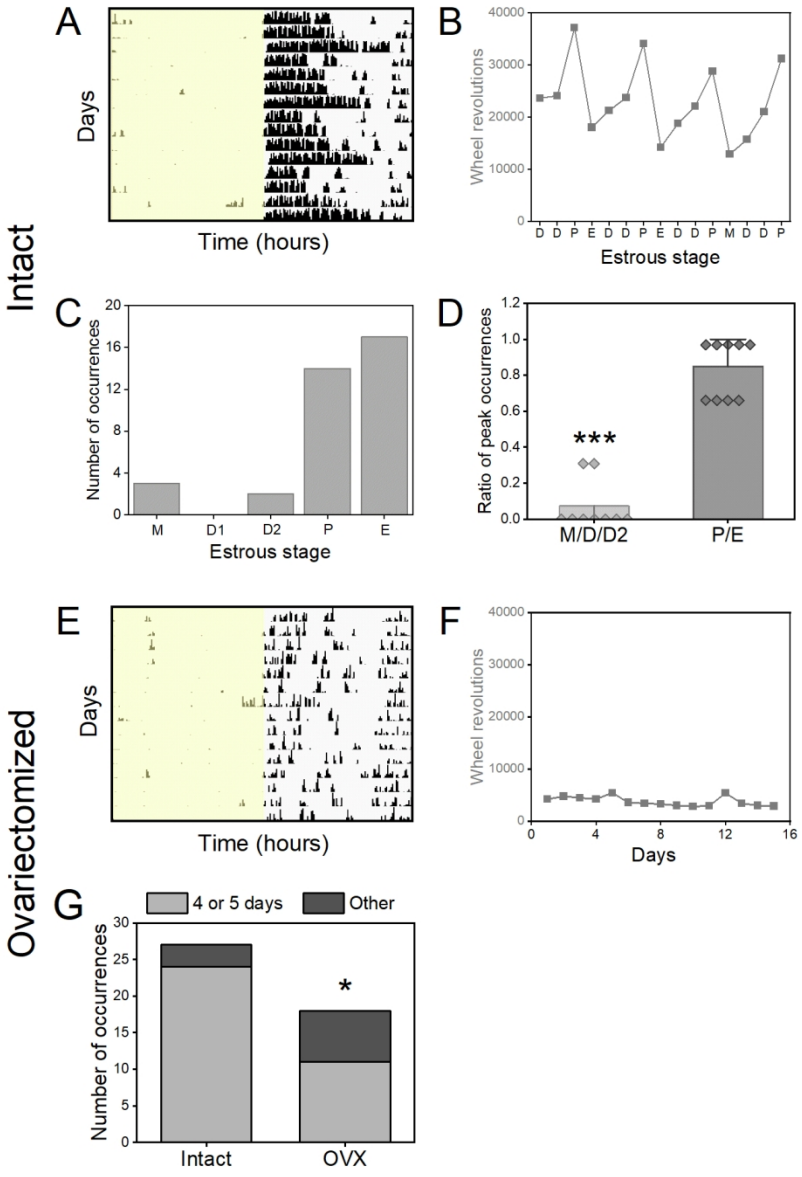


Figure 1. Wheel-running activity is regulated by the estrous cycle. Representative actograms of wheel-running activity of gonadally-intact (A) and ovariectomized (D) female mice housed in 12L:12D. The stage of the estrous cycle was determined by vaginal lavage (B,C,D,G) and plotted relative to wheel revolutions per day (B) and peak wheel revolutions (C,F) in gonadally-intact (B,C) and ovariectomized (E,F) female mice. The ratios of wheel-running activity peaks that occurred in metestrus (M) and diestrus (D) compared to proestrus (P) and estrus (E) were analyzed across 3 estrous cycles in gonadally intact mice (D, ratio paired t-test *p<0.0001). Wheel-running activity peaks were more likely to occur every 4 to 5 days than at other intervals in gonadally-intact compared to ovariectomized females (G, n=9/group across 15 to 16 days per mouse, *p=0.03, Pearson's chi-squared test).

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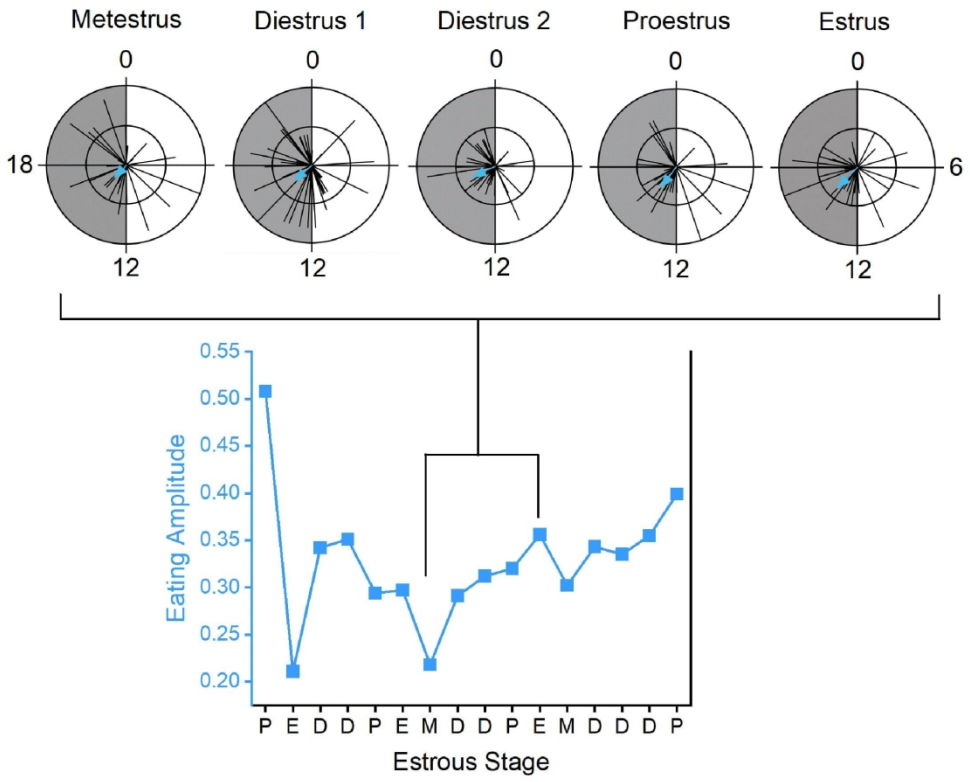


Figure 2. The amplitude of the eating behavior rhythm fluctuates every 4- to 5-days in female mice. Estrous cycle stages were determined from cytology of vaginal lavage samples collected daily at ZT2 to 3 (P: proestrus, E: estrus, D: diestrus, M: metestrus). Eating behavior was monitored using infrared video cameras and amplitudes and phases of eating behavior rhythms were determined from the lengths and directions, respectively, of vectors from Rayleigh statistics (top panel).

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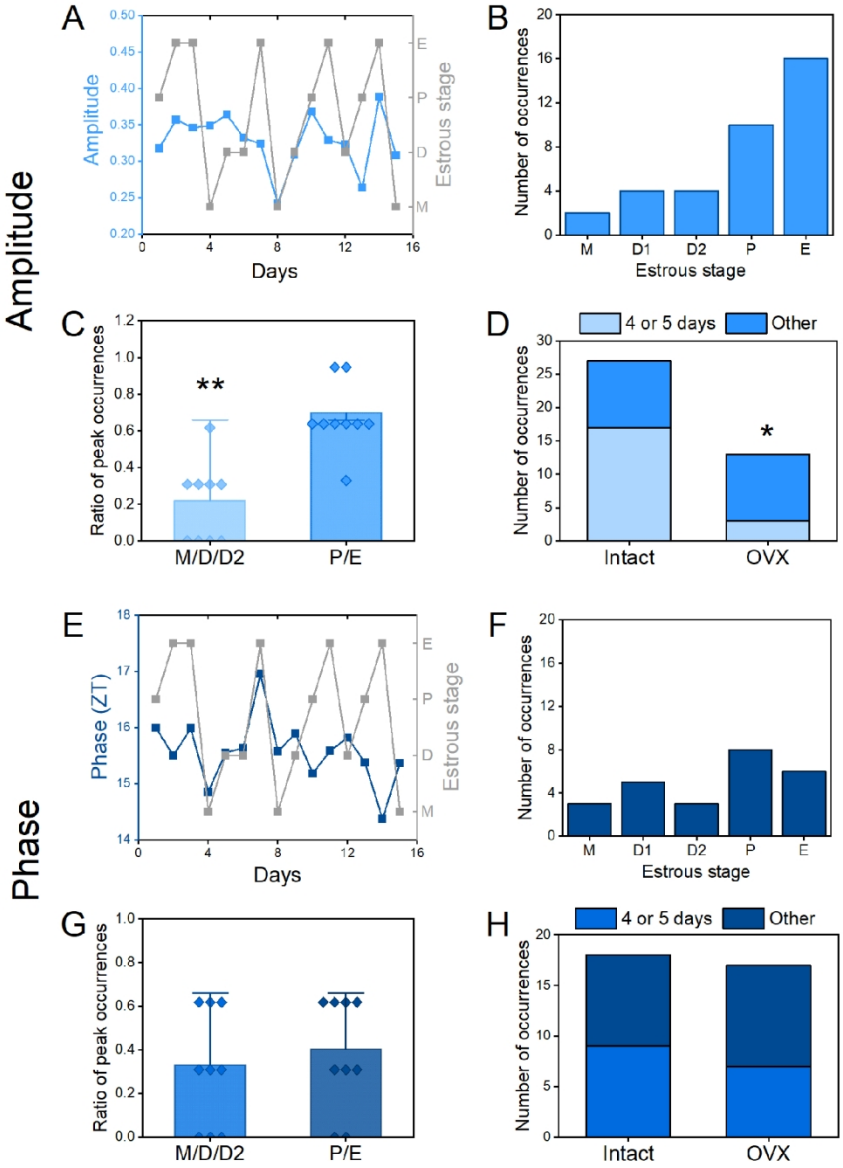


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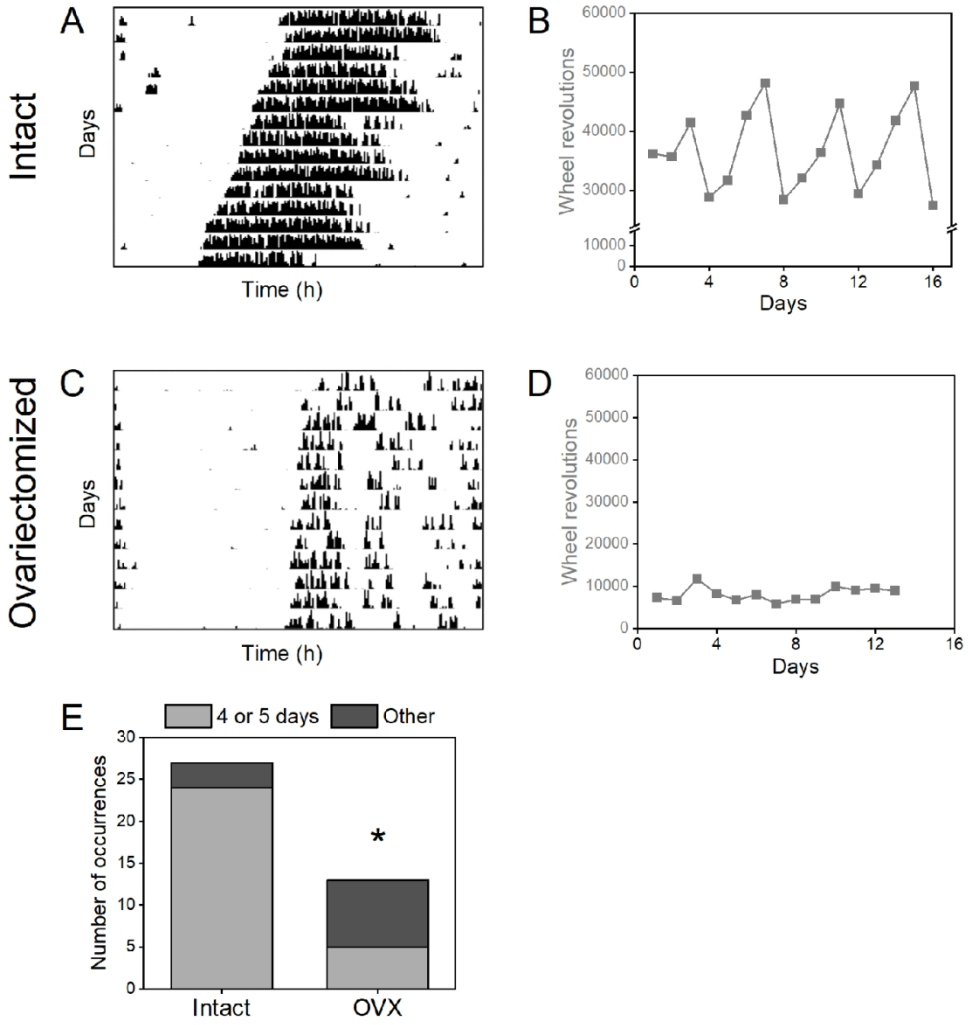


Figure 4. Circadian wheel-running activity rhythms are regulated by the estrous cycle in constant darkness. Representative actograms (A,C) and daily wheel revolutions (B,D) from a gonadally- intact (A,B) and ovariectomized (C,D) mouse in constant darkness. The proportion of 4-and 5-day cycles of wheel running activity in intact (n=9) and ovariectomized (n=7) mice (E, Pearson's chi-squared test, *p<0.001).

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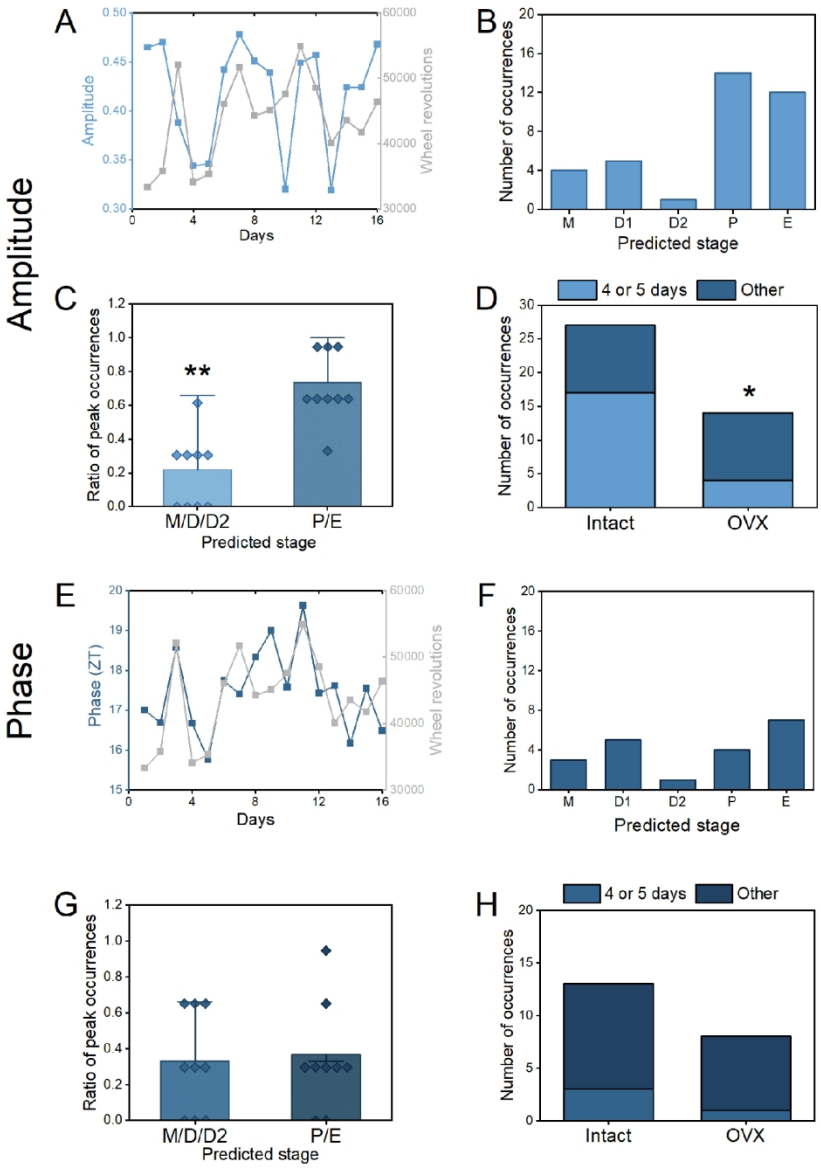


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## Supplemental Information

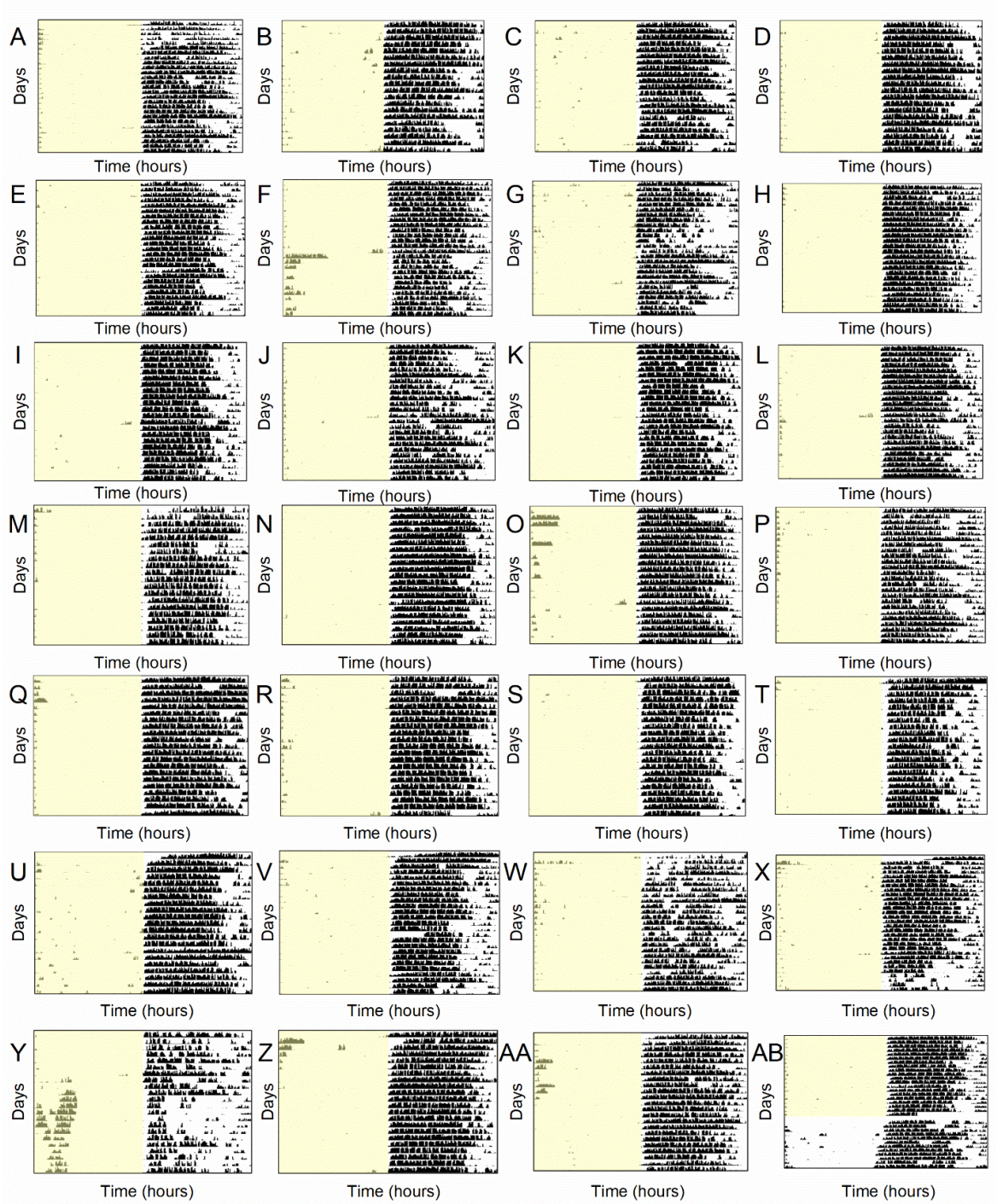


**Figure S1. Most mice exhibit clear 4- to 5-day cycles in wheel running activity rhythms.** All mice were initially housed in LD for 3-5 weeks to acclimate to the running wheel and to ensure that mice had clear 4- to 5-day wheel running cycles prior to collecting eating behavior data. Then mice were housed in LD (A-I), DD (J-R), or dim red light (S,T) for experiments. Some mice were excluded due to irregular wheel running activity rhythms during acclimation (U-X, AA), unusual activity caused by handling (Y), or irregular wheel running activity rhythms after being placed in DD (AB). All mice were wild-types, except those in B and G were heterozygous PER2::LUC.

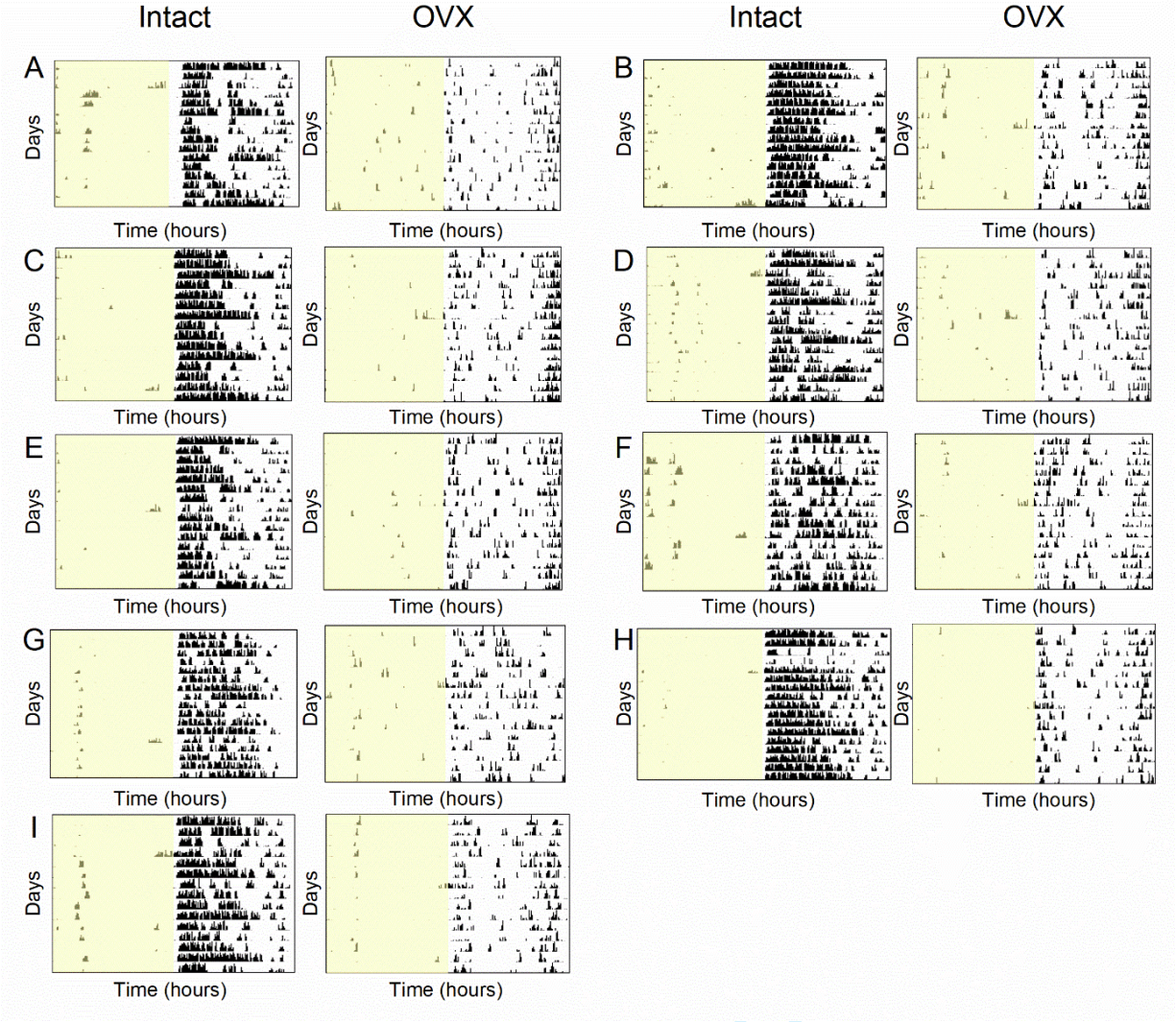


**Figure S2. Actograms of individual gonadally-intact and ovariectomized female mice in 12L:12D.** Actograms of wheel-running activity rhythms of all individual gonadally-intact female mice that were vaginally lavaged for 15 days (A-H: left panels) and then ovariectomized and lavaged for 15 more days (A-H: right panels). Days 3 and 4 in actogram H had low activity due to a stuck running wheel. The intact mouse in C and ovariectomized mouse in D are shown in Fig. 1A and 1D, respectively. All mice were wild-types, except those in B and G were heterozygous PER2::LUC.

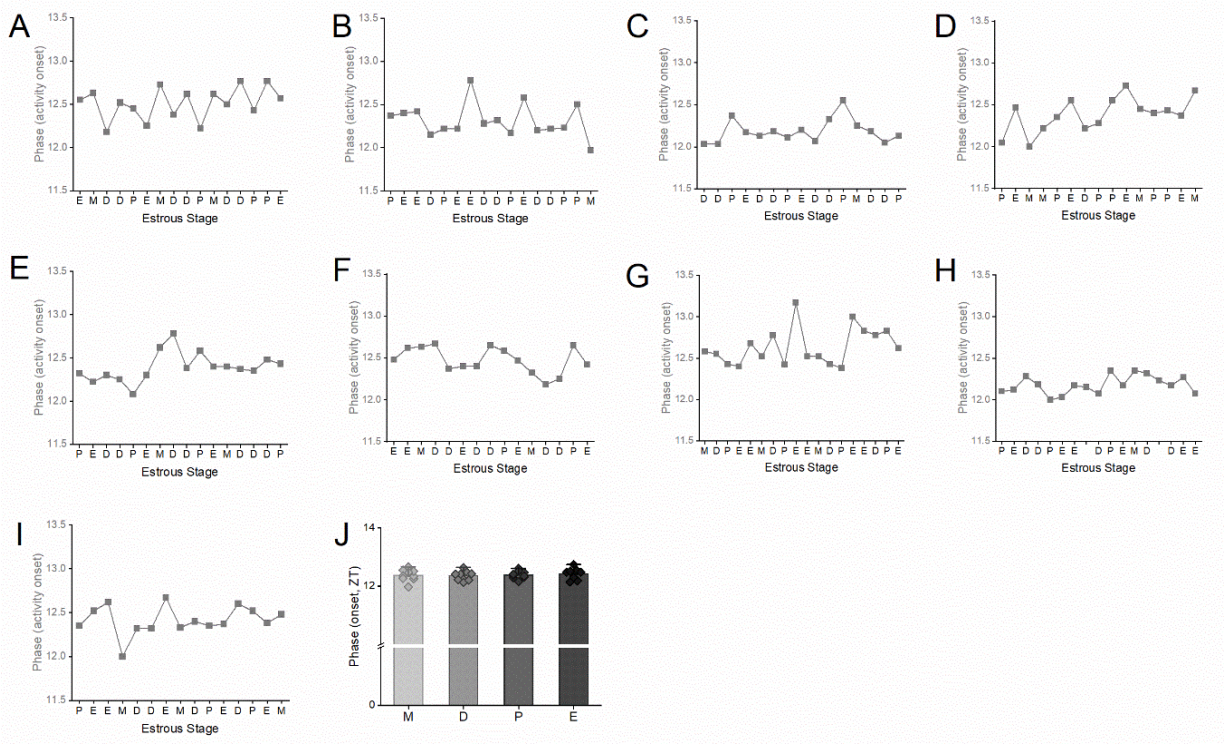


**Figure S3. The phases of the wheel running activity rhythms do not fluctuate with estrous cycle stages in 12L:12D.** Wheel-running activity phases were measured using activity onset (in ZT where ZT12 is lights off) and compared to estrous cycle stages for each mouse (A-I). Mice were housed in 12L:12D cycle. There were no significant changes in phases across the estrous cycle stages (J, one-way ANOVA with repeated measures, $p$=0.37). All mice were wild-types, except those in B and G were heterozygous PER2::LUC.

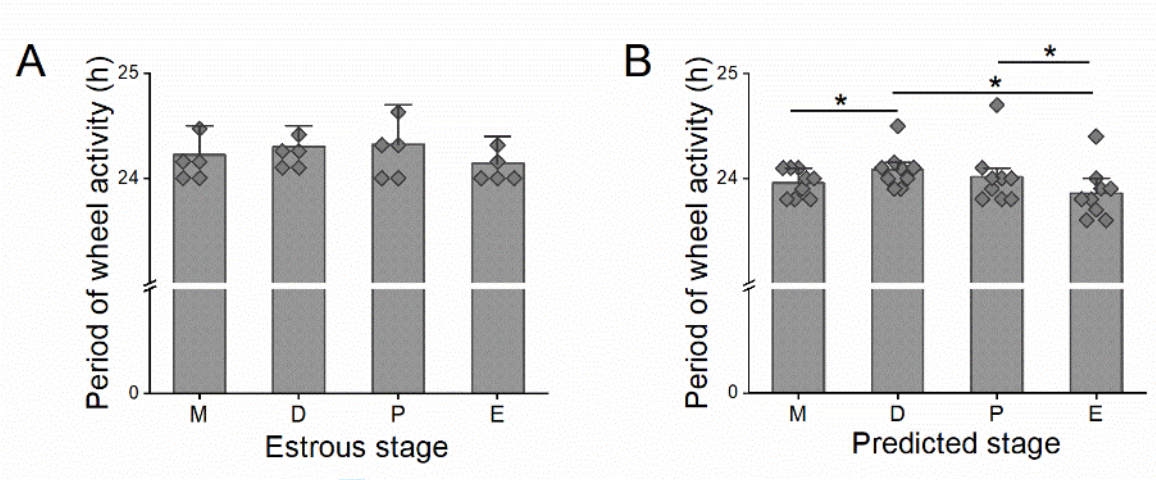


**Figure S4. Periods of wheel-running activity rhythms change across the estrous cycle in constant darkness, but not in 12L:12D.** Periods (in the circadian range) of wheel-running activity rhythms were analyzed using wavelet analysis for each day of the first complete infradian cycle for each mouse (one data point is one cycle from one mouse) in 12L:12D (A, n=5 for LD because only 5 mice had one complete estrous cycle in LD) and constant darkness (B). There were no significant differences in periods across estrous cycle stages in 12L:12D (A, one-way ANOVA with repeated measures, $p$=0.21). Mice housed in DD had the shortest period during estrus compared to diestrus and proestrus (B, one-way ANOVA with repeated measures, $p$=0.02; post-hoc LSD post-hoc: *$p$<0.05)

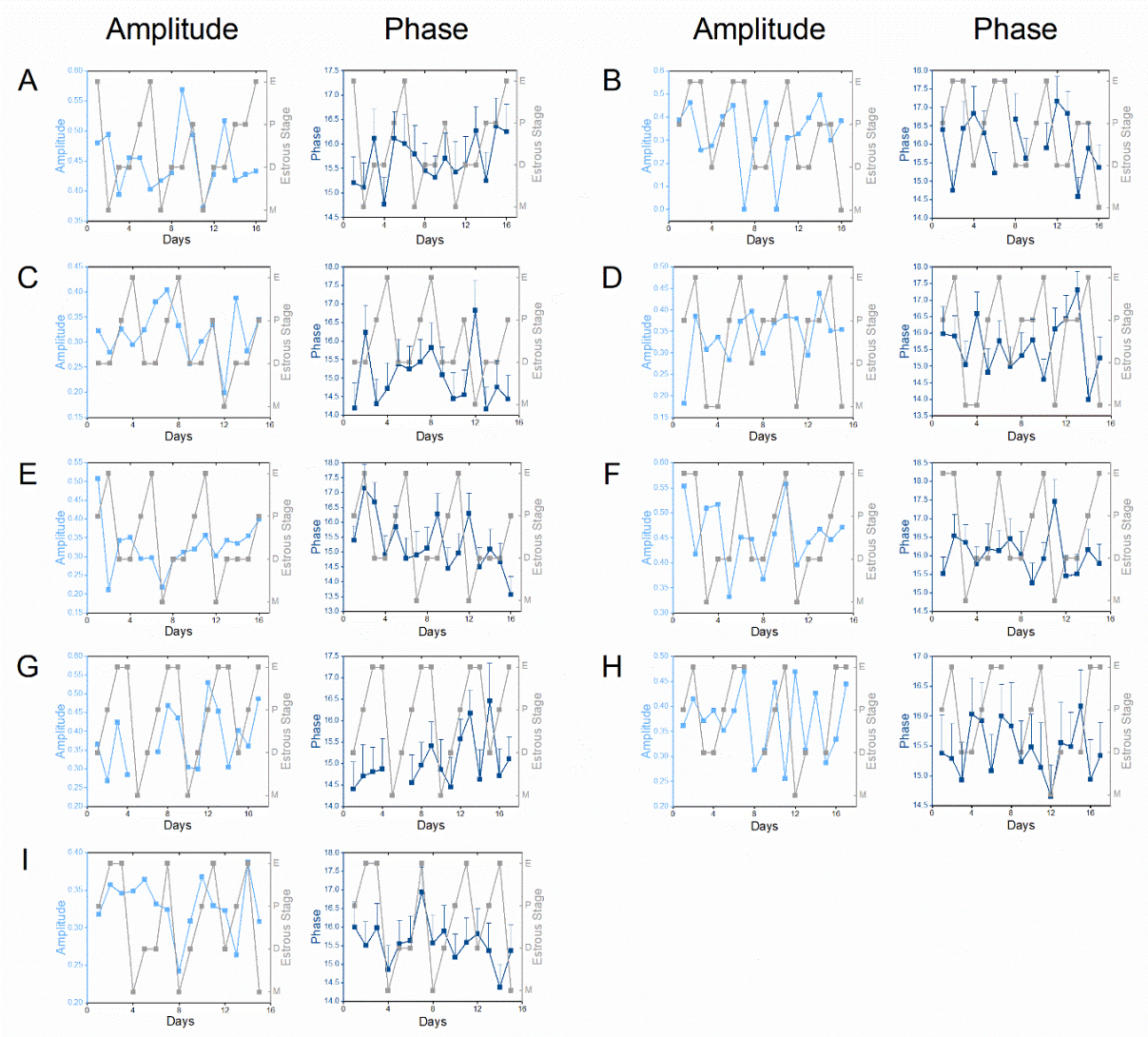


**Figure S5. Eating behavior rhythm amplitudes, but not phases, change systematically across estrous cycles in gonadally-intact female mice in the light-dark cycle**. Eating behavior rhythm amplitudes (light blue, left panels) and eating behavior rhythm phases (dark blue, right panels) were plotted with estrous cycle stages for individual gonadally-intact female mice in 12L:12D. Vaginal lavages were collected at ZT2-3 for 15-16 days. Error bars for phases represent the circular variances. Amplitude and phase figures from panel I are used in Fig. 3A and 3D, respectively. All mice were wild-types, except those in B and G were heterozygous PER2::LUC.

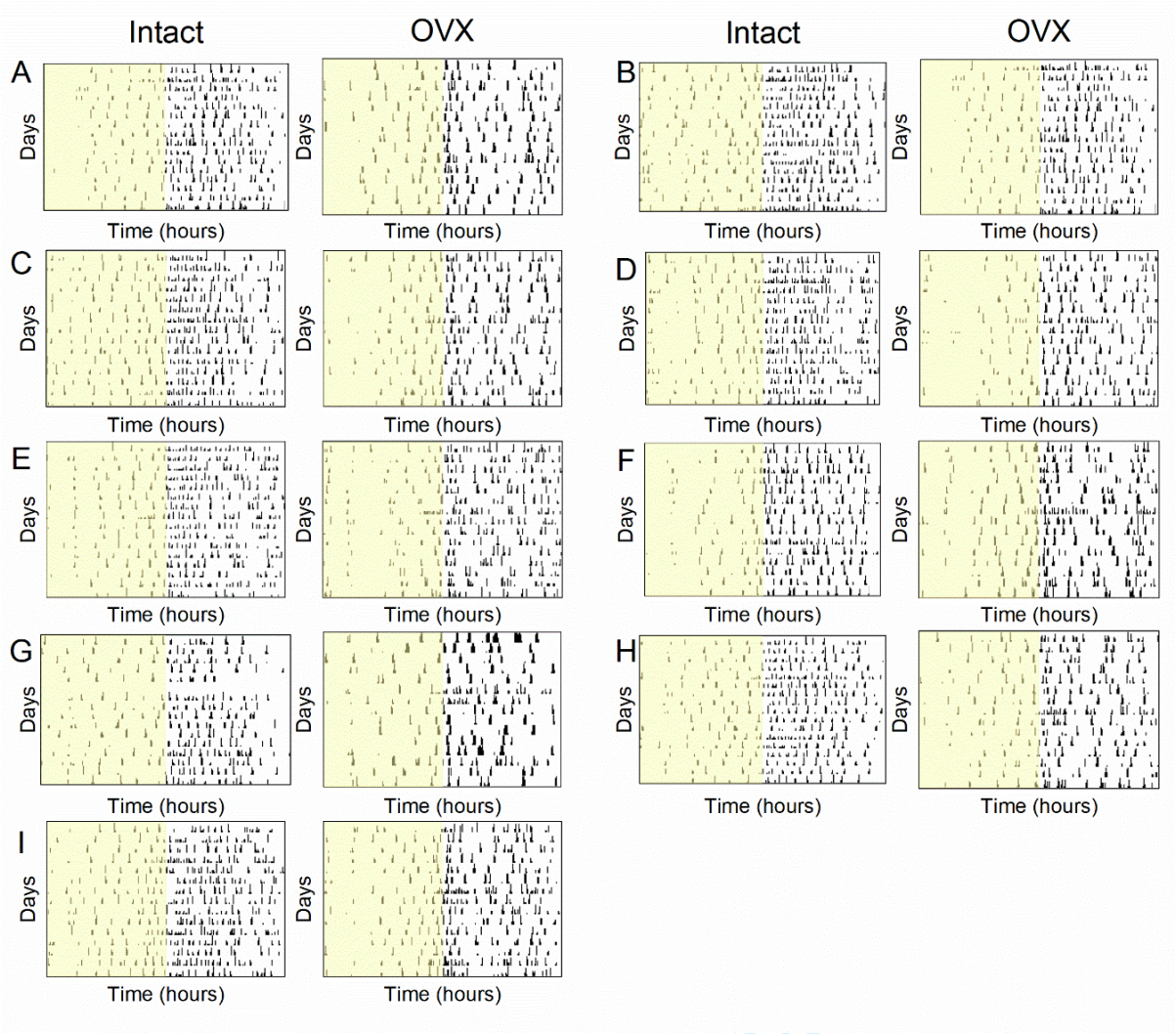


**Figure S6. Actograms of eating events from gonadally-intact and ovariectomized female mice in 12L:12D.** Each black tick represents an eating event in actograms from all individual mice before (left panels) and after (right panels) ovariectomy (A-I). All mice were wild-types, except those in B and G were heterozygous PER2::LUC.

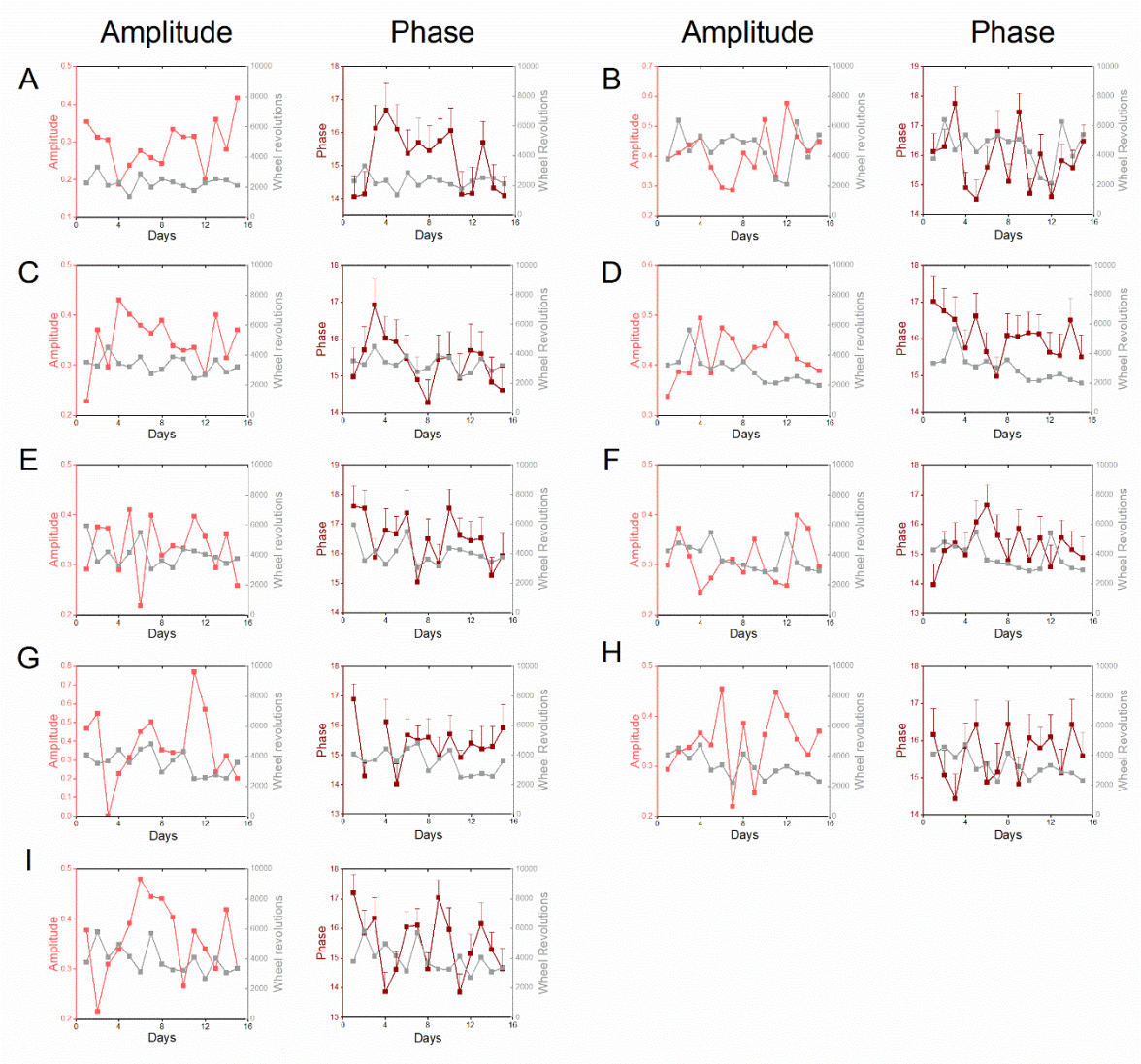


**Figure S7. Ovarian hormones are necessary for infradian cycles of eating behavior amplitude in the light-dark cycle.** Eating behavior rhythm amplitudes (light red, left panels) and eating behavior rhythm phases (dark red, right panels) plotted with wheel revolutions (light gray) for individual ovariectomized mice in 12L:12D (A-I). Vaginal lavage was performed from ZT 2-3 for 15 days. All mice were wild-types, except those in B and G were heterozygous PER2::LUC.

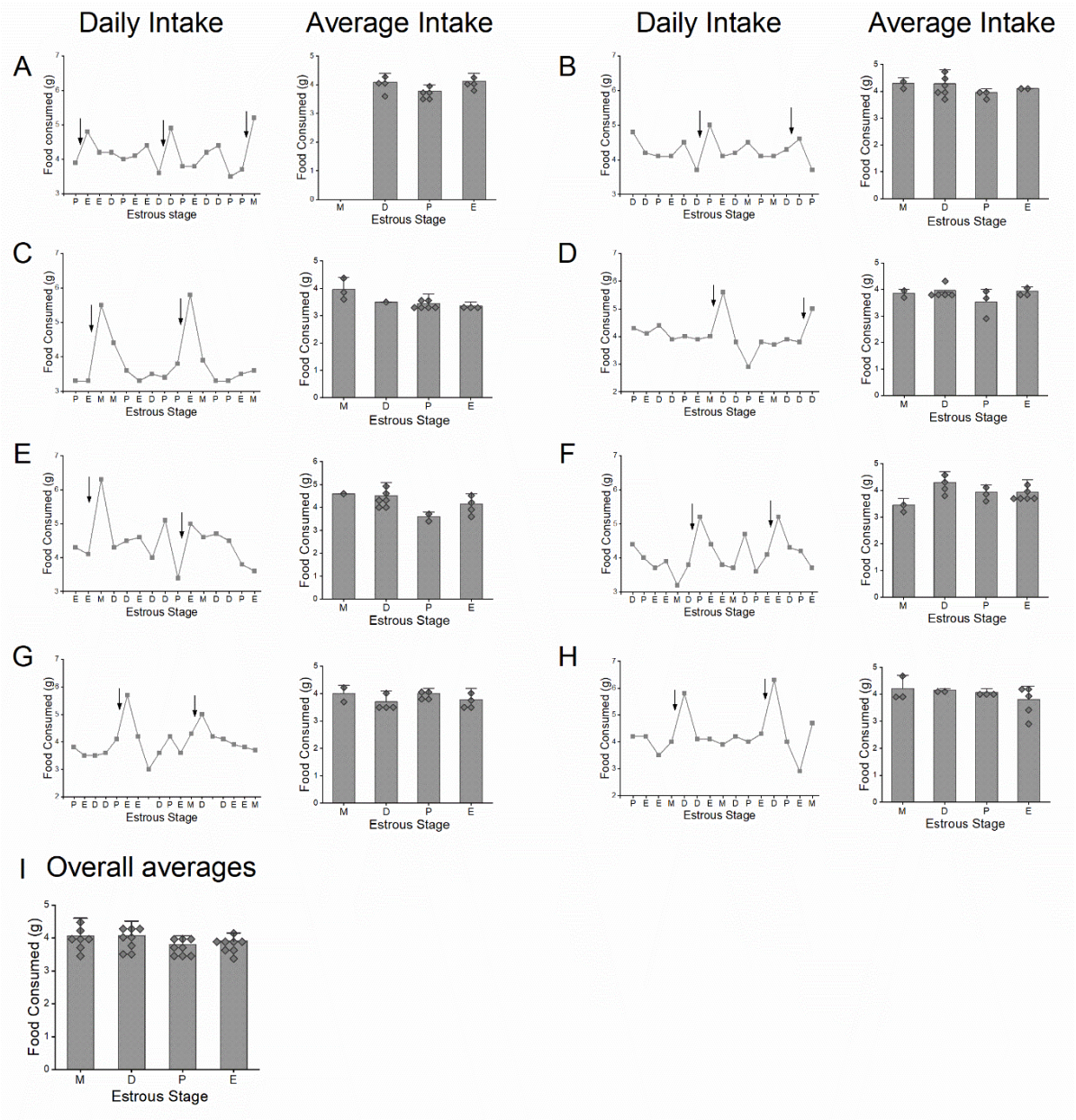


**Figure S8. Daily eating events do not fluctuate across the estrous cycle in gonadally-intact female mice.** The number (left) and averages across the experiment (right) of daily eating events were plotted with estrous cycle stages for each mouse (A-H). Each eating event was 1 minute when the mouse was eating (note that these data are not a measure of food intake since eating behavior was analyzed by video). The arrows show when food was weighed weekly and replaced between ZT 9-12. We did not include the days of food change in the analysis. There was no significant difference in eating events across stages of the estrous cycle (I, one-way ANOVA with repeated measures $p$=0.23). All mice were wild-types, except those in A and F were heterozygous PER2::LUC.

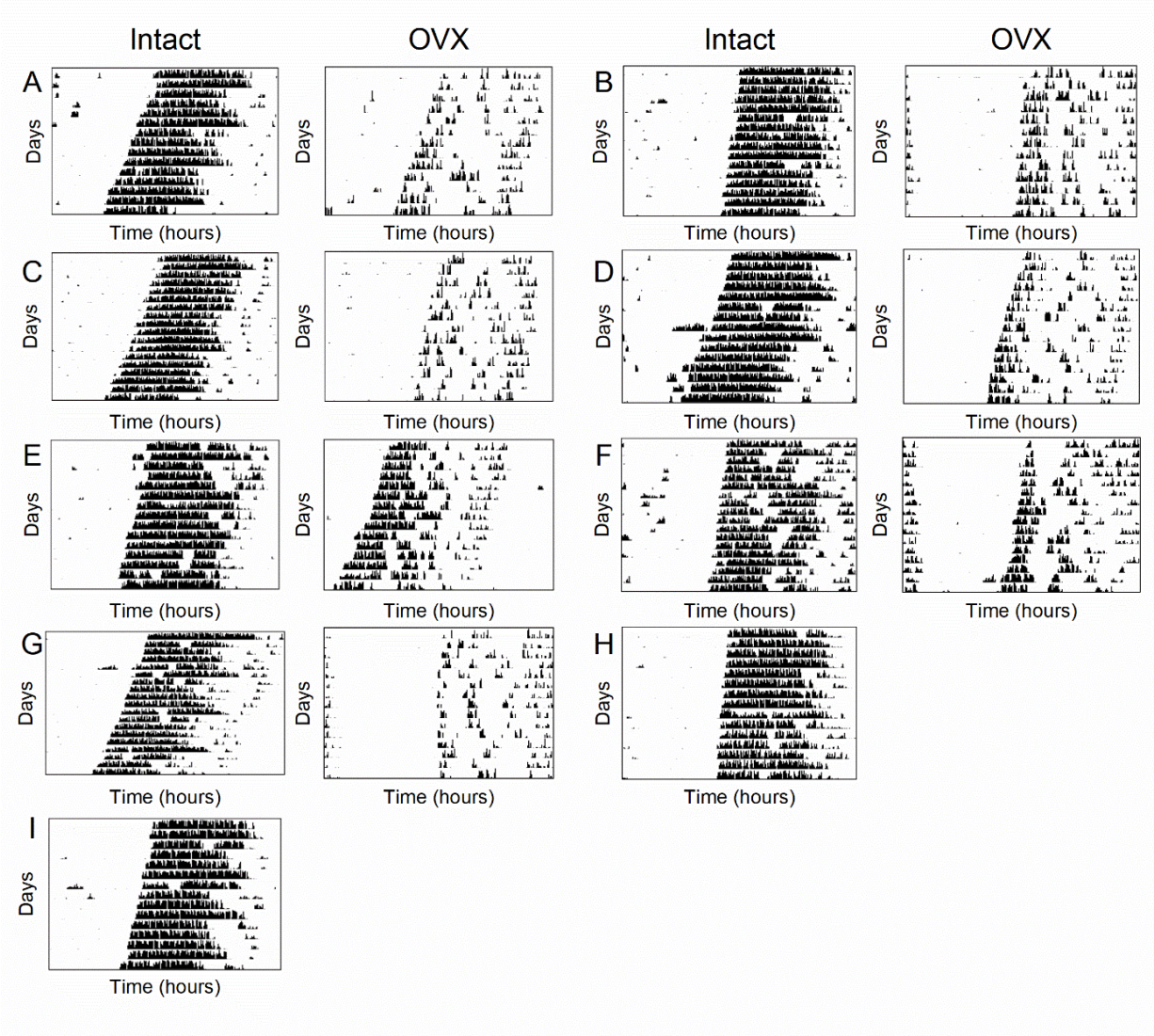


**Figure S9. Actograms of wheel-running activity from gonadally-intact and ovariectomized female mice in constant darkness (DD).** Mice were acclimated to a running wheel in LD before being released to DD. Eating behavior data were collected simultaneously with wheel-running activity in intact (A-H, left panels) and ovariectomized (A-H, right panels) mice. The intact mouse in A and ovariectomized mouse in B are shown in Fig. 4A and 4C, respectively.

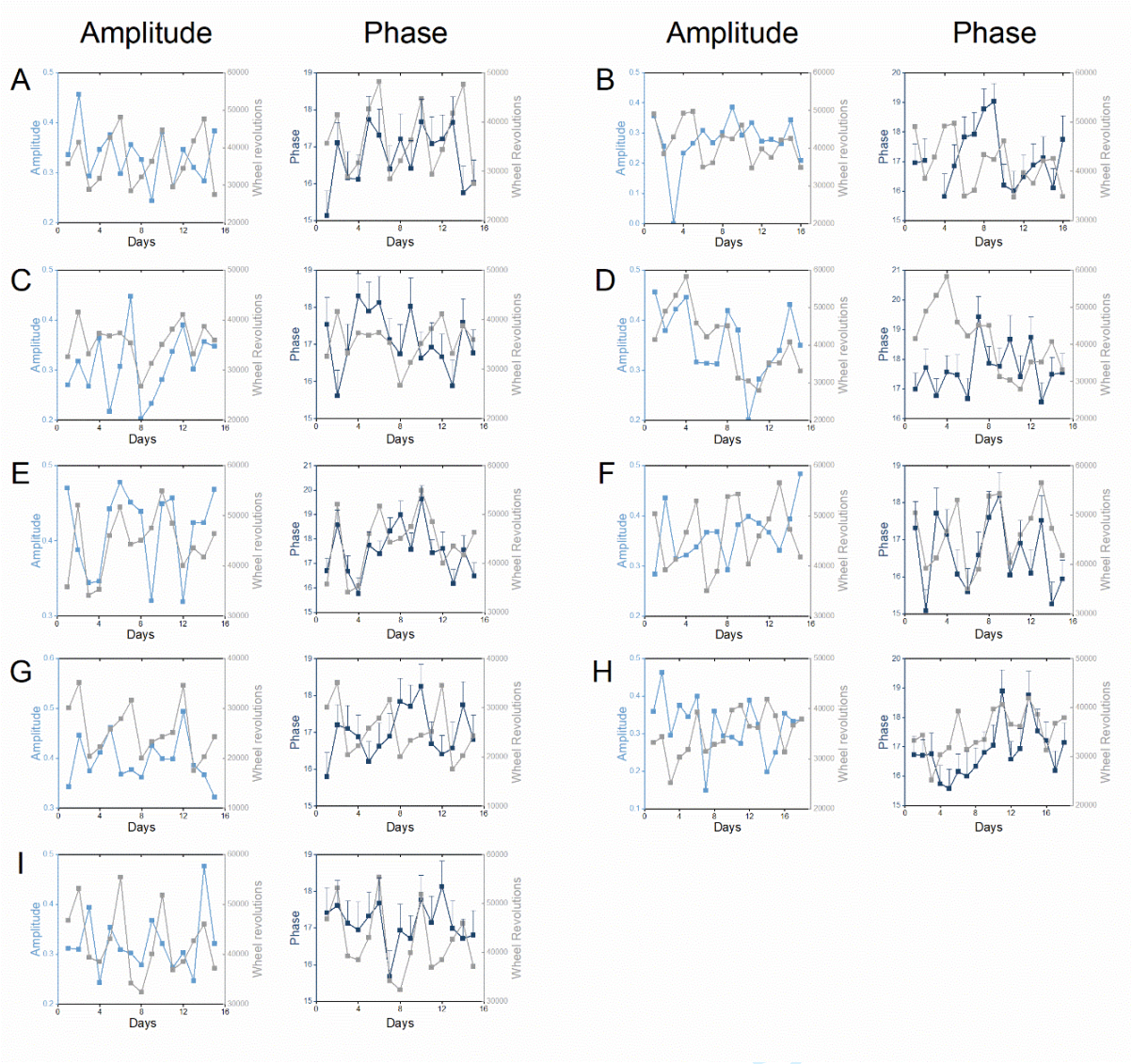


**Figure S10. Eating behavior rhythm amplitudes, but not phases, are correlated with wheel revolutions in gonadally-intact female mice in constant darkness.** Eating behavior rhythm amplitudes (light blue, left panels) and phases (dark blue, right panels) for all individual gonadally-intact female mice in constant darkness. Eating behavior rhythms and daily wheel revolutions were collected for 15-16 days. Amplitude and phase figures from E are shown in Fig. 5A and 5D, respectively.

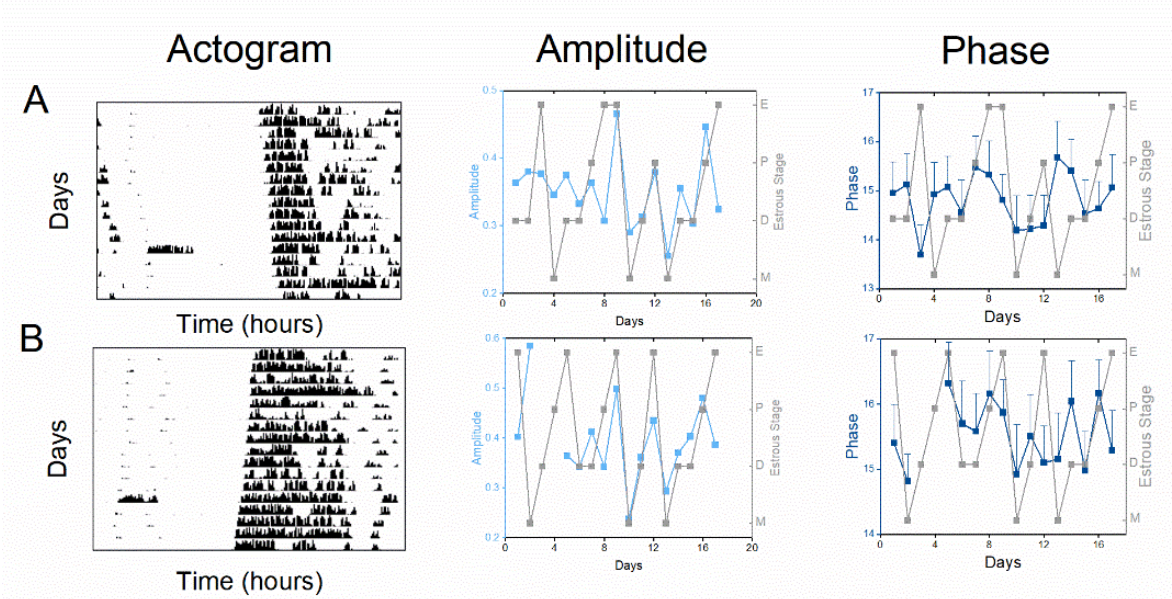


**Figure S11. Eating behavior rhythm amplitudes and wheel revolutions correlate with the estrous cycle in gonadally-intact female mice in constant dim red light.** Two gonadally-intact mice were single housed in constant dim red light (A-B). Vaginal lavage was performed daily at CT 2-3 for 21 days. The error bars for phase represent circular variances.

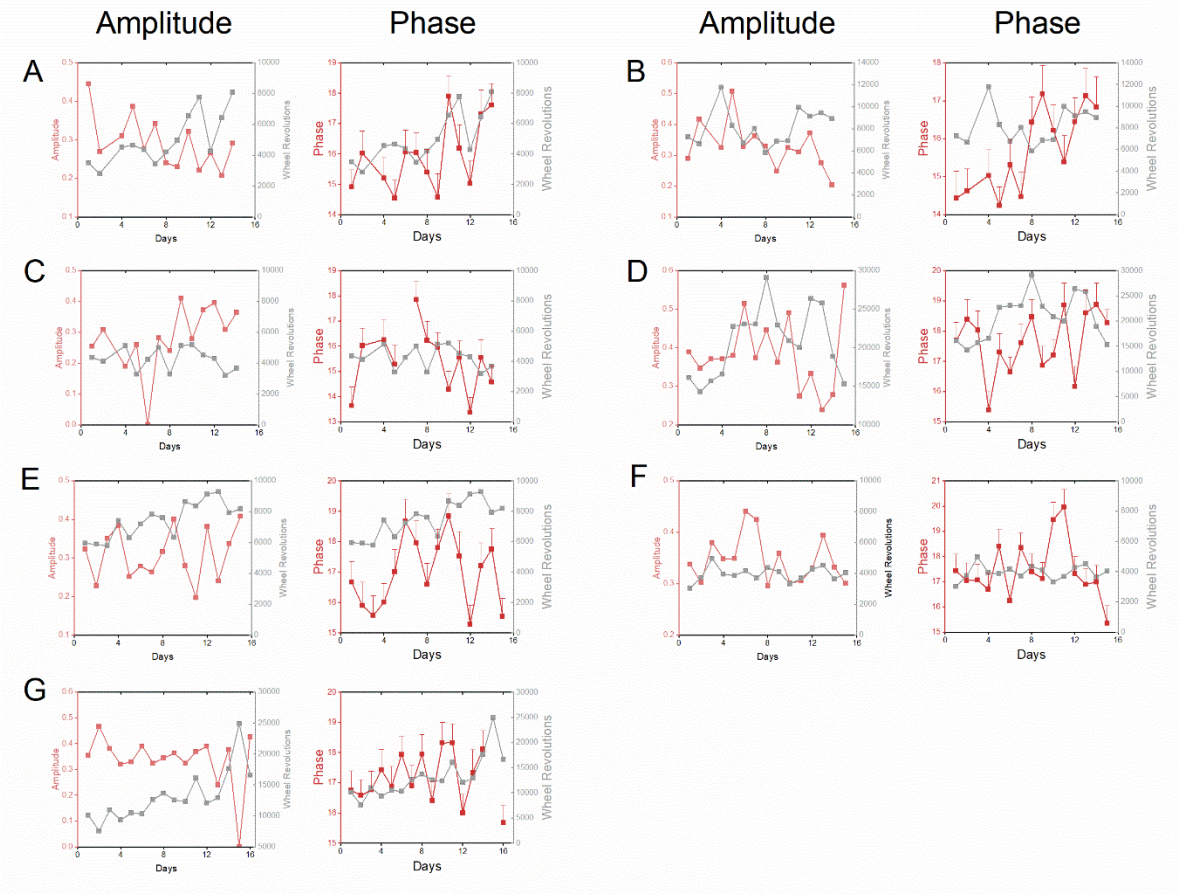


**Figure S12. Ovarian hormones are necessary for infradian cycles of eating behavior amplitude in constant darkness.** Eating behavior rhythm amplitudes (light red, left panels) and eating behavior rhythm phases (dark red, right panels) plotted with wheel revolutions (light gray) for individual ovariectomized mice in constant darkness (A-G).

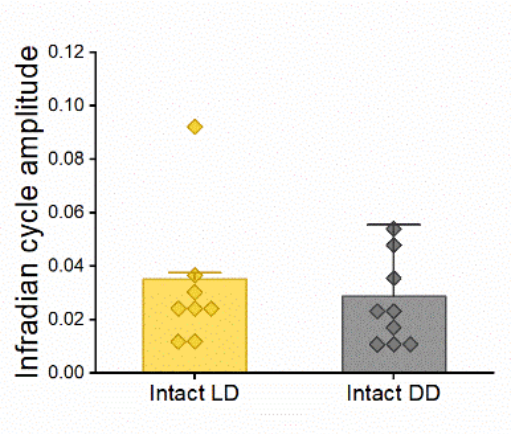


**Figure S13. The amplitudes of infradian eating rhythm cycles do not differ in 12L:12D and constant darkness.** Cosinor analysis (periods set to 96-h or 120-h for 4- or 5-day estrous cycles, respectively) was used to measure the amplitudes of the infradian eating behavior rhythm amplitude cycles. The amplitudes of infradian eating rhythm cycles from intact mice housed in LD did not differ from intact mice in DD (unpaired *t*-test, $p$=0.55).



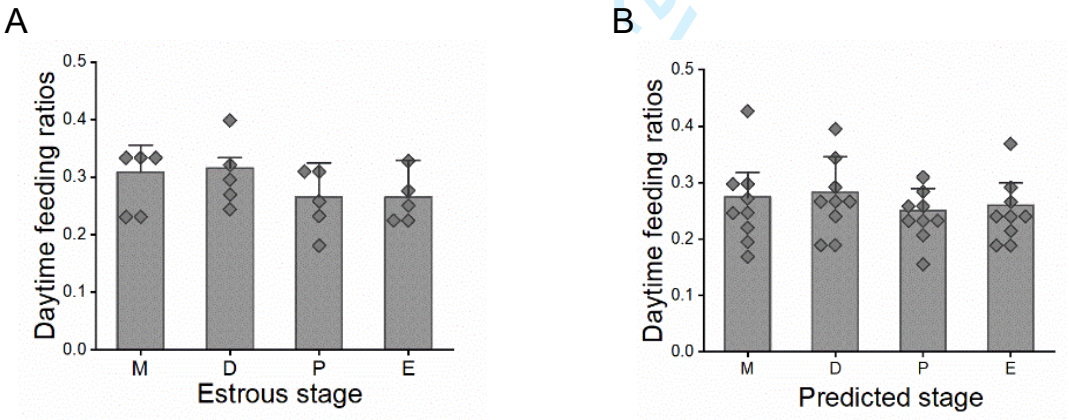


**Figure S14. Daytime feeding ratios did not differ by estrous cycle stage in 12L:12D or constant darkness.** The number of eating events during the light phase (in 12L:12D, A) or the subjective light (in constant darkness, B) relative to the total daily eating events was analyzed for the first complete cycle (one mouse is one data point). Daytime feeding ratios were not significantly different between estrous cycle stages in mice housed in 12L:12D (A, one way ANOVA with repeated measures, $p$=0.68) or constant darkness (one-way ANOVA with repeated measures, $p$=0.19).