Regulation of maternal and feeding behaviors in lactating mice by the circadian clock

授乳期マウスにおける
母性行動と摂食行動の概日時計による制御

大阪大学　理学研究科　生物科学専攻

生命機能グループ

村上　温美

Table of contents

I. Abstract 1

II. Introduction 4

III. Materials and Methods 9

IV. Results 18

V. Discussion 59

VI. References 67

VII. Acknowledgment 73

I. Abstract

The behavior of raising pups by the mother is called maternal behavior, and it's essential for the survival of pups. For species whose pups are born immature, maternal behavior is particularly important for the survival and sustainability of the species. Maternal behavior is widely observed in invertebrates and vertebrates, but is most developed in mammals such as mice, rats, and humans. Licking the placenta and other stains immediately after delivery, retrieving pups moved out of the nest, crouching to lactate and sustain pups' body temperature, and grooming pups are known as specific behaviors in the maternal behavior of rodents.

Dams are fully committed to the survival and maturation of their pups, but they require huge amounts of energy and nutrients for their own survival and milk production, and must also feed to meet these demands. Moreover, their body temperature increases as a result of increasing feeding and milk production. This research aims to clarify how the circadian clock regulates the dam's body and is compatible with several behaviors in this physiological state, which changes dramatically.

The circadian clock plays a crucial role for living organisms on the earth, functioning as a synchronizer of physiological phenomena and behavior to the light-dark cycle of the environment. In mammals, the suprachiasmatic nucleus (SCN) at the bottom of the brain works as a central circadian clock, and produces endogenous rhythms of about 24 hours via a transcriptional/translational feedback loop (TTFL) of clock genes like *Period* and *Bmal1*. Mice are nocturnal animals. Therefore, they are active at night and rest during the day when housed under 12 hours of light and 12 hours of dark (LD) cycles and show clear spontaneous locomotor activity. Additionally, they can maintain spontaneous locomotor activity rhythms under 24-hour constant dark (DD) conditions because the circadian clock

keeps endogenous rhythms without external time cues.

We observed the crouching behavior of dams, which is the posture covering pups for lactating and maintaining pups' body temperature. This behavior was consistent for several weeks from delivery until weaning. Analysis of its circadian rhythmicity showed that wildtype dams exhibited diurnal rhythm in crouching behavior under both LD cycles and constant darkness (DD). This rhythmicity of maternal behavior was disrupted in *Per*-null（*Per1*$^{-/-}$, *Per2*$^{-/-}$, *Per3*$^{-/-}$）. Furthermore, when wild-type dams or *Per*-null dams were housed with *Per*-hetero (*Per1*$^{+/-}$, *Per2*$^{+/-}$, *Per3*$^{+/-}$) pups, the observed maternal behavior rhythm was determined by the dam's genotype (wild-type or Per-null), suggesting that crouching rhythm is regulated by the dam's circadian clock. In the crouching of wild-type dams, long-lasting crouching appeared in the late half of the dark phase (crouching-dominant time), which was characteristic. This crouching-dominant time was consistent with the timing of decreased body temperature in both virgin mice and dams, suggesting that this body temperature-decreasing timing is suitable for concentrated crouching. Moreover, we analyzed feeding behavior of dams. The feeding time increased about five times during the light phase, but feeding during the early dark phase, usually observed during the non-lactation period, still remained. When we took away pups from dams during the lactating period, the feeding behavior rhythm of dams was back to that of virgin mice (feeding time in the light phase was decreased). This suggests that increasing feeding behavior in the light phase was not regulated by the circadian clock, but is a response to increasing demand for energy. And when crouching behavior and feeding behavior actograms were overlapped, these two behaviors occupied 80-90 % of the dam's behavior of a day. Therefore, it was revealed that they use time of day effectively regulated by the dam's circadian clock, with concentrating maternal behavior (crouching)

in the late half of the dark phase and feeding behavior in the first half of the dark phase, and during the light phase, two behaviors alternately appeared.

Clock gene expression rhythm in the SCN of dams maintained strong amplitude, as well as that of virgin mice, not affected by lactation. On the other hand, downstream genes related to carbohydrate and lipid metabolism were suppressed, and enzymes related to fatty acid synthesis were increased in the dam's liver, where metabolism and feeding patterns were changed for the production of milk. It is considered that metabolic rhythm is out of regulation of the circadian clock and altered to match the physiological state of dams.

These results suggest that while lactating mice show altered behavior and physiological states, both the central clock of the SCN and the peripheral clock continue to maintain a strong oscillation rhythm. Under the circadian clock, maternal behavior and feeding behavior, which are important in this period, appear at the time of day suitable for each behavior. In addition, it is clarified that there are behavioral components for the survival of pups and response to increasing energy demand during the lactation period.

II. Introduction

i. Circadian clock

The circadian clock plays crucial role for living organisms on the earth, as it synchronizes the circadian rhythms of various physiological phenomena and behaviors with the external light–dark (LD) cycle of the environment [1-4]. For human, wake-sleep cycle, hormonal secretion and body temperature show circadian rhythm, these disturbance is considered to be a cause of life-related disease and mental illness [5]. This circadian clock oscillates autonomously with a period of about 24 hours, which enables it to maintain physiological phenomena at a certain rhythm without external LD cycle information. In the circadian clock, rhythms are produced by transcription factors called clock genes. Transcription of *Period*, one of the clock genes, is promoted by BMAL1-CLOCK complex, other clock gene products. Translated PERIOD makes complex with CRY, another clock gene product, and suppresses its own transcription through suppressing BMAL1-CLOCK complex. This system forms a transcriptional/translational feedback loop (TTFL) composed of clock genes [6]. Furthermore, strong oscillation of the circadian clock with an approximately 24 hours period is supported by interlocked loops with other clock-related genes such as *Dbp* and *Rev-erbα* [2].

Mice are active at night and rest during the day when kept in LD cycles because they are nocturnal animals. During these cycles they exhibit clear spontaneous locomotor activity. Additionally, even under 24-hour constant darkness (DD) conditions, they can maintain spontaneous locomotor rhythms because their circadian clock keeps internal rhythm without external time cues [7]. *Per*-null（$Per1^{-/-}$, $Per2^{-/-}$, $Per3^{-/-}$）mice, which knock out all period genes, indicate apparent behavioral rhythms due to masking effects under LD cycles, but it is completely disrupted under DD conditions [8]. Thus, clock

genes play an important role in the temporal control of animal behavior.

In mammals, Suprachiasmatic nucleus (SCN) which is nucleus with a diameter of about 300 μm, carries on the center of this circadian clock mechanism. SCN-removed rats disrupt spontaneous locomotor rhythm, but when SCN is transplanted from another rat, it can show locomotor rhythm again [9]. And the circadian clocks also exist as peripheral clocks in peripheral organs besides the central clock in brain, they set the rhythm for the organs to work appropriate time. The peripheral clock is considered under regulation of the central circadian clock, but it is known that they are affected by external cues such as feeding behavior [10, 11].

ii. Maternal behavior

The maternal behavior is the actions of raising pups by mothers that are essential for the survival of pups. This plays a crucial role for species whose pups are born immature for the strategy of survival and sustainability of the species. Therefore, dams begin caring for their offspring immediately after delivery, continuing until weaning. This activity increases the survival rate of offspring and enable the transfer of genetic information to the next generation [12, 13]. Maternal care is observed in both invertebrates and vertebrates, but it is most developed among mammals[14]. The examples of well-known maternal behaviors of rodents are licking the placenta and other stains immediately after delivery, retrieving pups moved out of the nest, crouching to give milk and sustaining pups' body temperature, and grooming [15]. The mother-pup relationship through maternal behavior is the first social interaction for pups, and it affects their social development after weaning [16-18]. It is known that maternal behavior is quickly triggered by recognition of the pups' presence through smell and the pups' voice after

birth, when the dam's endocrine status is ready during gestation [19, 20]. The regulation of maternal behavior is centered in the medial preoptic area (MPOA), which integrates this information. In MPOA, receptors of hormones which is important for a perinatal period, such as estrogen and prolactin, are expressed [21-23]. Moreover, neurons in the MPOA are activated during maternal behavior [24], and the destruction of MPOA disrupts maternal behavior in multiparous mice [25].

iii. Previous research

As the timing of parturition exhibits a diurnal rhythm, it is considered that behaviors during the perinatal period are affected by the circadian system [26]. Is the maternal behavior regulated by the circadian clock? In the past, research has well established the circadian rhythm of maternal behavior in rats. The experiments with dual-chambered apparatus were carried out in the 1970s [27, 28]. These results reported that maternal behavior shows a circadian rhythm, with being more exhibited in the light phase and less in the dark phase in rats. However, only the duration during which dam and pups were in the nest at the same time was measured in these experiments; it is uncertain that maternal behavior was really exhibited.

In mice, there are bimodal rhythms in which maternal behavior was reduced at the transition from light to dark phase [29]. These studies were conducted only under light–dark cycles; the observations were not conducted under constant dark (DD) conditions in which the lights were off all day. Therefore, the possibility that they were influenced by light-dark cycles cannot be ruled out. Moreover, there is the circadian rhythm of body temperature, and a lot of research about the relationship between the lactating behavior of dams and body temperature are carried out. During the lactating behavior, it is

considered that the body temperature of the area in contact with the pups increase, and when the body temperature reaches the limit of heat dissipation, lactation is suspended [30]. When the dam's back was shaved or the tail was cut, which carried on heat dissipation, the duration of lactation was altered, as evidenced [31, 32]. From these results, it is believed that the body temperature rhythm of dams plays an important role in the regulation of maternal behavior.

iv. Aim of this research

During the lactation period, dams must manage not only their own needs, feeding, drinking, sleeping, and engaging in spontaneous activities around the cage, but also build nests and care for their pups. Additionally, they need huge energy and nutrients to produce and secrete breast milk. Food intake is increased to satisfy this higher demand in mother rats [33]. But mice cannot eat large amounts of solid food at once because their body and stomachs are smaller than those of rats [34], so it is considered that they need more time to eat than rats. That is, dams have to achieve both rearing their pups and feeding behavior to fulfill nutrient demands for their own energy and milk production. And as a subproduct of these increased feedings, the dam's body temperature rises [35, 36].

In dams with such dramatic changes of physiological state, how the circadian clock contributes to it is unclear. In this research, we simultaneously observed maternal and feeding behaviors both under the light–dark cycles and the constant dark conditions to clarify the allocation of time for these two behaviors. We made crouching behavior defined as a nursing posture in which the dam covers her litter and performed for about 3 weeks from birth to weaning as an index of maternal behavior because long-term data was needed to assess circadian rhythm. Next, we measured the rhythm of body

temperature in dams to explore its relationship with lactating behavior, which is believed to be influenced by temperature.

Furthermore, we analyzed the presence or absence of change in the peripheral clock in lactating dams. It is known that the peripheral clocks present in the peripheral organs are affected by external factors such as feeding, besides regulation of the central clock in the SCN [10, 11]. There is a possibility that clock gene expression in liver playing a central role in metabolism is influenced by increased feeding behavior in dams. Therefore, we investigated the role of clock in gene expression levels in the liver of dams by analyzing the expression rhythm of clock genes and metabolism-related genes using quantitative real-time PCR.

III. Materials and Methods

i. Animals

BALB/c background mice were purchased from Nihon SLC (Shizuoka, Japan) as wild-type mice. *Per*-null（*Per1*$^{-/-}$, *Per2*$^{-/-}$, *Per3*$^{-/-}$）mice were generated by crossing single knockout mice (*Per1*-knockout [37], *Per2*^{Brdm1}-mutant [38] and *Per3*-knockout [39], all of these mice had C57BL/6 background) that were backcrossed onto a BALB/c background for at least ten generations. *Per*-null mice completely disrupt circadian locomotor activity and eating and drinking rhythms under constant darkness (DD); however, locomotor rhythms are observed under a 12 h/12 h light/dark (LD) cycle due to the masking effect of the environmental lighting condition [8]. *Per*-null mice did not exhibit significant developmental abnormalities when provided a regular diet during standard LD cycles.

Mice were housed in an isolated light-controlled box under a 12-h light (-200 lux fluorescent light)/12-h dark cycle (LD) with access to food (ORIENTAL YEAST CO., LTD.) and water ad libitum. Before the experiments, mice were kept under LD cycle for at least 2 weeks to synchronize their circadian clock to the ambient LD cycle and subsequently transferred to each experimental condition. We use over 8-weeks old mice for experiments, particularly mother mice that were over 12 weeks old at parturition, and they were primiparous.

Time is expressed as zeitgeber time (ZT), with ZT12 as light-off time under LD, or circadian time (CT), with CT12 as the onset of locomotor activity under DD. All animal experiments were approved by the Animal Experimentation Committee of Osaka University (approval No.: FBS-18-002-1 and FBS-23-002).

ii. Circadian behavioral rhythm analyses

Mice were housed and mated in groups under LD. Pregnant female mice were transferred into an isolated light-controlled box (mentioned above) and reared under LD or DD. Locomotor activity rhythms were examined in each mouse from day 13 of the pregnancy until delivery, and the postnatal lactation period for 7 days, totaling 15 days. Some of the mice were housed under constant dark conditions (DD) throughout the experiment. Locomotor activity was individually recorded by passive infrared sensors (FA-05F5B; Omron, Japan) every minute using CLOCKLAB software (Actimetrics; Wilmette, IL, USA) and analyzed using CLOCKLAB Analysis software. χ^2 periodogram was analyzed using the CLOCKLAB Analysis software as previously described [40, 41]. Animal behaviors in a 7-day interval of the 7 days before and 7 days after the delivery were based on for χ^2 periodogram calculation. The relative power spectral density (rPSD) was calculated to quantify rhythmicity in these stages using the function embedded in the CLOCKLAB Analysis software. The power spectrum was calculated after applying the Blackman-Harris window. The strength of rhythmicity was expressed as rPSD, which corresponds to the relative signal strength of the frequency close to the circadian period (0.035-0.048 cycles/h) divided by the cumulative strength of all frequencies (0-1 cycles/h). The rPSD was calculated for 7 days during pregnancy (gestation days 13~19) and parenting (from the day after delivery to day 7) in mother mice.

iii. Video analyses of crouching and feeding behavior

Wild-type and *Per*-null females were mated with males of the same genotype under LD conditions. In the experiment in which dams reared *Per*-hetero ($Per1^{+/-}$ $Per2^{+/Brdm1}$ $Per3^{+/-}$) pups, wild-type females with *Per*-null males or *Per*-null females with wild-type males

were mated, and *Per*-hetero pups were obtained. When pregnancy was confirmed, females were transferred into individual cages, and they raised their pups after delivery. The experimental cages were placed in a stainless-steel box (with ventilation and lighting control) to block ambient light. An infrared dark-field camera (MK-0323E 270,000 pixel Sharp CCD with infrared LED, Mintron Enterprise Co., Ltd., Taipei, Taiwan) was positioned 60 cm above the bottom of the cage to record the behavior of the mice within the cage. Behavioral data of dams kept under LD (fluorescent light 200 lx, 8:00 on a 20:00 off lighting schedule) or DD (constant darkness) conditions were recorded using a digital video recorder (RH05 and RLC010R, SREE Co., Ltd., Tokyo, Japan). Video data were recorded during late gestation, 3-5 days before delivery, and approximately 3 weeks after delivery. Fourteen wild-type dams, 11 *Per*-null dams, and 4 non-lactating wild-type mice (assessed only feeding) were used.

We focused on and analyzed crouching behavior as a maternal behavior. A nursing posture in which the dam covers her litter was defined crouching behavior. The start time was when the animal covered the litter and stopped moving, whereas the end time was when the animal started moving from that posture and ultimately moved away from the litter. The measurement time was divided into 1-min segments, and a CSV file was created in which, in the 1-min bin, one was entered for each minute the animal was crouching, and zero was entered when no crouching occurred. In addition, one for the start time and zero for the end time of the behavior were entered. The CSV file was visualized as actograms using the KD graph program (Kyoto Densoku, Kyoto, Japan). In the analysis of crouching behavior under LD conditions, Zeitgeber Time (ZT) 0 is defined as when light is on (8:00 a.m.), and ZT12 is when light is off (8:00 p.m.). In the analysis of crouching time under DD, 8:00 a.m. on the day of delivery was considered the start time

(Time 0) of the actogram. The feeding behavior time was analyzed in a similar manner using video data. The measurement time was divided into 1-min segments, and 1 and 0 were entered in a 1-min bin in the CSV file based on the feeding behavior when a mouse came into contact with the food and moved away from the food, respectively.

The rhythmicity of crouching behavior was analyzed using data for 10 days, from day 4 to day 13 (day 0 was the day of delivery) after delivery, by χ^2 periodogram program [42] ranging from 1 to 30 h; this was calculated using Excel (Microsoft). The duration of crouching behavior was summed the crouching time over a 4-hour period divided by the total daily crouching time for 10 days after the delivery (day 4 to day 13). Similarly, the bar graph comparing feeding time was calculated by summing the time that mice had contact with the food every 4 hours for 10 days after parturition (day 4 to day 13) in dams and for any 5 days in non-lactating mice. For individuals, the hourly percentages of time spent crouching or feeding were calculated separately, and the 24-h data were divided into hourly intervals and represented in a stacked graph. The percentages of time spent crouching and feeding were averaged over the same period per day for 10 days from day 4 after delivery in LD and for 5 days from day 4 after delivery in DD. Crouching-dominant time was defined as the period in which more than 60% of crouching lasted more than three consecutive hours.

iv. Body weight of *Per*-hetero pups

We measured the weight of the *Per*-hetero pups reared by wild-type or *Per*-null dams approximately once a week. Then, we performed a linear regression and calculated the correlation coefficient.

v. Correlation between behavioral time and amount of physiological data

We measured the weight of the pups at 6 points (ZT0.5, 3.5, 8, 12, 21, 24) on the fifth day after birth (day 5, assuming the day of birth as day 0), and the crouching behavior of the mother during that time was simultaneously recorded with the method described above. The duration of crouching and the increase in weight of the pups during that time were calculated and plotted. Additionally, the amount of food in the cage was measured at 5 points (ZT0, 4, 8, 12, and 24) on the eighth day after birth, and the behavior was recorded simultaneously. The duration that the mother mice contact with the food and the amount of food intake were plotted. Linear regression was performed to calculate the correlation coefficient.

vi. Body temperature recordings

Virgin female mice (8-week-old) and pregnant mice (day 14-17 pregnant) were anesthetized (0.45 mg/kg medetomidine, 2.4 mg/kg mitazolam, 3 mg/kg butorphanol) and subcutaneously implanted nanotags (KISSEI COMTECH Co., Ltd., Nagano, Japan) on their back side. Body temperature was recorded every 5 minutes, and a moving average of 65 min (30min before and after any point) was calculated. Crouching and feeding behaviors were recorded using an infrared camera simultaneously with the method previously described.

vii. Sampling of SCN and liver

Sampling was performed under LD and DD conditions. Virgin mice were 8~16 weeks old, while dams were 7 to 12 days post-partum. Mice were kept under LD or DD to eliminate the influence of ambient light on the SCN and liver. For the DD sampling, mice

kept under LD were released into DD for thirty-six hours. Mice were killed by cervical dislocation, and the brain and liver samples were collected every 4 h [43]. In DD, sampling was conducted under a safe red light. The brain that removed from the skull was immediately frozen on dry ice. Two coronal brain sections (250 μm thick) containing the SCN were prepared using a cryostat microtome (CM3050S, Leica). The bilateral SCN was collected from the frozen sections using a 0.75 mm diameter stainless steel pipe [44]. A piece of liver was taken from the abdominal cavity and quickly frozen in dry ice.

viii. Quantitative real-time PCR

Total cell RNA was extracted from the SCN using the RNeasy Micro Kit (Qiagen). RNA was then converted into cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR of the individual cDNAs was performed as described previously [45, 46], using THUNDERBIRD SYBR qPCR Mix (TOYOBO CO., LTD., Osaka, Japan) with StepOnePlus (Applied Biosystems, Tokyo, Japan) and analyzed by a standard curve method. Data were normalized using the relative expression of the housekeeping gene, 36b4 (NM_007475). The primer sets used were following.

Gene	Fw	Rv
36b4 (NM_007475)	5'-CTCACTGAGATTCGGGATATG-3'	5'-CTCCCACCTTGTCTCCAGTC-3'
Per1 (NM_011065)	5'-TGGCTCAAGTGGCAATGAGTC-3'	5'-GGCTCGAGCTGACTGTTCACT-3'
Per2 (NM_011066)	5'-CCATCCACAAGAAGATCCTAC-3'	5'-GCTCCACGGGTTGATGAAGC-3'
Bmal1 (NM_007489)	5'-CCTAATTCTCAGGGCAGCAGAT-3'	5'-TCCAGTCTTGGCATCAATGAGT-3'
Reverbα (NM_145434)	5'-ACAGCAGCCGAGTGTCCC-3'	5'-ACACAGTAGCACCATGCCATTC-3'
Dbp (NM_016974)	5'-AATGACCTTTGAACCTGATCCCGCT-3'	5'-GCTCCAGTACTTCTCATCCTTCTGT-3'
Tef (NM_017367.3)	5'-ATCTTTCAGCCCTCGGAAAC-3'	5'-GGTCTCCCTCTCCTTTTCCA-3'
Hlf (NM_172563.3)	5'-CGTCTCCGAACTGTATGCAGAG-3'	5'-GGTCAATGGGACTCGGTGTATT-3'
Ppaaα (NM_011144)	5'-CCTCAGGGTACCACTACGGAGT-3'	5'-GCCGAATAGTTCGCCGAA-3'
Gys2 (NM_145572)	5'-CCAGCTTGACAAGTTCGACA-3'	5'-ATCAGGCTTCCTCTTCAGCA-3'
Pygl (NM_133198)	5'-CCTATGGCTACGGCATTCGT-3'	5'-TCTCCCAAGGGTTTCCATGC-3'
G6Pase (NM_008061)	5'-GGGCATCAATCTCCTCTGGG-3'	5'-GTCCAGGACCCACCAATACG-3'
Fasn (NM_007988)	5'-CCCTTGATGAAGAGGGATCA-3'	5'-CAAGGCGTTAGGGTTGACAT-3'
Elovl6 (NM_130450)	5'-CCCGAACTAGGTGACACGAT-3'	5'-CCAGCGACCATGTCTTTGTA-3'
Cpt1a (NM_013495.2)	5'-CCAGGCTACAGTGGGACATT-3'	5'-GAACTTGCCCATGTCCTTGT-3'
Acot1 (NM_012006)	5'-GACTGGCGCATGCAGGAT-3'	5'-CCAGTTTCCATAGAACGTGCTTT-3'
Acot3 (NM_001346701)	5'-GGTGGGTGGTCCTGTCATCT-3'	5'-TGTCTTCTTTTTGCCATCCAAAT-3'
Lpl (NM_008509)	5'-GGGCTCTGCCTGAGTTGTAG-3'	5'-AGAAATCTCGAAGGCCTGGT-3'

ix. Statistical analysis

A paired t-test was used to compare rPSD (Figure 2C, 3C) and body temperature (Figure 25B), Welch's t-test for locomotor activity period of Per-hetero, one-way ANOVA as used to analyze the period length of crouching (Figure 13) and difference in feeding time between non-lactating female mice and lactation mice removed pups (Figure 24C), and two-way ANOVA RM was performed to assess crouching duration (Figure 9) and the change in feeding time (Figure 15C, 15D, 24B). Differences in the cumulative probability of duration of a crouching event were compared with the pairwise Kolmogorov-Smirnov test (Figure 19). To evaluate the difference in mRNA expression level (Figure 28, 29, 30), we conducted two-way ANOVA. Sidak's multiple comparison test was applied to compare multiple conditions, following one-way or two-way ANOVA tests. We also analyzed with compareRhythms (http:// github.com/bharathananth/compareRhythms) [47] with R studio (version 4.4.1; http://www.rstudio.com/) in Table 1. Program settings are; method = dodr, amp_cutoff = 0.2, rhythm_fdr = 0.01, compare_fdr = 0.05. Linear

regression was performed to correlate between crouching duration and pups weight gain, as well as food contact time and food intake (Figure 23). Sample numbers and statistical results are shown in the figure legends. Data are presented as the mean ± SE, and p-values <0.05 were considered to indicate significance. P values are represented as *$p < 0.05$, **$p < 0.01$ or $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$. Statistical analyses were performed with GraphPad Prism software (GraphPad software, LLC, version 7.0).

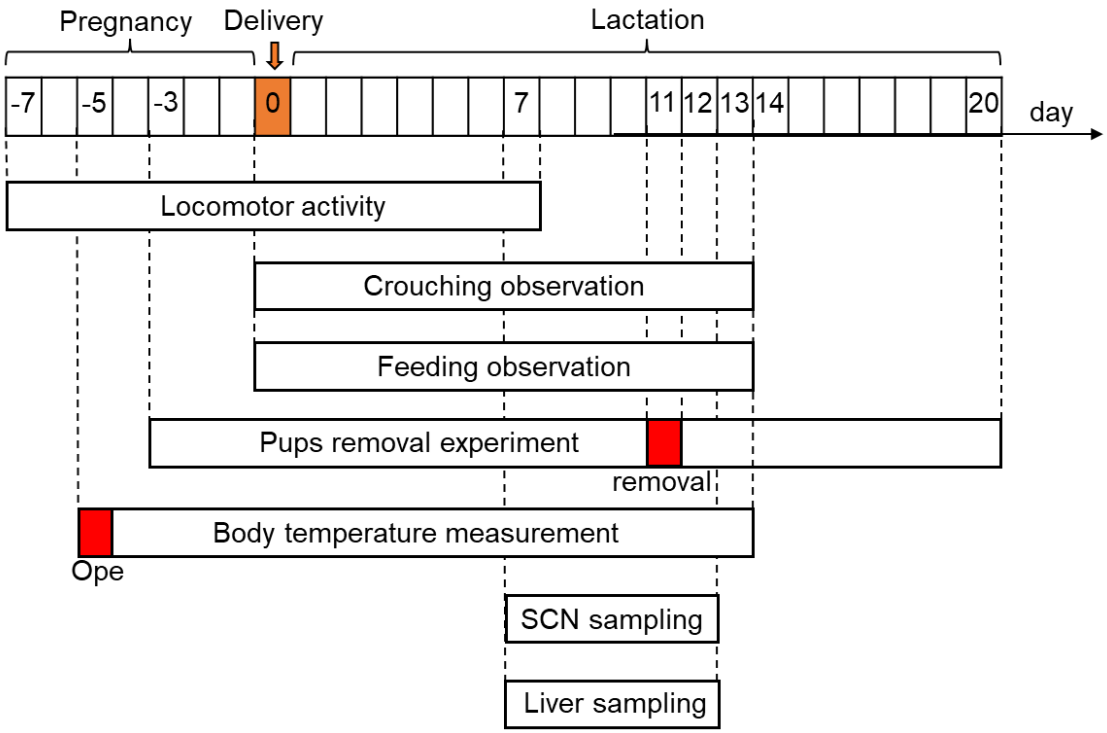

Figure.1 Time course of experiments

The horizontal axis represents time passage, a rectangle indicates a day. The condition of dam is written on the upper side of the rectangle, while the scheduled experiments are written on the lower side. The word “removal” indicates the day of separation of mother mice and pups, “ope” indicates the day of the surgery of embedding nanotags into mother mice.

IV. Results

i. Locomotor activity rhythms in mice became weakened during lactation

BALB/c strain mice are active at night and rest during the day under LD cycles because they are nocturnal. During days 12 to 17 of pregnancy, pregnant mice exhibited higher amounts of activity and robust nocturnal activity rhythms detected by passive infrared sensors than during late pregnancy and lactation. However, at the end of pregnancy (i.e., 2 days before delivery), the locomotor activity of the pregnant dams decreased (Figure 2A).

In the actual video, mice tended to stay still in the newly formed nest. Chi-square (χ^2) periodograms and the relative power spectral density (rPSD) analysis also quantitatively revealed the prominent decrease of locomotor activity (Figure 2B, C).

To determine whether the decrease in locomotor activity rhythmicity from the end of pregnancy and during lactation was affected by the presence or absence of ambient LD cycles, pregnant mice were placed under constant dark (DD) conditions, which is all day darkness. We found that under DD conditions, locomotor activity exhibited a prominent circadian rhythm during pregnancy; however, a sudden loss occurred around the delivery date and during lactation periods (Figure 3). These findings indicate that locomotor activity rhythms are generated by the endogenous circadian clock. However, special events such as delivery and lactation may disturb the expression of the circadian rhythmicity of locomotor activity.

ii. Wild-type dams showed a clear circadian rhythm of crouching behavior

The amount and circadian rhythms of spontaneous behavioral activity decrease during the lactation period; however, they should engage in behaviors to rear pups, which are

essential for the survival of pups. We next examined whether maternal behavior shows circadian rhythm. Some kinds of maternal behaviors, such as nest building, grooming, and retrieval, are known. These behaviors occur only at certain times during the post-delivery period, although they need long-term data to evaluate their rhythmicity. Therefore, we observed and analyzed the crouching posture, covering over pups with an arched back (Figure 4A) for maintaining the body temperature and lactation of pups for approximately 3 weeks after birth until weaning. An infrared dark-field camera was installed on the ceiling of the box (Figure 4B) to record video data of the cage from above and analyze the crouching behavior.

Firstly, we assessed the periodicity of crouching behavior under the LD condition. For several days after giving birth, dams spent most of their time crouching throughout the day. Thereafter, a periodicity was observed, and intensive crouching appeared in the second half of the dark phase, whereas short crouching occurred in the light phase. χ^2 periodogram analysis showed a clear 24-h periodicity under LD cycles（Figure 5A, 6 left）.

Crouching under LD cycles was periodic, which may reflect the effect of masking on the light-dark cycle. Therefore, next, we recorded and analyzed the crouching under DD condition. Crouching under DD was also observed throughout the day for several days after the delivery. Thereafter, the crouching periodicity began to appear. χ^2 periodograms revealed a periodicity of 23.8 h (Figure 5B, 6 right）.

iii. *Per*-null dams disrupted circadian rhythm of crouching behavior

It is well known that all circadian clocks oscillate based on the TTFL of clock genes [2]. Therefore, to clarify whether the crouching rhythms observed in wild-type dams were

controlled by a circadian clock, we observed the rhythm of crouching in *Per*-null (*Per1*$^{-/-}$, *Per2*$^{-/-}$, *Per3*$^{-/-}$) dams. Their behavioral rhythm is known to be disrupted under DD conditions due to loss of the main clock genes [8]. *Per*-null dams exhibited fragmented crouching throughout the day under both LD and DD cycles, χ^2 periodogram analysis didn't show a specific periodicity (Figure 7, 8). In addition, *Per*-null crouching was not affected by the masking effect of light (200 Lux, fluorescent light).

We compared the daily rhythms of crouching behavior of wild-type and *Per*-null mice over a 4-h period in both LD and DD cycles (Figure 9). The timing of crouching was not considered significantly shifted because all dams under DD conditions gave birth within one week of being transferred to DD conditions. Wild-type mice spent more time on crouching behavior at the late 4 h of the daily cycles, that is, ZT20-24 or CT20-24. On the other hand, Per-null mice exhibit crouching behavior at the same level throughout the day, no rhythms were detected.

iv. Dam's circadian clock regulates crouching behavior

The experiments used the same genetic background: wild-type dams and pups, or Per-null dams and pups. It is known that tactile stimulation from pups is a critical sensory input for maternal behaviors [48], there is the possibility that the crouching rhythm was influenced by the endogenous rhythm of the pups. To elucidate whether crouching rhythm is driven by dams' clock or pups' clock, we observed dams with *Per*-hetero pups by mating wild-type females and *Per*-null males, or *Per*-null females and wild-type males (Figure 11A). *Per*-hetero mice exhibit shorter periodicity of behavioral rhythm than wild-type mice under DD conditions (Figure 10; wild-type; 23.5±0.07h, n=5, *Per*-hetero; 22.8±0.04h, n=8, $p<0.01$, Welch's t-test). If pups' clock influences crouching behavior,

wild-type dams should show a short crouching rhythm, and *Per*-null dams should show rhythmicity of crouching behavior. Or, if pups' circadian clock doesn't develop enough, wild-type dams would disrupt crouching periodicity.

In wild-type dams, crouching rhythms were virtually the same both the pups were wild-type or *Per*-hetero (Figure 11B, 12, 13; Wild-type dam with wildtype pups in LD; 24.0±0.02, n=5, Wild-type dam with wildtype pups in DD; 23.7±0.07, n=5, wildtype dam with *Per*-hetero pups in DD;23.8±0.09 hr, n=4、P>0.05 by Sidak's multiple t-test after one-way ANOVA）. *Per*-null dams exhibited no periodicity, regardless of whether the pups were *Per*-null or *Per*-hetero (Figure 11B). These results indicate that the dam's clock endogenously drives the crouching rhythm. In this condition, the maternal genotypes differed, pups' weight or daily crouching time did not differ (Figure 14).

v. Feeding pattern of dams are altered during lactation period

During the lactation period, dam's feeding must be increased due to the demand for nutrition for milk production [49]. Therefore, we concurrently observed the feeding behavior and crouching behavior of dams. Female mice not in the lactating stage feed mainly in the dark phase, like the locomotor activity rhythm, particularly in the first half of the dark phase of the LD cycles (Figure 15A). However, in the lactation period, wild-type dams markedly increased the feeding time, especially in the whole duration of the light phase (Figure 15B). When we compared dams and non-lactating female mice regarding daily changes in feeding behaviors over a 4-h period in LD cycles, we demonstrated that feeding behavior was enhanced in both the light and dark phases. Importantly, the feeding behavior of dams maintained the highest level from ZT0 to ZT20, and it decreased at the last section of the dark phase (Figure 15C). As a result, the total

amount of the feeding time increased in dams by about three times, especially during the light phase, resulting in an approximately 5-fold increase (Figure 15D).

vi. There is a crouching-dominant time at the second half of dark phase

Importantly, under our experimental conditions, they could not simultaneously access the feeding boxes and lactating nests, so mice could not perform crouching and feeding behaviors at the same time. By overlaying the actogram of crouching with that of feeding behavior, we confirmed that crouching and feeding were completely separated (Figure 16A; left). Moreover, there is intensive crouching during the latter half of the dark phase (crouching-dominant time) of the LD cycles, during this time, the dams spent the largely time crouching. When the time for crouching and feeding behaviors was summed hourly, dams spent 80%–90% of the day on these behaviors (Figure 16A; right, 17). Importantly, we could observe the crouching-dominant time also under DD, crouching is a clock-controlled phenomenon (Figure 16B, 18). When we examined the duration of a crouching event, the crouching duration in crouching-dominant time (the late half of the dark phase) is longer than that in the light phase (Figure 19). From these findings, it is clarified that dams concentratedly perform intensive crouching at late half of the dark phase. On the other hand, *Per*-null dams didn't exhibit crouching-dominant time in both LD condition and DD condition (Figure20, 21, 22).

vii. There was a correlation between video analysis and behavior

We confirmed strong correlations between the crouching time and the litter's milk intake, determined by their weight gain (Figure 23A). Thus, crouching behavior observed in the video should involve lactating. Furthermore, we also confirmed correlations between the

feeding time and the food intake of lactating mice. Therefore, the time when mice come into contact with food pellets is when they actually eat food (Figure 23B). From these results, we can assume that crouching behavior and feeding behavior we observed in the video represent actual lactating and eating.

viii. Feeding behavior in light phase was immediately decreased after removing pups

We next analyzed whether the change in the daily pattern of feeding behavior in dams was because of lactation. We separated the pups from their dams on day 11 of lactation, when the nutritional intake of pups is still completely dependent on their dams' milk, and dams are considered to need the most nutrients for lactation. The feeding behavior of dams during the light phase decreased from the day the pups were removed from the dam's cage (Figure 24A). When we compared before and after removing pups regarding the every 4-h profile of feeding behavior, the duration of feeding behavior was significantly reduced during the first 8 hours of the light phase after removing pups (Figure 24B). And after separating pups, an average of the 4-day feeding time in the light phase was almost lost without the pup-stimulation, similar to that during the non-lactating or pregnancy period (Figure 24C). However, the active feeding behavior during the early stage of the dark phase remained even after the pups were removed.

ix. The timing of body temperature decreasing matched crouching-dominant time

Under LD conditions, the body temperature of wild-type virgin mice showed a diurnal rhythm, peaking at the beginning of the dark phase and decreasing toward the beginning of the light phase (Figure 25A). After they transferred to DD, this diurnal rhythm continued, despite a decrease in the daily maximum and average temperatures (Figure 25B). Thus, body temperature is regulated by the circadian clock.

The body temperature of pregnant mice exhibited circadian rhythms similar to those of virgin mice under DD, although the basal level of body temperature was elevated at 1.0 °C. Under DD conditions, the body temperature of rearing dams began to increase after delivery, with peak temperature on day 7 of lactation; this temperature remained high (Figure 26A). By comparing the body temperature at the late stage of pregnancy (days 15–17 of gestation) to that during days 10–12 of lactation in the same mice, a difference of 2.0 °C in average temperature was observed per daily cycle (Figure 26B). When daily body temperature changes were overlapped on crouching and feeding behaviors in the same animal, body temperature tended to increase during the feeding behavior and fall during the crouching-dominant time (Figure 27; left).

Crouching-dominant time was present at the time of decreasing body temperature at late half of dark phase. There was a possibility that body temperature was dropping due to reduced movement, importantly, decreasing body temperature occurred preceding the onset of crouching-dominant time (Figure 27; right).

x. SCN of dams maintained robust oscillation of clock gene expression

From the results so far, changes in some activities with pregnancy and delivery were observed in dams. To clarify these changes occur at the level of the central clock in the SCN, we examined the circadian expression of clock genes in the SCN and compared it between lactating mice and virgin female mice (Figure 28, Table 1).

Notably, in the SCN of dams and virgin female mice under LD and DD conditions, robust circadian expression of the main clock genes was observed. Both mice exhibited peak expression of *Per1*, *Per2*, *Dbp*, and *Rev-erb*α during the subjective day with a trough during the subjective night and *Bmal1*, in opposite phase, peaked during the subjective

night with a trough during the subjective day. There were significant main effects of time for all genes. However, there were no main effects between virgin mice and dams except for *Per1* in LD, nor time vs condition (virgin mice or dams) interaction for most genes (see Table S1). Notably, the robust circadian rhythmicity of clock gene expression was maintained in the SCN of lactating mice. These results revealed that the circadian central clock oscillated at almost the same level as that in virgin female mice (Figure 28 and Table 1).

xi. The expression rhythm of metabolism-related genes was altered in dam's liver

Dams exhibited profound metabolic changes in many organs of the body with the initiation of lactation after delivery [50-52]. Particularly liver works as the central organ of body metabolism on carbohydrate and lipid metabolism. In this metabolic loading stage, how is the circadian system contributing? The mammalian peripheral organs are known to be influenced by the circadian central clock in the SCN and clocks located in peripheral organs [4]. As shown previously, clock gene expression profiles of the SCN of lactating dams were virtually unaltered (Figure 28). Therefore, next, we examined how circadian gene expression rhythms operate in the liver of wild-type lactating mice under LD and DD using qPCR (Figure 29, Table 1). In the liver of lactating mice, the expression profiles of *Per1* and *Per2* were slightly flattened but oscillated in LD. *Per1*, *Per2*, and *Bmal1*, the core clock genes, exhibited clear circadian rhythms similar to those in wild-type virgin mice in DD (Figure 29). These results suggest that core clock oscillation was maintained in liver of lactating dam. Otherwise, the expression amplitude of accessory loops of core clock genes, *Rev-erb* α and *Dbp* were diminished in lactating mice both in LD and DD. *Tef* and *Hlf* also attenuated in LD condition. These findings suggest that

accessory loops of core clock oscillation in the liver were affected during lactation.

We examined the circadian expression profiles of important for carbohydrate and lipid metabolism regulated by clock genes in the liver (Figure 30, Table1). *Pparα*, the transcription factor which regulates genes involved in lipid uptake and catabolism, had diminished its expression rhythm in LD condition, and decreased expression level throughout the day in DD condition. The gene expression of *Gys2*, a rate limiting enzyme for glycogen synthesis regulated by E-box and PPRE [45, 52], and *Cpt1a*, a rate limiting enzyme in FA β -oxidation which promotor also have PPRE [53] were markedly damped in LD, decreased expression level was observed throughout the day in DD condition. In addition, the nighttime increase in gene expression of *G6pase*, a rate-limiting enzyme for glycogenolysis, was also attenuated. Although there were changes in *Pygl* at ZT0 and ZT4, the rhythm and expression level did not change significantly. The gene expression of FA metabolism was also impaired. Interestingly, the mean gene expression levels of *Fasn* (FA synthase) [54] and *Elovl6* (FA elongase) [55] were always increased in lactating mice under LD and DD for the whole day. The gene expression of acyl-CoA thioesterase 3 (*Acot3*) was attenuated but not in its homolog, *Acot1*. And *Lpl*, an enzyme that hydrolyzes triglycerides, didn't change its expression rhythm.

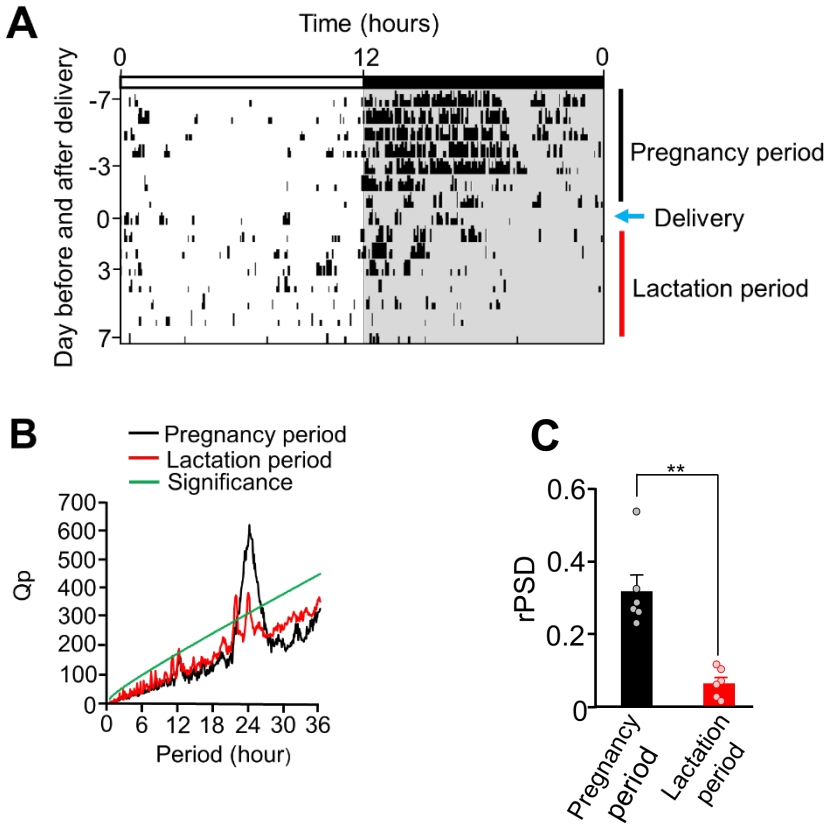

Figure 2. The spontaneous activity of the wild-type dam decreased after delivery and during lactation in LD

(A) Representative actogram of the spontaneous activity of a dam during the perinatal period. Dark periods are depicted by gray backgrounds. The vertical axis shows the days before and after delivery, with day 0 representing delivery day. The horizontal axis shows Zeitgeber time. Time 0 corresponds to light-on time (ZT0, 8:00 a.m.), and Time 12 to light-off time (ZT12, 8:00 p.m.).

(B) Comparison of $\chi 2$ periodograms for the dam in the pregnancy period (black line) and lactation period (red line). The calculation was conducted over 7 days of activity rhythms as indicated by a black and red vertical line in (A). The green line indicates a threshold depicted by $p = 0.001$.

(C) rPSD analysis (see text for detail) of dams (n=6) in the pregnancy period (indicated by black line in (A)) and lactation period (indicated by red line in (A)) housed under LD. The rhythmicity of dams was weakened (during pregnancy; 0.317 ± 0.046, during lactation; 0.062 ± 0.016, n=6, $^{**}p < 0.01$, Paired-samples t-test.)

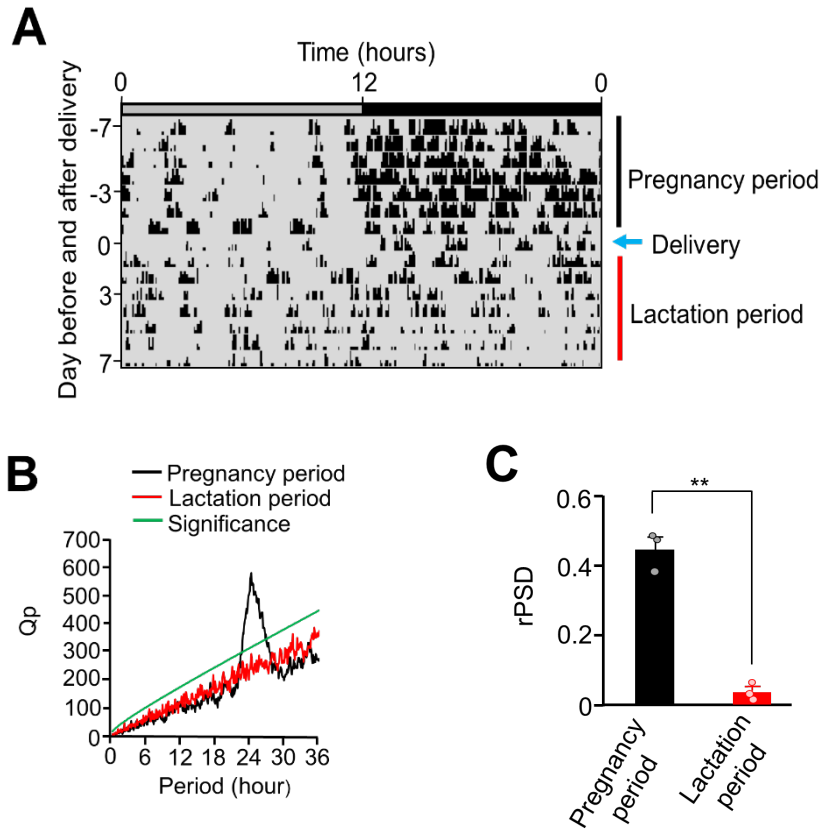

Figure 3. The spontaneous activity of the wild-type dam decreased after delivery and during lactation also in DD

(A) Representative actogram of spontaneous activity of a dam housed under DD. Dark periods are depicted by gray backgrounds. The vertical axis shows days before and after delivery, with day 0 representing delivery day. The horizontal axis indicates time (Time 0 corresponds to 8:00 a.m.). The spontaneous activity of the dam decreased after delivery and during lactation.

(B) Comparison of χ^2 periodograms for dam in the pregnancy period (black line) and lactation period (red line). The calculation was conducted over 7 days of activity rhythms as indicated by the black and red vertical line in (A). The green line indicates a confidence interval depicted by p = 0.001.

(C) rPSD analysis (see text for detail) on dams (n=3) in the pregnancy period (indicated by black line in (A)) and lactation period (indicated by red line in (B)) under DD. The rhythmicity of spontaneous activity of dams was also weakened in DD (during pregnancy; 0.451 ± 0.033, during lactation; 0.036 ± 0.016, n = 3, **p<0.01, Paired-samples t-test).

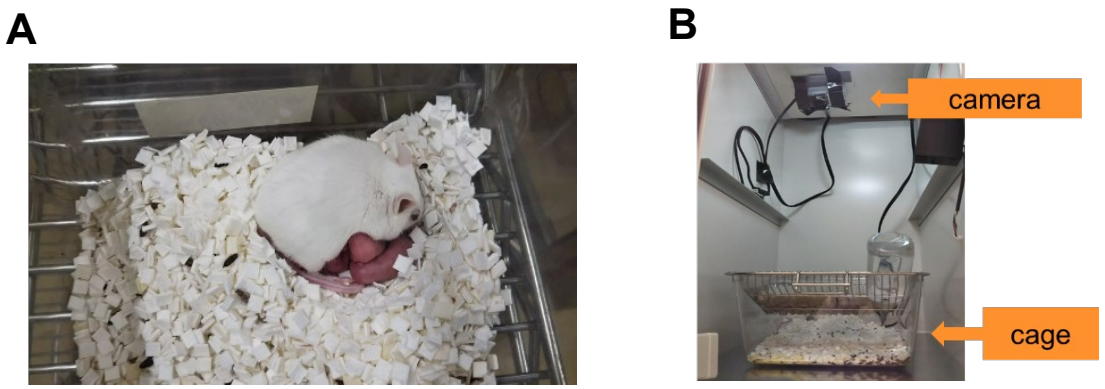

Figure 4. The Recording system and crouching behavior

(A) Video recording apparatus for dam in the cage using an infrared dark-field camera set in a light-tight box. Video data were recorded using an HD recorder.

(B) The picture of crouching behavior of a dam. She covers her pups to nurse and maintain their body temperature.

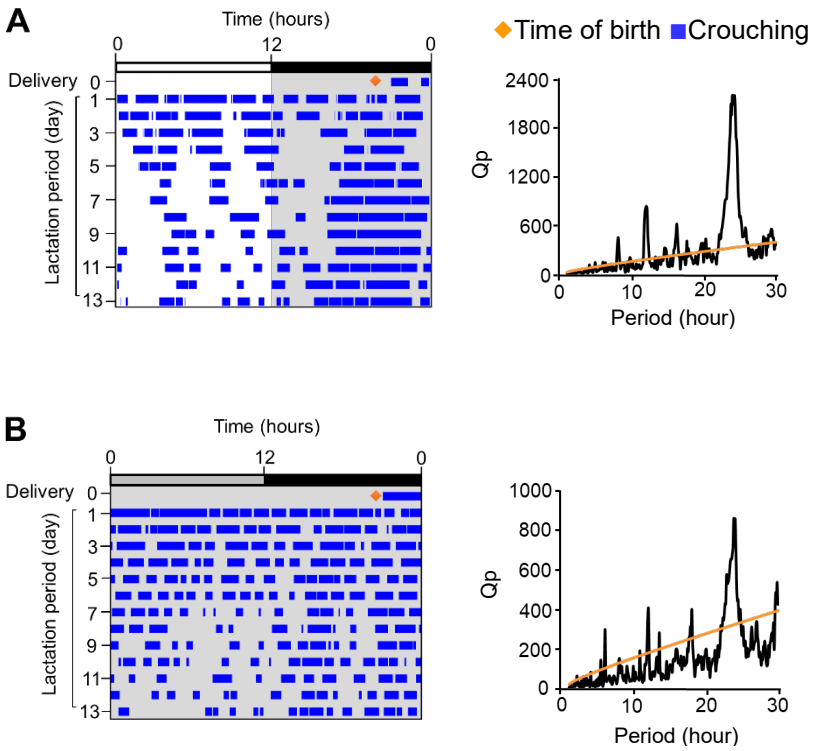

Figure 5. Crouching behavior of wild-type dam

(A, B) Representative actograms of crouching behavior (left) and its χ2 periodograms (right) of wild-type (WT) dams under LD (A) and DD (B). The vertical axis of the actogram of crouching behavior shows the delivery day (0) and subsequent days of the lactation period (day 1-13). The horizontal axis represents Zeitgeber time (Time 0 corresponds to lights on, 8:00 a.m.) in (A) and time (Time 0 corresponds to 8:00 a.m.) in (B). The blue bars show the duration of crouching and the orange diamonds show the estimated delivery timing. Events longer than 1 minute were considered crouching. χ2 periodograms were calculated for 10 days (day 4 to day 13) as indicated by a red vertical line in the crouching actogram with the orange line representing $p = 0.001$. For WT mice, the marked peak in χ2 periodograms under LD was exactly 24.0 h while that under DD was 23.8 h.

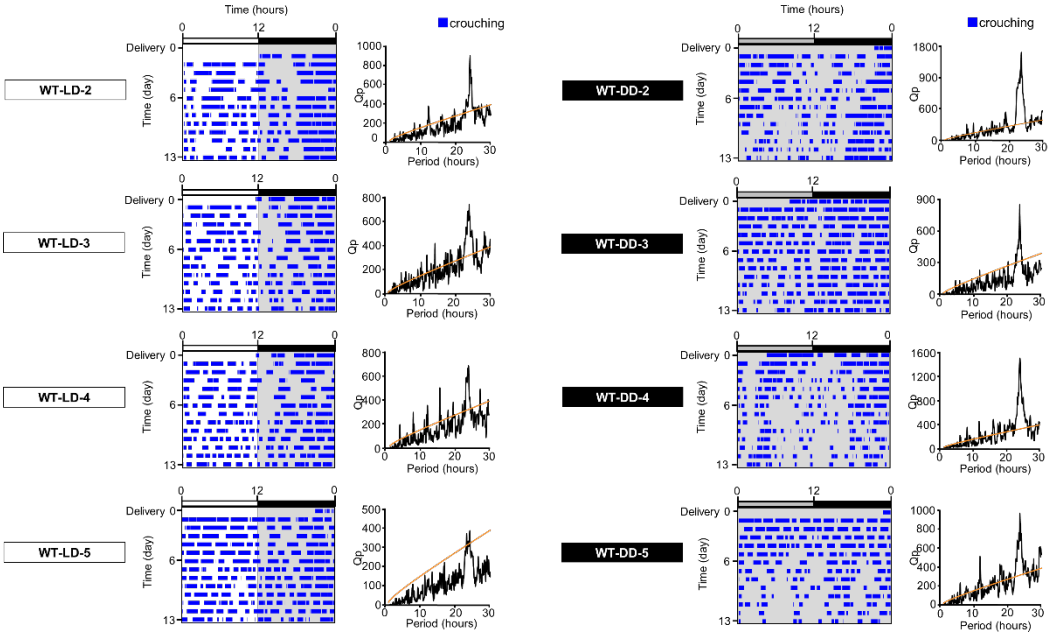

Figure 6. Actograms of crouching behavior of all individual wild-type dams except those shown in Figure 5.

Actograms of crouching behavior in wild-type (WT) dams under LD (n = 5; WT-LD2, WT-LD3, WT-LD4, WT-LD5, and WT-LD1: Figure.5A) and DD (WT-DD2, WT-DD3, WT-DD4, WT-DD5, and WT-DD1: Figure.5B). In each graph, crouching actogram (left) and χ^2 periodogram (right) are shown. The vertical axis of actograms indicates the number of days from the delivery day (day 0). The horizontal axis of actograms indicates Zeitgeber time. Time 0 corresponds to ZT0 (lights on, 8:00 a.m.) for LD (left), and Time (Time 0 corresponds to 8:00 a.m.) for DD (right). In actograms, blue bars show the crouching time for more than 1 min. χ^2 periodograms were calculated for 10 days (day 4 to day 13), with the orange line representing p = 0.001. WT-LD2 missed day 0~1 data owing to a camera malfunction.

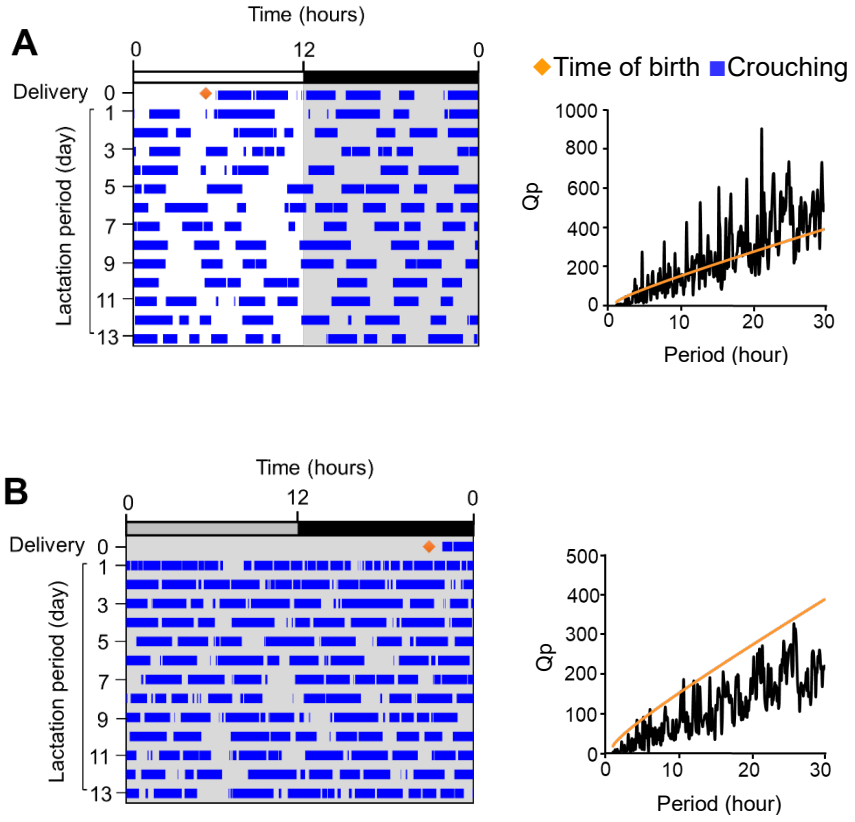

Figure 7. Crouching behavior of *Per*-null dam

(A, B) Representative actograms of crouching behavior (left) and its χ2 periodograms (right) of *Per*-null dams under LD (A) and DD (B). The vertical axis of the actogram of crouching behavior shows the delivery day (0) and subsequent days of the lactation period (day 1-13). The horizontal axis represents Zeitgeber time (Time 0 corresponds to lights on, 8:00 a.m.) in (A) and time (Time 0 corresponds to 8:00 a.m.) in (B). The blue bars show the duration of crouching and the orange diamonds show the estimated delivery timing. Events longer than 1 minute were considered crouching. χ2 periodograms were calculated for 10 days (day 4 to day 13) as indicated by a red vertical line in the crouching actogram with the orange line representing $p = 0.001$. For *Per*-null dams, no marked peak was found under either LD or DD.

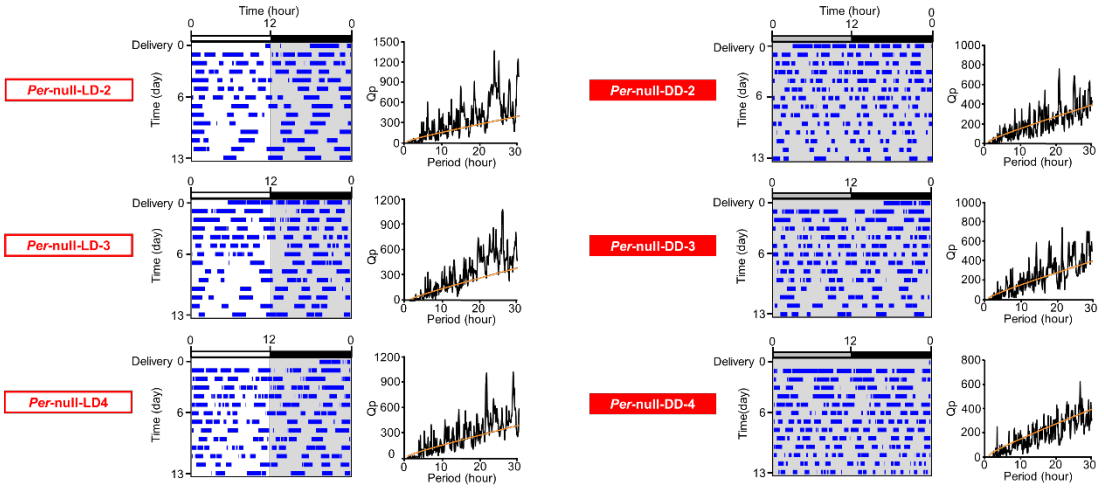

Figure 8. Actograms of crouching behavior of all individual *Per*-null dams except those shown in Figure 7.

Actogram of crouching behavior in *Per*-null dam under LD (n = 4; *Per*-null-LD2, *Per*-null-LD3, and *Per*-null-LD4, and *Per*-null-LD1: Figure.7A) and DD (*Per-null*-DD2, *Per*-null-DD3, *Per*-null-DD4, and *Per*-null-DD1: Figrue.7B). In each graph, crouching actogram (left) and χ^2 periodogram (right) are shown. The vertical axis of actograms indicates the number of days from the delivery day (day 0). The horizontal axis of actograms indicates Zeitgeber time. Time 0 corresponds to ZT0 (lights on, 8:00 a.m.) for LD (left) and time (Time 0 corresponds to 8:00 a.m.) for DD (right). In actograms, blue bars show the crouching time for more than 1 min. χ^2 periodograms were calculated for 10 days (day 4 to day 13), with the orange line representing p = 0.001.

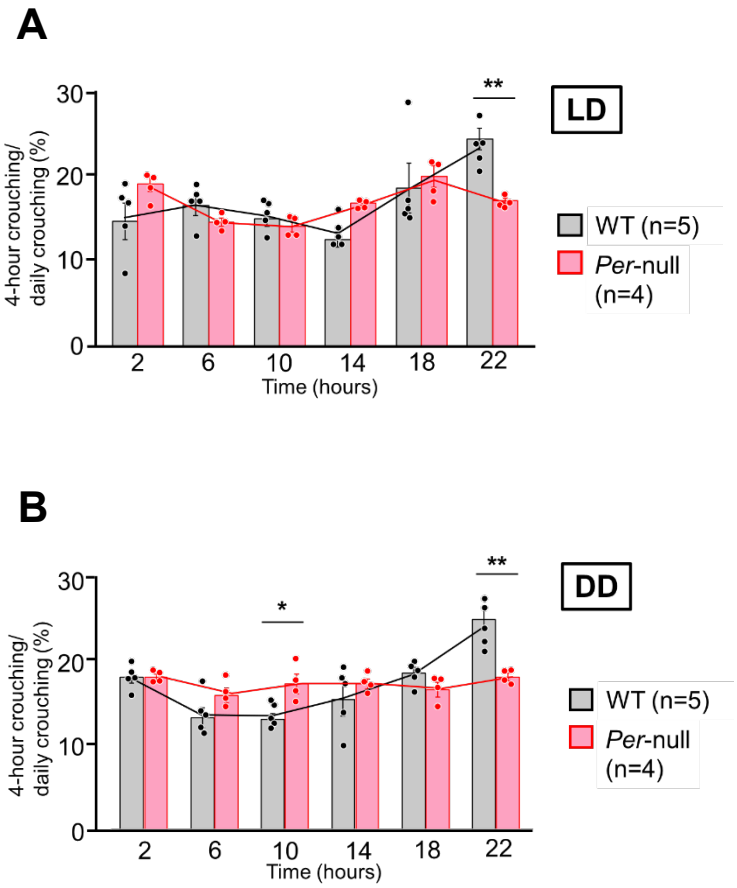

Figure 9. Wild-type dams performed increased crouching behavior at the last 4 hours of the daily cycle

（A,B）Time of crouching behavior in LD (upper) and DD (lower). Each point represents an individual data point. The time spent crouching over 4 hours was normalized by the total time spent crouching in a day, and the average for the 10 days after delivery (from day 4 to day 13) was calculated. Time 0 corresponds to ZT0 (lights on in LD) and 8:00 a.m. in DD, and the horizontal axis represents a time of the midpoint of 4-hour duration. WT is represented in black, while Per-null is shown in red (mean ± SE, *p<0.05, **p<0.01, Sidak's multiple comparison after two-way ANOVA RM).

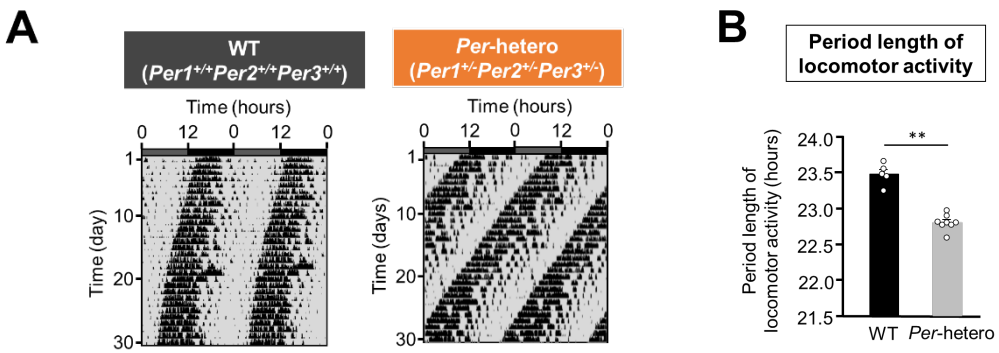

Figure 10. Spontaneous activity rhythm of *Per*-hetero mice

(A) Locomotor activity rhythm of wild-type (left) and *Per*-hetero (right) under DD. Gray backgrounds depict dark periods. The vertical axis shows time (days) and the horizontal axis shows double-plotted time (hours).

(B) The period length of locomotor activity. The free-running period was determined by eye-fitting regression in ClockLab Analysis. The period length of locomotor rhythms of *Per*-hetero mice was significantly shorter than those of WT mice (WT; 23.5 ± 0.07, n = 5, *Per*-hetero; 22.8 ± 0.04, n = 8, **p < 0.01, Welch's t test).

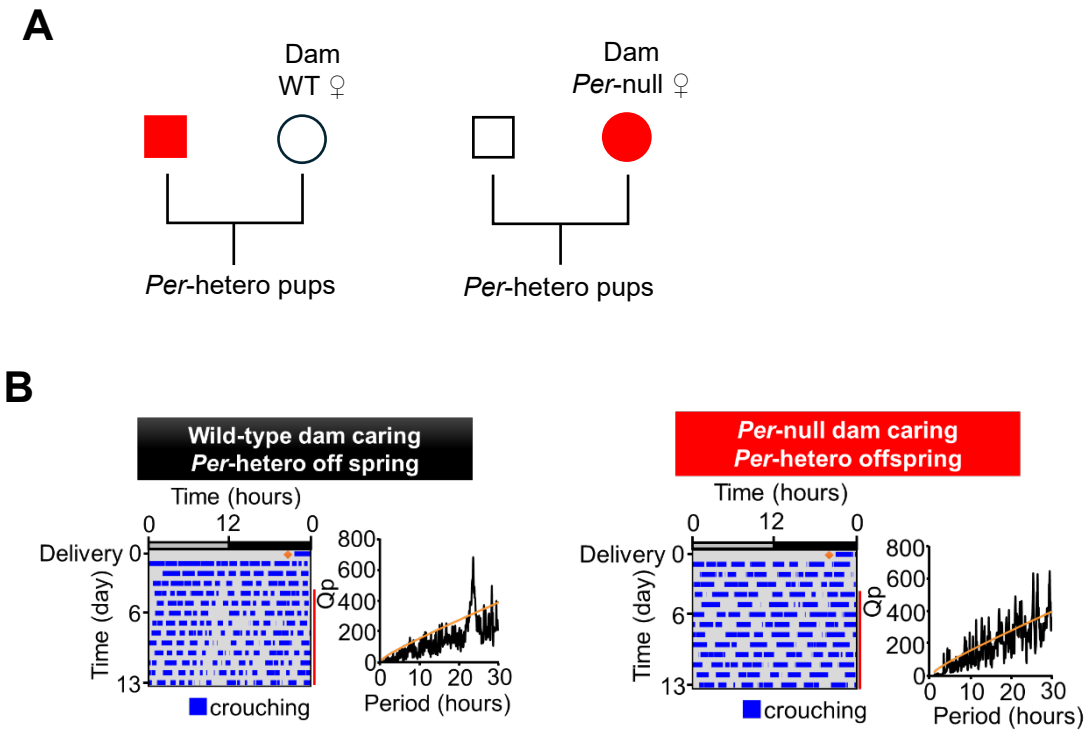

Figure 11. The crouching rhythm is regulated by circadian clock of dams

(A) Wild-type females with *Per*-null males or *Per*-null females with wild-type males were mated and both produced *Per*-hetero offspring. Both of wild-type and *Per*-null dams raised *Per*-hetero pups.

(B) Representative actograms of crouching behavior and its χ^2 periodograms in Wild-type or *Per*-null dams raising *Per*-hetero pups under DD. The vertical axis of the crouching actogram shows the delivery day (day 0) and subsequent days of lactation period (day 1 to day 13). The horizontal axis shows time (Time 0 corresponds to 8:00 a.m.). The blue bars show the duration of crouching and the orange diamonds show the estimated birth timing. χ^2 periodograms were calculated for 10 days (day 4 to day 13) with the orange line representing $p = 0.001$. Wild-type mice, but not *Per*-null mice, had a marked peak in χ^2 periodograms.

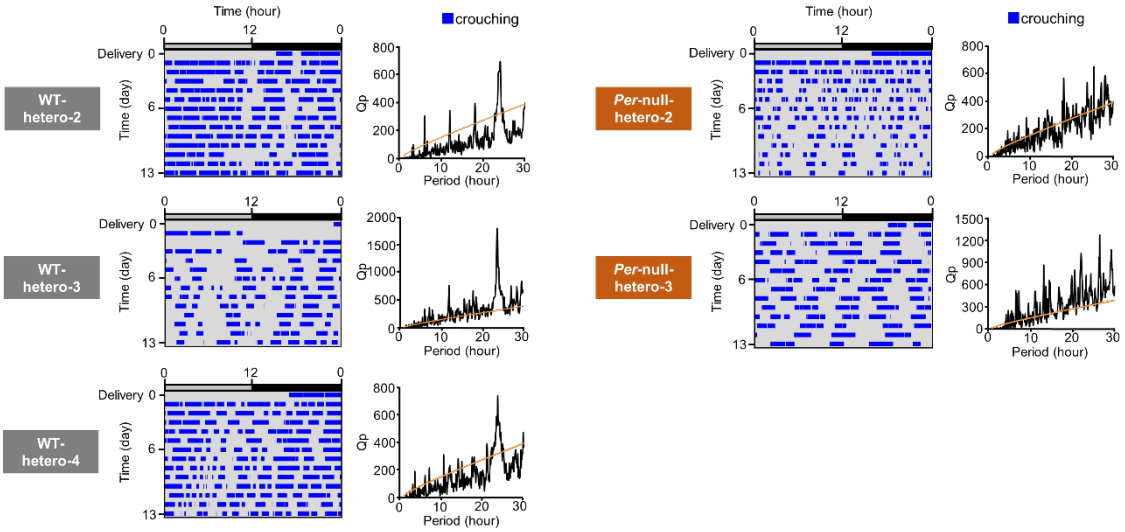

Figure 12. Actogram of crouching behavior of dams rearing *Per*-hetero pups under DD

Crouching behavior of all dams, except that presented in Figure 11 for WT dams with her own *Per*-hetero pups (WT-hetero-2, WT-hetero-3, WT-hetero-4, and WT-hetero-1: Figure.11) and *Per*-null dams with their own *Per*-hetero pups (*Per*-null-hetero-2, *Per*-null-hetero-3, and *Per*-null-hetero-1: Figure.11) under DD. In each graph, crouching actogram (left) and χ^2 periodogram (right) are shown. The vertical axis of the actograms indicates the number of days from the delivery date, defined as day 0. The horizontal axis indicates time (hours), with 8:00 a.m. defined as Time 0. In actograms, blue bars show the crouching time for more than 1 min. χ^2 periodograms were calculated for 10 days, from day 4 to day 13, with the orange line representing p = 0.001. WT-hetero-3 missed day 1-2 data owing to a camera malfunction.

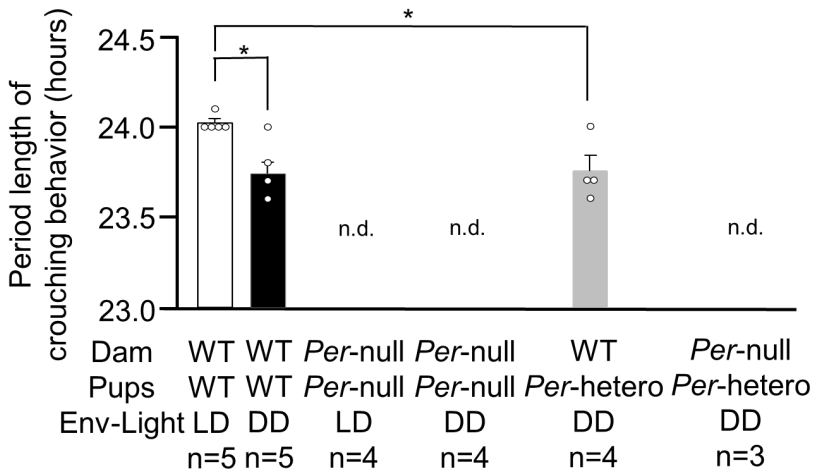

Figure 13. Comparison of period length of crouching behavior

Period length of crouching behavior rhythms in all conditions. The period length of crouching behavior of wild-type mother mice under DD was a little shorter than 24.0 h, either cohabitating with Wild-type pups or *Per*-hetero pups. (Wild-type dam with wildtype pups in LD; 24.0±0.02, n=5, Wild-type dam with wildtype pups in DD; 23.7±0.07, n=5, wildtype dam with *Per*-hetero pups in DD;23.8±0.09 hr, n=4、P>0.05 by Sidak's multiple t-test after one-way ANOVA.）

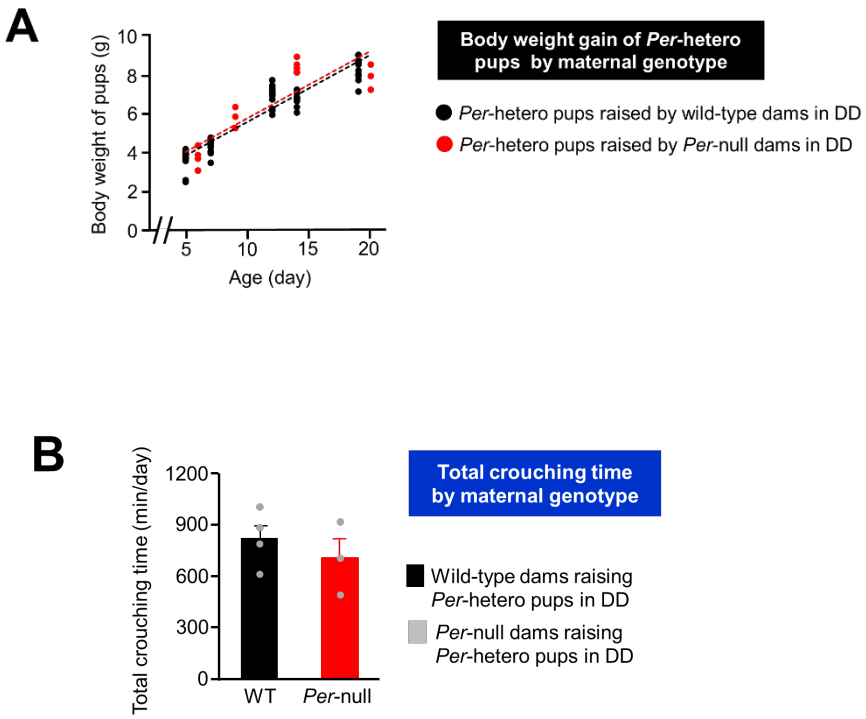

Figure 14. The growth of pups and total crouching time were not affected by differences in dams' genotype

(A) Increase of body weight of *Per*-hetero pups raised by wild-type dams or *Per*-null dams. We measured the body weight of pups approximately once per week. No difference was found between them (Wild-type dam; 25 pups from 3 dams, slope = 0.35, intercept = 2.1, *Per*-null dams; 8 pups from 2 dams, slope = 0.35, intercept = 2.3, slope; $p = 0.97$, intercept; $p = 0.38$, Linear regression with F test).

(B) Total crouching duration per day when Wild-type and *Per*-null dams raised their own *Per*-hetero pups under DD. The total crouching duration from day 4 to day 13 was averaged; thus, the birth date (delivery day) was defined as day 0. Crouching time did not differ between Wild-type and *Per*-null dams (WT; 810 ± 82 min, n = 4, *Per*-null: 693 ± 122 min, n = 3, $p = 0.47$, Welch's t-test).

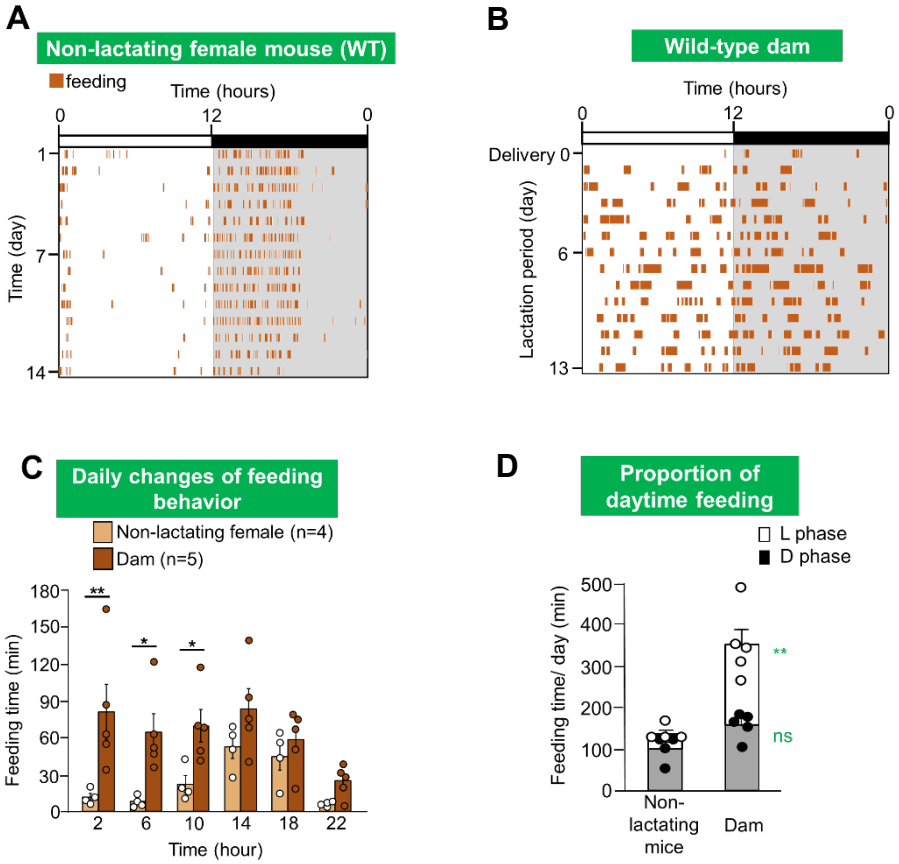
A
Non-lactating female mouse (WT)
feeding
Time (hours)
0
12
0
1
7
14
Time (day)
B
Wild-type dam
Time (hours)
0
12
0
Delivery 0
6
13
Lactation period (day)
C
Daily changes of feeding behavior
Non-lactating female (n=4)
Dam (n=5)
180
150
120
90
60
30
0
Feeding time (min)
**
*
*
2
6
10
14
18
22
Time (hour)
D
Proportion of daytime feeding
L phase
D phase
500
400
300
200
100
0
Feeding time/ day (min)
**
ns
Non-lactating mice
Dam

Figure 15. The feeding behavior of dams increased in light phase

(A) The actogram of feeding behavior (orange bars) of a non-lactating female mouse under LD. The vertical axis of the non-lactating actogram indicates any 14 days. The horizontal axis shows Zeitgeber time. Time 0 corresponds to light-on time (ZT0, 8:00 a.m.). Feeding behaviors lasting more than 1 min were recorded, and less than 1 min were not counted.

(B) The actogram of feeding behavior (orange bars) of a dam under LD. The vertical axis of the dam actogram shows the lactating period for 14 days after delivery day (day 0). The horizontal axis shows Zeitgeber time. Time 0 corresponds to light-on time (ZT0, 8:00 a.m.). Feeding behaviors for more than several minutes in one episode were observed in dam; however, this pattern was not observed in non-lactating female mice.

(C) Duration of feeding behavior per 4-hour in non-lactating female mice and dams. Each point represents an individual data point. The vertical axis shows the total time spent on feeding behavior over a 4-hour period. The horizontal axis represents time of midpoint of 4-hour duration. Non-lactating female mice are represented in light brown, while dams are shown in dark brown (mean ± SE, **$p<0.01$, Sidak's multiple comparison after two-way ANOVA RM).

(D) The feeding behavior duration in the light phase and dark phase in non-lactating female mice and dams. Data were averaged for any 14 days in non-lactating female mice and for post-delivery 13 days (day 1 to day 13) in dams. In a stacked bar chart, the feeding behavior time in the light phase was shown by white areas, and that in the dark phase was shown by gray areas (non-lactating multiparous female mice; light phase: 37.6 ± 10.0 min, dark phase: 100.4 ± 16.4 min, n = 4, mother mouse; light phase: 193.8 ± 37.5 min, dark phase: 158.6 ± 14.0 min, n = 5, **$p < 0.01$ for non-lactating mice in light phase vs. mother mice in light phase, Sidak's multiple comparison after two-way ANOVA RM).

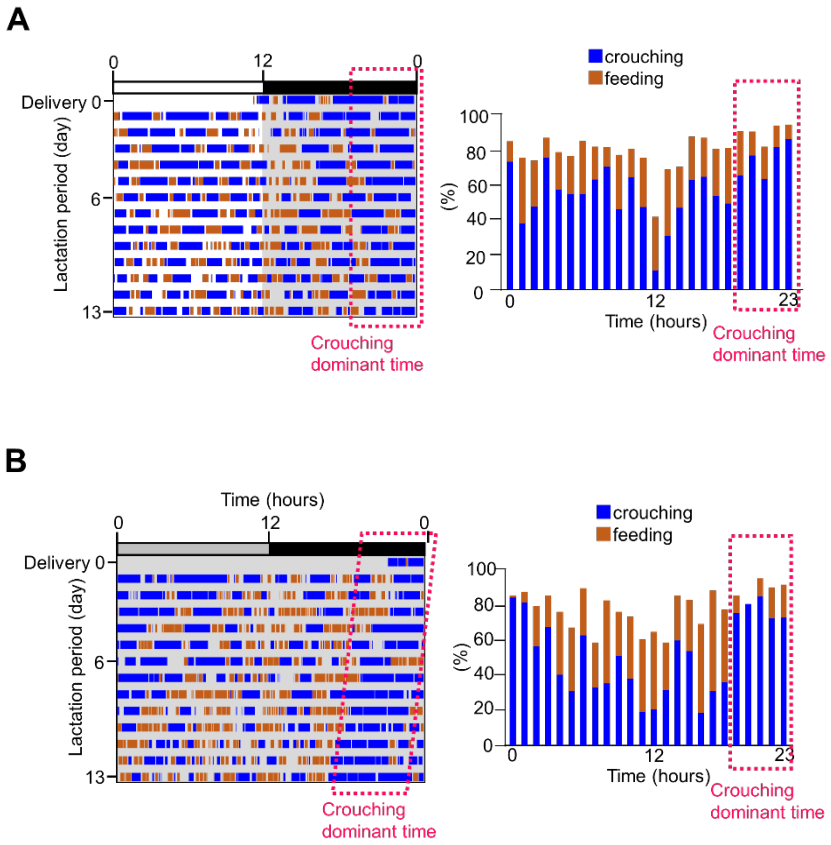

Figure 16. Dams spent over 80% of their day for feeding and crouching behavior

(A, B) Left; actograms merged with the crouching actogram (blue bars) and feeding behavior actogram (orange bars) of WT dam under LD (A) and DD (B). The vertical axis shows the post-delivery days, and the horizontal axis shows time (A; Time 0 corresponds to ZT0 (lights on, 8:00 a.m.), B; Time 0 corresponds to 8:00 a.m.). A crouching-dominant time was indicated by pink rectangle.

Right; Stacked graphs of crouching and feeding behavior at each hour. The horizontal axis shows time (A; Time 0 corresponds to ZT0 (lights on, 8:00 a.m.), B; Time 0 corresponds to 8:00 a.m.). Values were averaged over 10 days from day 4 to day 13 for LD (A) and over 5 days from day 4 to day 8 for DD (B, due to crouching being free-run and shifted). Crouching-dominant time was defined as a period in which more than 60 % of crouching lasted more than 3 consecutive hours. During the lactation period, dams spent 80 to 90 % of the 24-h period on either crouching or feeding behaviors every day.

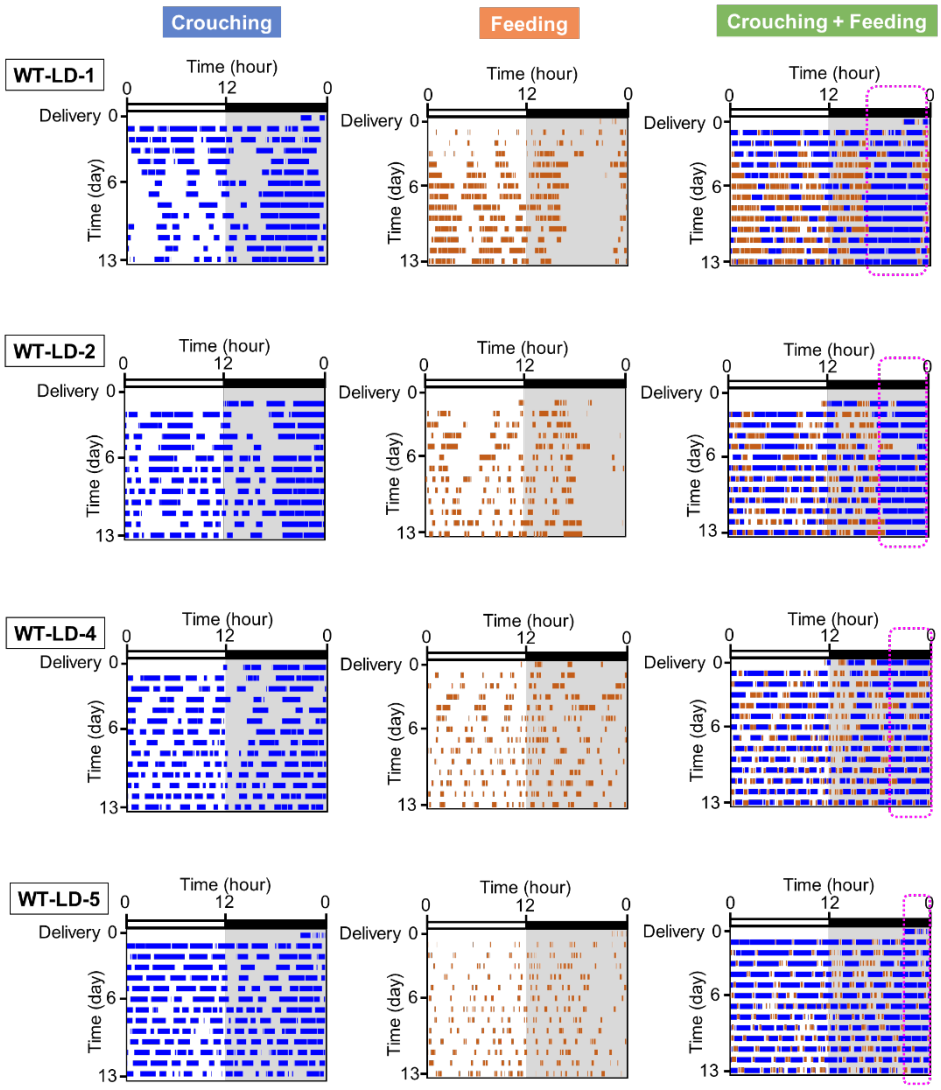

Figure 17. Actogram of crouching and feeding behavior of wild-type dams under LD

Actogram of crouching behavior, feeding behavior, and merged in WT dams (WT-LD2, WT-LD3, WT-LD4, WT-LD5, crouching actogram is same as that in Figure 6, and WT-LD1: Figure. 16A) under LD cycles. The vertical axis of the actogram indicates the number of days from the delivery date, defined as day 0. The horizontal axis indicates Zeitgeber time. Time 0 corresponds to ZT0 (lights on, 8:00 a.m.). In crouching actograms, blue bars show the crouching time, and in feeding actograms, orange bars show the feeding time for more than 1 min. The pink dashed open square indicates crouching-dominant time, defined as a period in which more than 60% of crouching lasted more than 3 consecutive hours. WT-LD2 missed day 0-1 data owing to a camera malfunction.

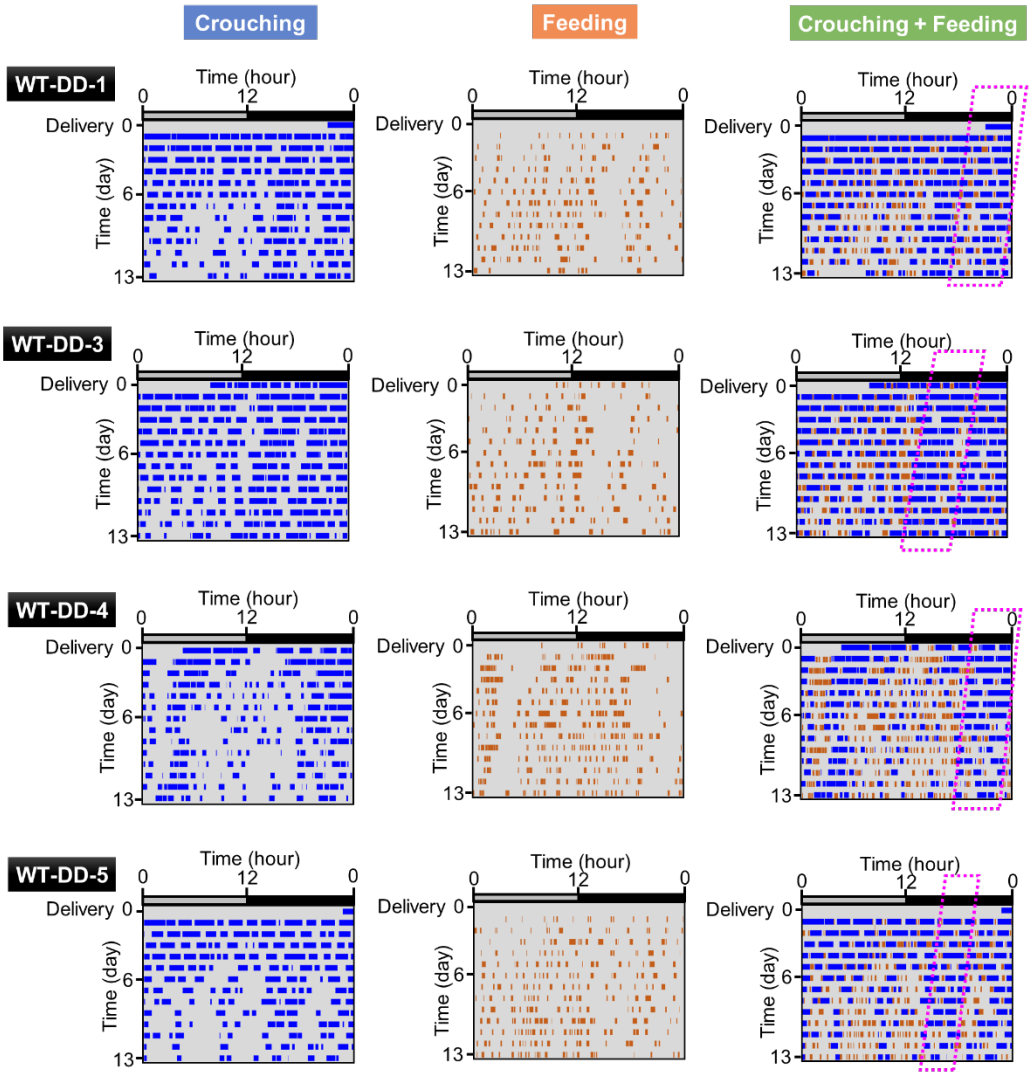

Figure 18. Actogram of crouching and feeding behavior of Wild-type dams under DD

Actogram of crouching behavior, feeding behavior, and merged in WT dams (WT-DD1, WT-DD3, WT-DD4, WT-DD5, crouching actogram is same as that in Figure 6, and WT-LD2: Figure. 16B) under DD cycles. The vertical axis of the actogram indicates the number of days from the delivery date, defined as day 0. The horizontal axis indicates time (hours), with 8:00 a.m. defined as Time 0. In crouching actograms, blue bars show the crouching time, and in feeding actograms, orange bars show the feeding time for more than 1 min. The pink dashed open square shows crouching-dominant time, defined as a period in which more than 60% of crouching lasted more than 3 consecutive hours.

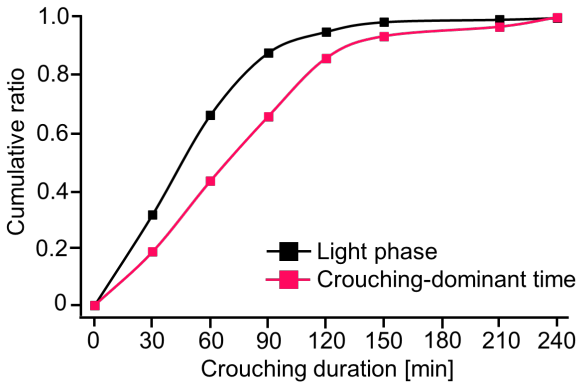

Figure 19. The crouching time for one event was longer in crouching-dominant time than in light phase

Cumulative probability of duration of a crouching event in light phase (ZT0~12) and crouching-dominant time (ZT16~24). The horizontal axis indicates the cumulative ratio of crouching duration for a single event. The black line indicates the data in the light phase; the pink line represents that in the crouching-dominant time (light phase; 356 events, crouching-dominant time; 183 events from 5 mother mice, $p<0.01$, paired Kolmogorov-Smirnov test).

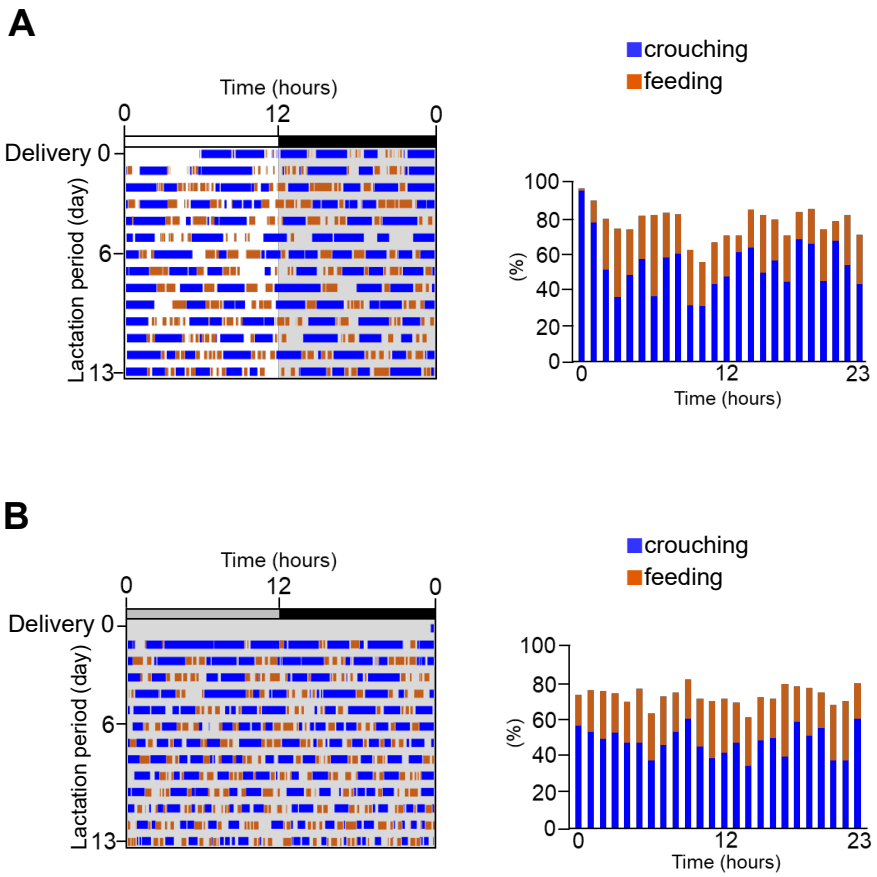

Figure 20. *Per*-null mother didn't show crouching-dominant time

(A, B) Left; actograms merged with the crouching actogram (blue bars) and feeding behavior actogram (orange bars) of *Per*-null dams under LD (A) and DD (B). The vertical axis shows the post-delivery days, and the horizontal axis shows time (A; Time 0 corresponds to ZT0 (lights on, 8:00 a.m.), B; Time 0 corresponds to 8:00 a.m.). A crouching-dominant time was indicated by pink rectangle.

Right; Stacked graphs of crouching and feeding behavior at each hour. The horizontal axis shows time (A; Time 0 corresponds to ZT0 (lights on, 8:00 a.m.), B; Time 0 corresponds to 8:00 a.m.). Values were averaged over 10 days from day 4 to day 13 for LD (A) and over 5 days from day 4 to day 8 for DD (B, due to crouching being free-run and shifted).

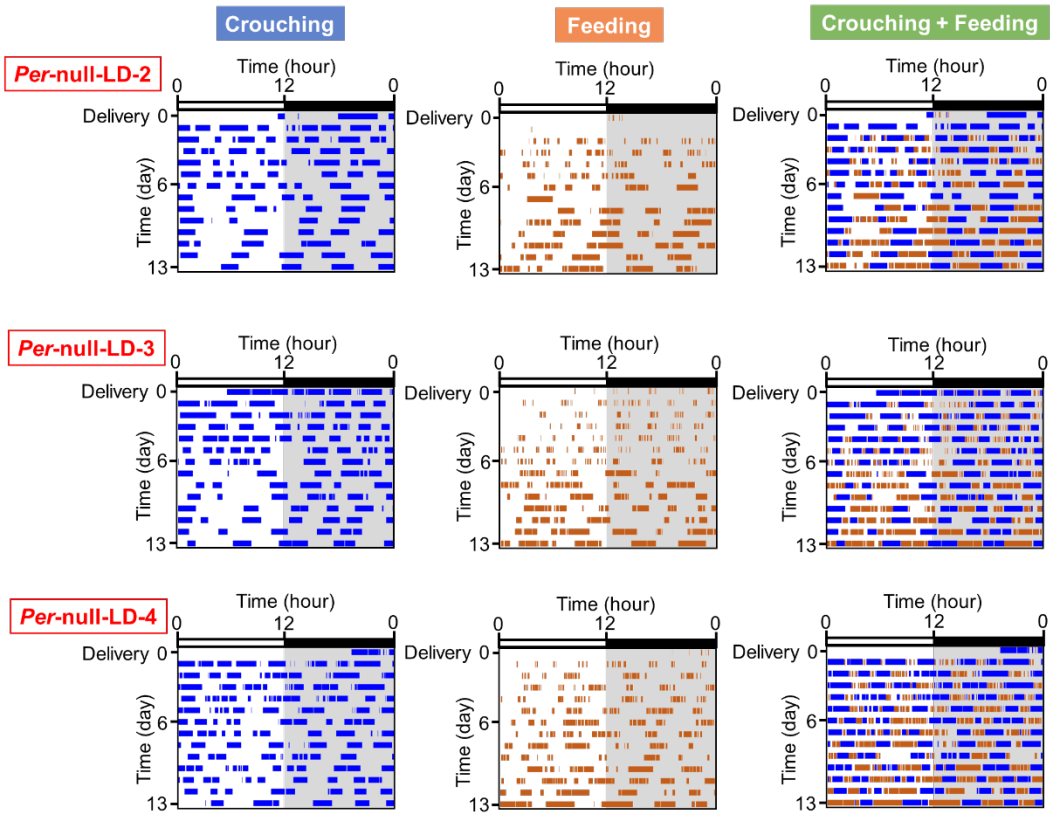

Figure 21. Actogram of crouching and feeding behavior of *Per*-null dams under LD

Actogram of crouching behavior, feeding behavior, and merged in *Per*-null dams (*Per*-null-LD2, *Per*-null-LD3, *Per*-null-LD4, crouching actogram is the same as that in Figure 8, and *Per*-null-LD1: Figure. 19A) under LD. The vertical axis of the actogram indicates the number of days from the delivery date, defined as day 0. The horizontal axis indicates Zeitgeber time. Time 0 corresponds to ZT0 (lights on, 8:00 a.m.). In crouching actograms, blue bars show the crouching time, and in feeding actograms, orange bars show the feeding time for more than 1 min. The pink dashed open square indicates crouching-dominant time. There wasn't crouching-dominant time in *Per*-null dams.

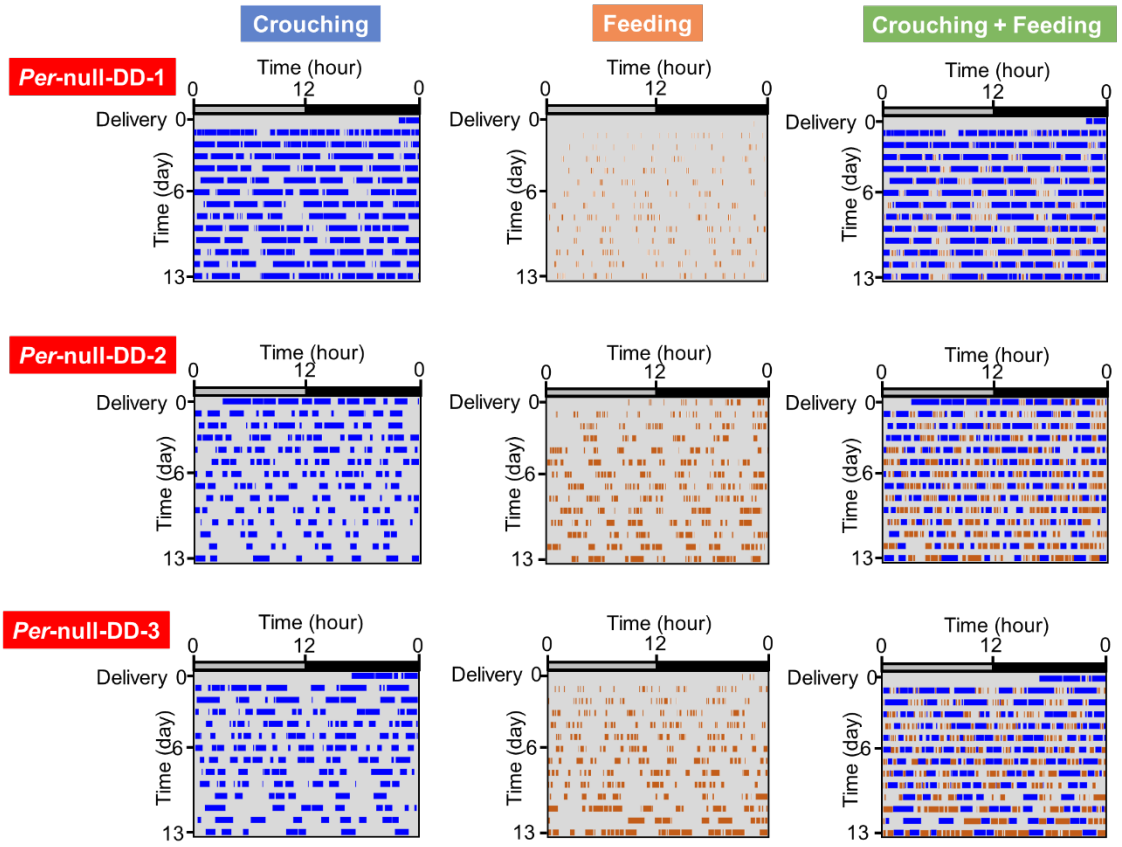

Figure 22. Actogram of crouching and feeding behavior of *Per*-null dams under DD

Actogram of crouching behavior, feeding behavior, and merged in *Per*-null dams (*Per*-null-DD1, *Per*-null-DD2, *Per*-null-DD3, crouching actogram is the same as that in Figure 8, and Per-null-DD4: Figure 19B) under DD. The vertical axis of the actogram indicates the number of days from the delivery date, defined as day 0. The horizontal axis indicates time (hours), with 8:00 a.m. defined as Time 0. In crouching actograms, blue bars show the crouching time, and in feeding actograms, orange bars show the feeding time for more than 1 min. There wasn't crouching-dominant time in *Per*-null dams.

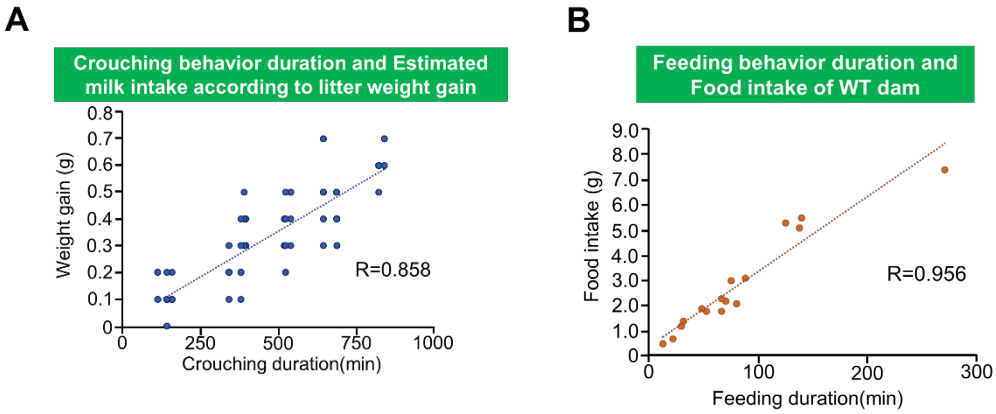

Figure 23. The results of video analysis were correlated with estimated milk intake of pups or food intake

(A) Correlation between crouching behavior duration and estimated milk intake of pups. The amount of estimated milk intake was according to litter weight gain. The vertical axis represents the weight gain of pups (20 pups from 3 dams, 5 points per pup), and the horizontal axis indicates crouching time determined by video analysis. The straight line represents linear regression, and R is the correlation coefficient (slope = 0.0007, intercept = 0.0127, R = 0.8584).

(B) Correlation between feeding behavior duration and food intake of lactating dams. The vertical axis represents the food intake of lactating mother mice (4 sections from 3 dams), and the horizontal axis indicates time of feeding behavior determined by video analysis. The straight line represents linear regression, and R is the correlation coefficient (slope = 0.0295, intercept = 0.4426, R = 0.9556).

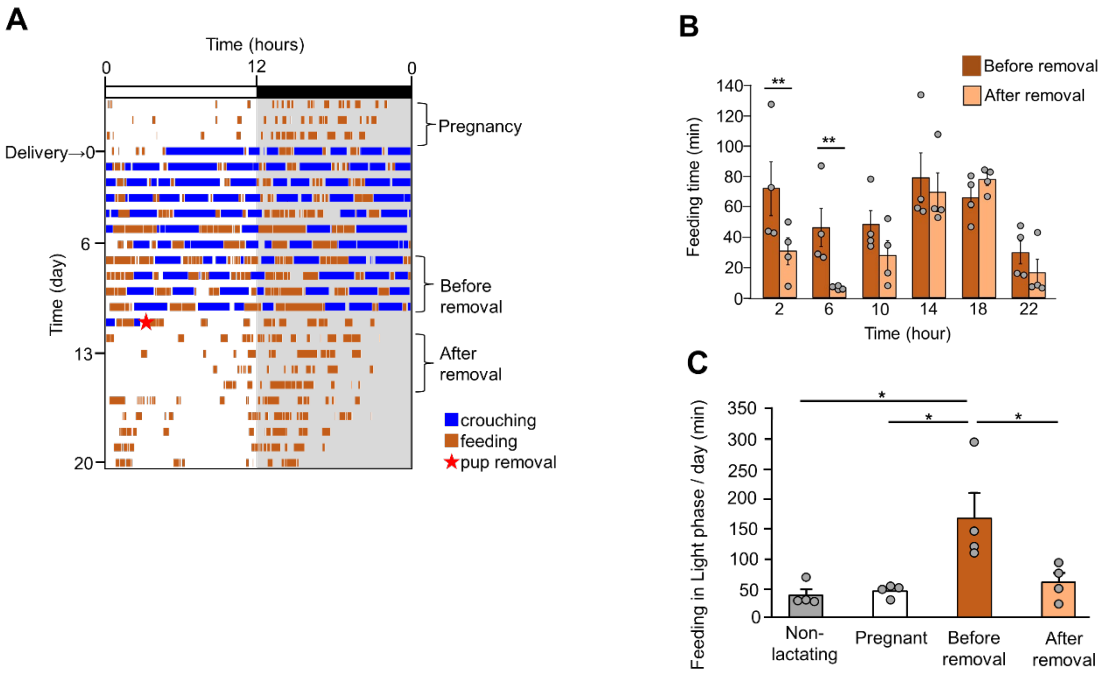
A
Time (hours)
0
12
0
Delivery→0
6
13
20
Time (day)
Pregnancy
Before removal
After removal
crouching
feeding
pup removal
B
Before removal
After removal
Feeding time (min)
140
120
100
80
60
40
20
0
**
**
2
6
10
14
18
22
Time (hour)
C
Feeding in Light phase / day (min)
350
300
250
200
150
100
50
0
*
*
*
Non-lactating
Pregnant
Before removal
After removal

Figure 24. Removal of pups reduced feeding behavior in light phase of dams

(A) The actogram of the crouching (blue bars) and feeding (orange bars) behaviors with pups removal at day 11 post-delivery. The vertical axis shows the number of days from the delivery (day 0), and the horizontal axis shows Zeitgeber time (ZT 0 corresponds to lights on, 8:00 a.m.). The blue bars indicate the time of crouching, the orange bars indicate the time of feeding behavior. Pups were removed at time shown by the red star in the actogram. A drastic change occurred in the daily pattern of feeding behavior after the removal of pups.

(B) Duration of feeding behavior before and after the removal of pups. The vertical axis shows the time spent on feeding behavior over a 4-hour period. The horizontal axis indicates time of midpoint of 4-hour duration. The duration of feeding behavior for 4-days of before and after pups removal was calculated. Each point represents an individual data point (mean ± SE, n=4, **p<0.01, Sidak's multiple comparison after two-way ANOVA RM).

(C) Feeding behavior in light phase of non-lactating female mice, pregnant mice, and dams before and after pup removal. After pups were removed, the time of feeding behavior during the light phase was comparable to that of non-lactating female mice or during pregnancy (Non-lactating female mice; 37.6 ± 10.0 min, Pregnant; 44.1 ± 5.2 min, Before removing pups; 166.8 ± 43.3 min, After removing pups; 58.9 ± 15.4 min, n = 4, *p < 0.05, One-way ANOVA with Sidak's multiple comparison).

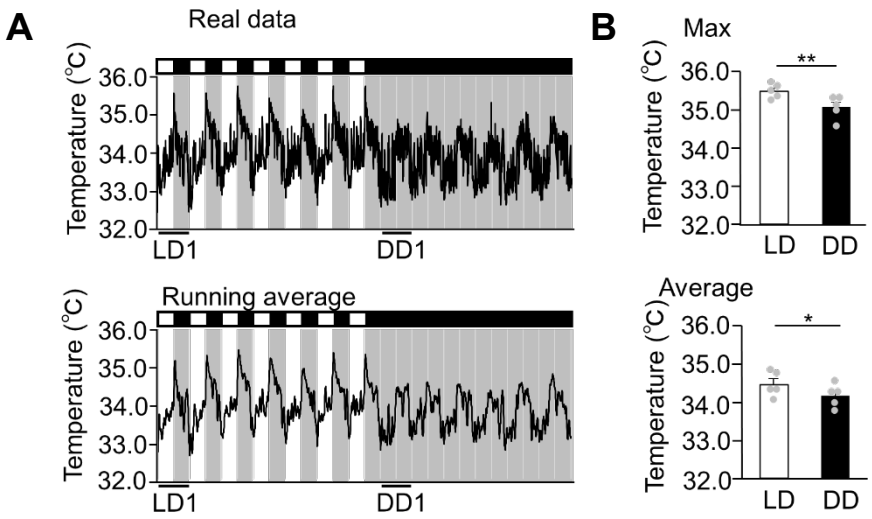

Figure 25. The body temperature of virgin mice exhibits circadian rhythms

(A)Body temperature of a virgin female mouse when transferred from LD into DD. Representative real data (upper) and its 65-min moving average data (bottom). Gray backgrounds depict dark phases.

(B)The comparison of max body temperature (upper) and average body temperature (bottom) under LD and DD (n = 5). The max and average values of each day were calculated and averaged over 7 days for the LD cycles and 5 days for the DD. The calculation was performed with the running average (Max value; LD: 35.5 ± 0.04 ℃, DD: 35.1 ± 0.10 ℃, average; LD: 34.5 ± 0.15 ℃, DD: 34.2 ± 0.14 ℃, *$p < 0.05$, **$p < 0.01$ by paired t-test).

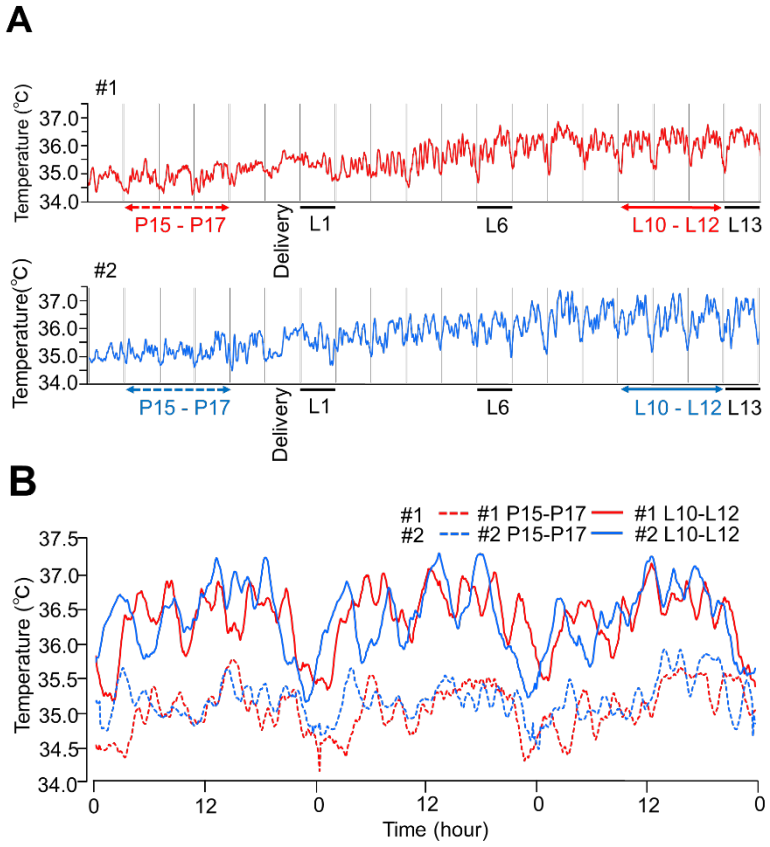

Figure 26. The body temperature increased during the lactation period

(A) Body temperature from late pregnancy to day 13 after delivery under DD in two dams. The horizontal axis indicates days before and after delivery. Gray vertical lines are drawn every 24 h (The period length of these mice was 24 h). P: Pregnancy, L: lactation.

(B) Expanded graph during P15-17 and L10-12 in (A). The horizontal axis represents time (Time 0 corresponds to 8:00 a.m.). The dashed lines indicate body temperature during pregnancy, and the solid lines indicate body temperature during lactation. Body temperature during lactation was higher than that during late pregnancy.

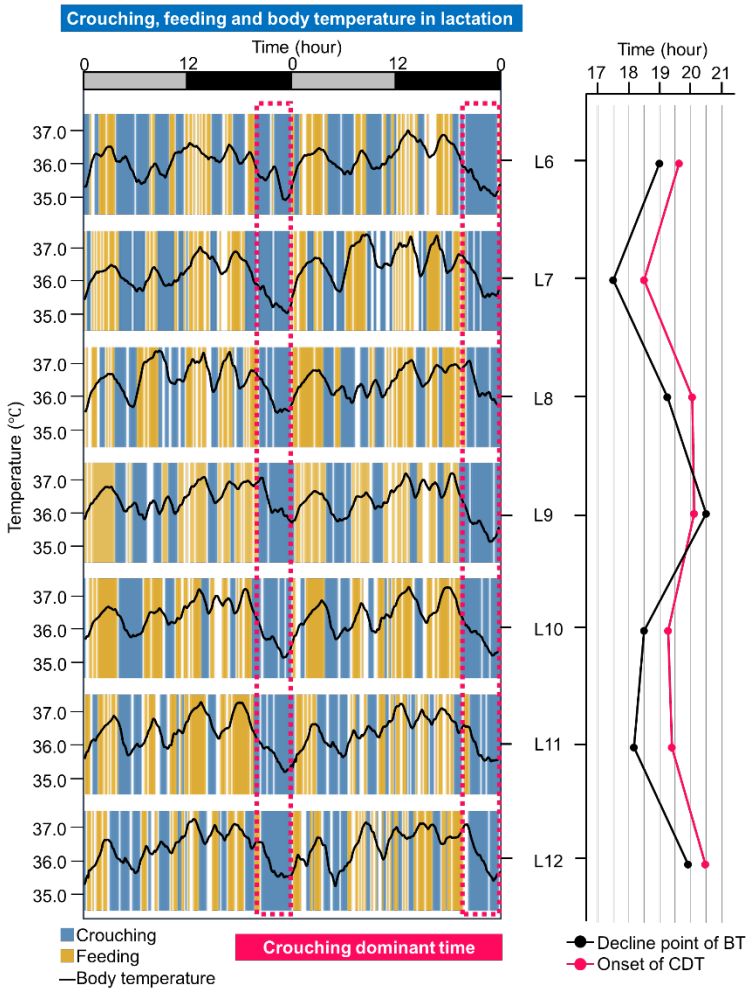

Figure 27. The times for crouching dominance and a decrease in body temperature corresponded

The actogram of crouching behavior and feeding behavior and graph of body temperature for L6-L12 were overlaid (left). The blue bars represent crouching, the orange bars represent feeding, and black lines represent body temperature, pink rectangle represents crouching-dominant time. The horizontal axis indicates time (Time 0 corresponds to 8:00 a.m.). The onset of crouching-dominant time (CDT) and decline point of body temperature (right). The decline point of body temperature refers to the highest temperature observed during the crouching-dominant time. The vertical light gray lines are drawn every 0.5-hour. The black line represents decline point of body temperature and the pink line represents the onset of crouching-dominant time.

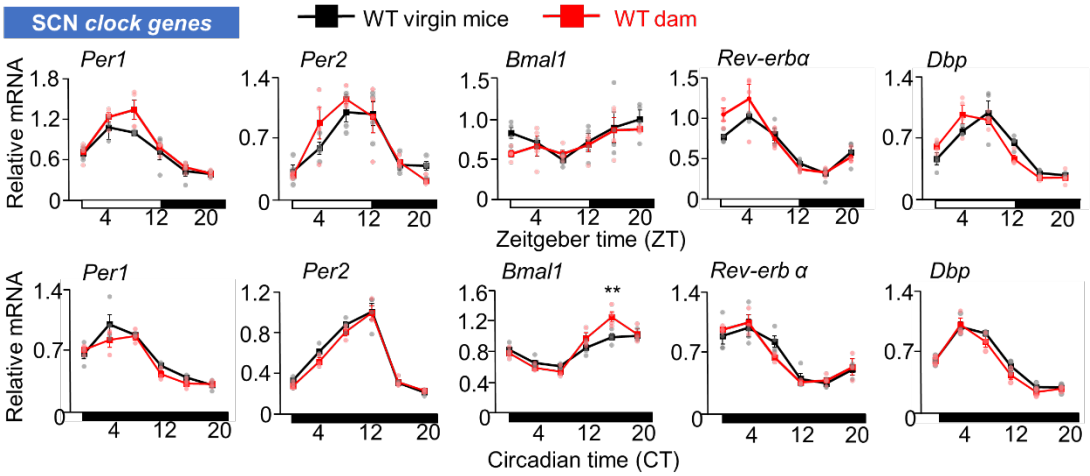

Figure 28. Clock genes oscillate in dams' SCN as well as virgin mice

Relative expression level of clock genes in the central clock SCN under LD and DD measured by real-time qPCR. Brains, including the SCN, were collected every 4 hours under LD and DD. The expression profiles in virgin mice were indicated by the black line, and those in dams were indicated by the red line. The peak of gene expressions in virgin mice was set to 1 (mean ± SEM, n = 3-4 for each time point, *p < 0.05, **p < 0.01, Sidak's multiple comparison after Two-way ANOVA).

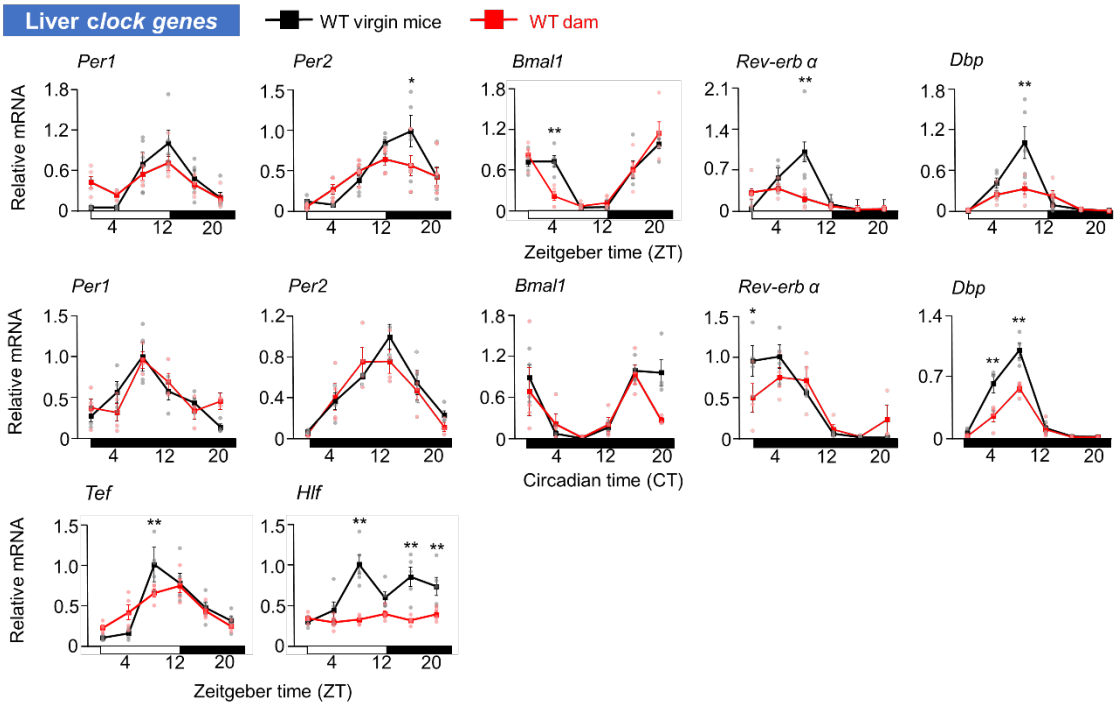

Figure 29. The expression of clock genes in the liver of dams

Relative circadian expression of clock genes in the liver of virgin mice (black) versus dams (red) under LD and DD measured by real-time qPCR. Liver was collected every 4 h under LD and DD, and qPCR was conducted. Each points represent individual data, and the peak of gene expression in virgin mice was set to 1 (mean ± SEM, n = 5 for each point of the LD cycle, n=3-4 for each time point under DD, *p < 0.05, **p < 0.01, Sidak's multiple comparison after two-way ANOVA).

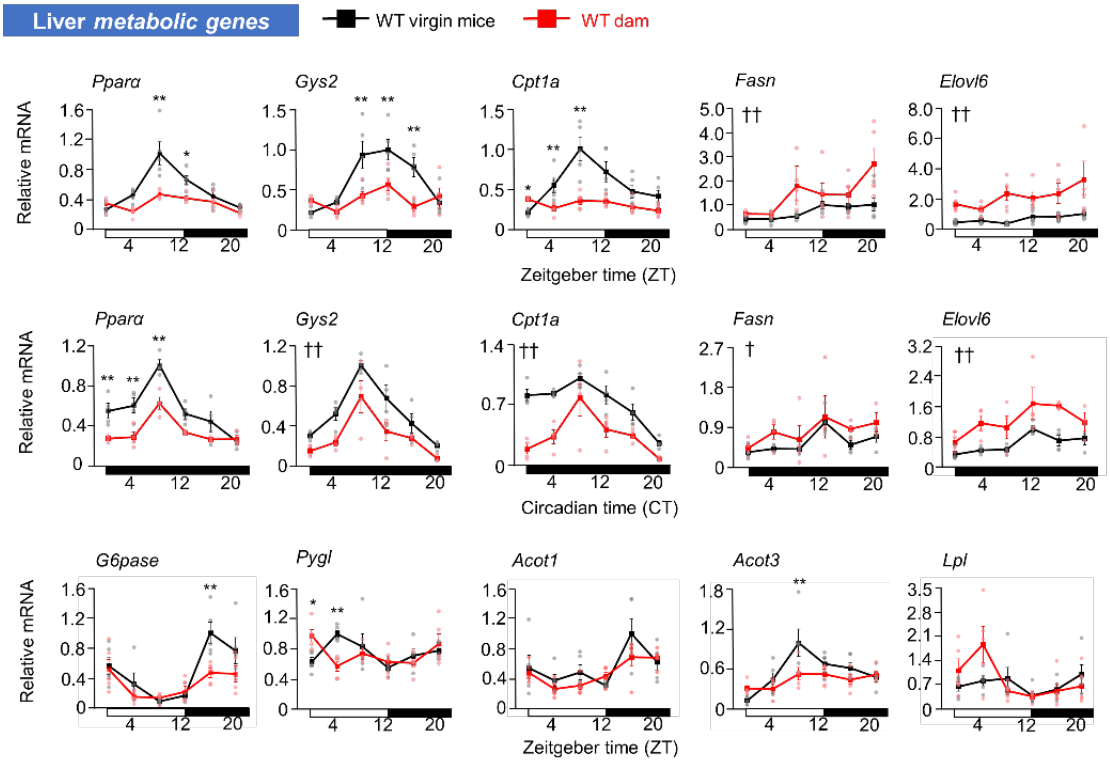

Figure 30. The expression of metabolism-related genes in dams

Relative circadian expression of metabolism-related genes in the liver of virgin mice (black) versus dams (red) under LD and DD measured by real-time qPCR. Sampling was performed every 4 h under LD and DD. Each points represent individual data, and the peak of gene expression in virgin mice was set to 1 (mean ± SEM, n = 5 for each point of LD cycle, n = 3-4 for each time point under DD, *p < 0.05, **p < 0.01, Sidak's multiple comparison after two-way ANOVA, †p < 0.05, ††p < 0.01, virgin mice vs. dam based on Two-way ANOVA).

Table 1. Statistics of gene expression rhythm related to Figures 28, 29 and 30

			Two-way ANOVA						Sidak's multiple comparisons test						compareRhythms							
			Time × condition		time		condition		Time (ZT in LD, CT in DD)						category	virgin			dam			adj_p_val
tissue	light	gene	F(DFn, DFd)	p value	F(DFn, DFd)	p value	F(DFn, DFd)	p value	0	4	8	12	16	20		phase	amplitude	adj_p_val	phase	amplitude	adj_p_val	_dodr
SCN	LD	Per1	F(5,35)=1.445	0.2328	F(5,35)=39.75	<0.0001	F(1,35)=6.229	0.0174							same	1.588677	0.7303185	3.96E-07	1.684591	0.9942471	2.32E-09	0.5263625
		Per2	F(5,35)=0.9758	0.4461	F(5,35)=23.31	<0.0001	F(1,35)=0.2364	0.6299							same	2.406092	0.7509746	5.11E-05	2.223563	0.9972942	3.04E-08	0.2910458
		Bmal1	F(5,35)=0.6052	0.6963	F(5,35)=4.244	<0.0040	F(1,35)=1.408	0.2434							same	5.108812	0.4518986	5.11E-05	4.584457	0.3007237	0.029775	0.2910458
		Rev-erb α	F(5,35)=2.183	0.0784	F(5,35)=34.88	<0.0001	F(1,35)=1.733	0.1966							same	0.951698	0.6553664	1.59E-10	0.770899	0.9562418	2.99E-09	0.098062
		Dbp	F(5,35)=2.568	0.0442	F(5,35)=43.16	<0.0001	F(1,35)=0.01196	0.9135	0.5097	0.2681	0.9232	0.2002	0.9886	0.9998	change	1.850229	0.7383611	1.3E-07	1.42591	0.8232819	1E-08	0.0096154
	DD	Per1	F(5,32)=1.325	0.2787	F(5,32)=53.21	<0.0001	F(1,32)=3.06	0.0899							same	1.421251	0.7023003	3.14E-10	1.290501	0.6290622	1.01E-05	0.4283415
		Per2	F(5,32)=0.7313	0.6052	F(5,32)=148.1	<0.0001	F(1,32)=2.578	0.1182							same	2.363697	0.7988498	1.27E-11	2.448558	0.7521617	8.26E-11	0.7189116
		Bmal1	F(5,32)=4.376	0.0038	F(5,32)=46.42	<0.0001	F(1,32)=2.116	0.1556	0.9772	0.9353	0.8978	0.4019	0.0008	0.9999	change	4.716163	0.4308005	9.6E-08	4.439027	0.7044786	3.39E-09	0.0141589
		Rev-erb α	F(5,32)=1.037	0.413	F(5,32)=37.87	<0.0001	F(1,32)=0.006788	0.9348							same	0.901348	0.719699	1.63E-07	0.641426	0.7493936	7.74E-06	0.1169826
		Dbp	F(5,32)=0.9689	0.4515	F(5,32)=133.6	<0.0001	F(1,32)=4.069	0.0521							same	1.481979	0.7780267	2.38E-09	1.343921	0.7814172	3.39E-09	0.1060288
Liver	LD	Per1	F(5,48)=2.724	0.0302	F(5,48)=14.63	<0.0001	F(1,48)=0.002482	0.9605	0.0784	0.7437	0.8987	0.2789	0.9879	>0.9999	change	3.102269	0.9538257	2.39E-09	2.812416	0.3849877	0.000126	0.0188004
		Per2	F(5,48)=3.028	0.0187	F(5,48)=19.35	<0.0001	F(1,48)=1.439	0.2361	0.9971	0.6099	0.9365	0.5621	0.0108	>0.9999	change	3.763151	0.9459646	9.16E-10	3.390288	0.5285317	3.2E-07	0.0188004
		Bmal1	F(5,48)=4.066	0.0037	F(5,48)=42.23	<0.0001	F(1,48)=0.3578	0.5526	0.9617	0.0006	>0.9999	0.9964	>0.9999	0.7342	same	5.747922	0.9289332	2.24E-08	5.401452	1.0986095	1.64E-09	0.0659389
		Dbp	F(5,48)=6.603	<0.0001	F(5,48)=21.07	<0.0001	F(1,48)=6.236	0.016	>0.9999	0.5968	<0.0001	0.8094	>0.9999	>0.9999	same	1.887309	0.8352578	5.24E-11	2.081913	0.3515884	3.3E-08	0.5928034
		Rev-erb α	F(5,48)=7.648	<0.0001	F(5,48)=14.35	<0.0001	F(1,48)=5.016	0.0298	0.2094	0.6954	<0.0001	>0.9999	>0.9999	>0.9999	change	1.716249	0.9753781	5.4E-07	0.973534	0.5172429	4.45E-09	0.0038475
		Tef	F(5,48)=4.361	0.0024	F(5,48)=31.4	<0.0001	F(1,48)=0.205	0.6528	0.7493	0.0703	0.0048	0.9998	0.9991	0.9823	same	2.870584	0.8165613	2.66E-10	2.70822	0.53844	3.94E-08	0.210687
		Hlf	F(5,48)=6.714	<0.0001	F(5,48)=6.738	<0.0001	F(1,48)=55.21	<0.0001	0.9989	0.6614	<0.0001	0.2825	<0.0001	0.009	same	3.329874	0.4369152	0.000686	4.307376	0.0540368	0.648456	0.0659389
		Ppar α	F(5,48)=6.919	<0.0001	F(5,48)=19.05	<0.0001	F(1,48)=28.74	<0.0001	0.8979	0.0585	<0.0001	0.0245	0.9338	0.9489	loss	2.433279	0.6394999	1.83E-09	2.754706	0.1765323	3.6E-05	0.0466313
		Gys2	F(5,48)=5.861	0.0003	F(5,48)=11.49	<0.0001	F(1,48)=19.39	<0.0001	0.7869	0.9173	0.0009	0.0054	0.0012	0.987	loss	3.036419	0.8769658	1.03E-08	3.335879	0.1587651	0.004507	0.0188004
		Pygl	F(5,48)=4.625	0.0016	F(5,48)=2.426	0.0486	F(1,48)=0.1225	0.7278	0.0358	0.0048	0.9756	0.9879	0.9609	0.9727	same	0.983463	0.2431538	0.001065	5.928005	0.2826059	0.054386	0.4635132
		G6pase	F(5,48)=2.482	0.0445	F(5,48)=12.71	<0.0001	F(1,48)=7.262	0.0097	0.9998	0.8272	0.9998	0.9996	0.0036	0.2092	same	5.03339	0.8243054	1.41E-07	5.206181	0.4198104	0.000937	0.5719726
		Cpt1a	F(5,48)=6.703	<0.0001	F(5,48)=7.995	<0.0001	F(1,48)=35.95	<0.0001	0.5098	0.0433	<0.0001	0.0045	0.3453	0.4139	loss	2.494713	0.6292336	2.24E-08	1.997511	0.0647048	0.259916	0.0310833
		Fasn	F(5,48)=1.553	0.1916	F(5,48)=5.928	0.0002	F(1,48)=14.37	0.0004							same	4.245738	0.7678101	0.000105	4.232717	1.1077516	0.004507	0.8541222
		Elovl6	F(5,48)=0.6162	0.688	F(5,48)=1.54	0.1951	F(1,48)=35.34	<0.0001							same	4.496474	0.5369548	0.000416	4.234413	1.0063618	0.373565	0.3992956
		Acot1	F(5,48)=1.184	0.3308	F(5,48)=7.529	<0.0001	F(1,48)=1.863	0.1786							same	4.695018	0.444713	0.005502	4.740534	0.4626184	2.66E-05	0.5719726
		Acot3	F(5,48)=2.764	0.0284	F(5,48)=8.157	<0.0001	F(1,48)=4.933	0.0311	0.6894	0.889	0.0052	0.8039	0.7416	>0.9999	same	2.823819	0.6299726	2.72E-07	3.501953	0.2081294	0.054386	0.0659389
		Lpl	F(5,48)=2.364	0.0537	F(5,48)=3.534	0.0084	F(1,48)=0.7532	0.3898							same	0.229416	0.3144594	0.069915	0.611226	1.2303542	0.005705	0.3992956
	DD	Per1	F(5,35)=1.778	0.1431	F(5,35)=13.2	<0.0001	F(1,35)=0.1963	0.6604							same	2.235899	0.7274915	2.13E-07	2.486949	0.5061624	0.003638	0.7717886
		Per2	F(5,35)=1.062	0.3981	F(5,35)=22.22	<0.0001	F(1,35)=0.707	0.4061							same	3.001999	0.816413	2.51E-11	2.699698	0.7850729	2.29E-06	0.7717886
		Bmal1	F(5,35)=1.807	0.1371	F(5,35)=13.62	<0.0001	F(1,35)=2.089	0.1572							same	5.141724	1.1965144	1.41E-07	5.023569	0.6624866	3.84E-05	0.7717886
		Dbp	F(5,35)=8.159	<0.0001	F(5,35)=79.43	<0.0001	F(1,35)=24.57	<0.0001	0.9958	<0.0001	<0.0001	>0.9999	>0.9999	>0.9999	same	1.746955	0.9406249	3.97E-10	1.905672	0.503404	2.2E-08	0.7717886
		Rev-erb α	F(5,35)=2.531	0.0467	F(5,35)=21.98	<0.0001	F(1,35)=0.4905	0.4884	0.0435	0.5328	0.922	0.9998	>0.9999	0.7557	same	0.873626	1.1561839	1.82E-09	1.126947	0.7816848	1.63E-06	0.7717886
		Ppar α	F(5,35)=2.933	0.0258	F(5,35)=22.79	<0.0001	F(1,35)=45.26	<0.0001	0.0078	0.0018	0.0001	0.1427	0.1664	>0.9999	same	1.8963	0.5627488	2.13E-07	2.172645	0.2791829	0.0006	0.7717886
		Gys2	F(5,35)=0.7011	0.6263	F(5,35)=20.61	<0.0001	F(1,35)=25.5	<0.0001							same	2.324218	0.7057588	6.27E-10	2.380129	0.4823153	2.29E-06	0.7717886
		Cpt1a	F(5,35)=1.728	0.154	F(5,35)=12.46	<0.0001	F(1,35)=47.1	<0.0001							same	1.884358	0.5794572	4.42E-08	2.344585	0.5536111	1.91E-05	0.1206788
		Fasn	F(5,35)=0.1884	0.965	F(5,35)=3.048	0.0218	F(1,35)=4.494	0.0412							same	3.649336	0.4431793	0.004746	3.694924	0.4490984	0.421498	0.7717886
		Elovl6	F(5,35)=0.6214	0.6843	F(5,35)=5.048	0.0014	F(1,35)=29.61	<0.0001							same	3.782537	0.5418494	0.000377	3.548601	0.8658948	0.001855	0.7717886

To assess gene expression rhythms, we conducted sidak's multiple comparison after two-way ANOVA and compareRhythms (http:// github.com/bharathananth/compareRhythms). This table contains F value and P value of two-way ANOVA, P value of Sidak's multiple comaprion, and results of compareRhythms (phase, amplitude, adjusted p-values for the rhythmicity of both virgin and dam, adjusted p value of differential rhythmicity, category).

V. Discussion

i. Circadian rhythm of maternal and feeding behavior

During the lactation period, dams have to satisfy the growing energy demand while raising pups, which is crucial for the species' survival. In this study, we conducted long-term video recordings of dams' behavior to clarify the time distribution of maternal behavior and feeding behavior during the lactation period.

The amount of locomotor activity of lactating dams was decreased both in LD condition and DD condition (Figure 2,3). A result that has been reported in rats and other strains of mice in LD [35, 56, 57], we found that dams remained in the nest to care for their pups in the dark phase, while virgin mice actively moved around. And they spent most of their time on maternal and feeding behaviors.

Although the voluntary movement rhythm became unclear, maternal behavior showed a clear rhythm. It is known that maternal behavior is quickly triggered by the pup's stimuli after birth when the dam's endocrine status is ready during gestation [18, 48]. Also, in this study, dams performed the crouching posture immediately after delivery and continuously kept this behavior for several days. Thereafter, a diurnal rhythm in crouching behavior gradually began to appear (Figure 5A, 6; left). We confirmed diurnal rhythm in crouching behavior by video analysis, which is consistent with previous reports [27-29]. Furthermore, we also clarified that there is a diurnal rhythm of crouching behavior in the DD condition in this study (Figure 5B, 6; right). The period is slightly shorter than 24 hours under DD conditions, about 23.7 hours, which is consistent with the characteristics of mouse locomotor activity [7]. Moreover, we also observed in *Per*-null dams. As a result, *Per*-null dams didn't exhibit rhythmicity of crouching behavior in both LD and DD condition (Figure7,8). Compared crouching duration every 4 hours, *Per*-null

represented crouching at the same level throughout a day, in contrast, wild-type exhibited intensive crouching in the late half of the dark phase (the expected time for this in DD) (Figure 9). These results suggest that the oscillation of the circadian clock, regulated by clock genes, drives the circadian rhythm of maternal behavior.

Then, which circadian clock, dam's or pups', contributes to the rhythmicity of maternal behavior? When both wild-type and *Per*-null dam reared their own *Per*-hetero pups under DD conditions, crouching behavior of wild-type mice represented a 23.7-hour period, while *Per*-null mice didn't show any periodicity (Figure 13). They showed the same results as when they raised pups of the same genotype, clarifying that the rhythmicity of crouching behavior is regulated by the dams' circadian clock.

Next, the feeding behavior of the lactating mice was significantly increased, and it was revealed that the feeding pattern was changed compared to when they were not raising their pups (Figure 14). In the first half of the dark phase, they fed a lot, as well as non-lactating mice (Figure 16, 17), in the late half of the dark phase, they spent a large time in crouching (crouching-dominant time). Dams need a huge amount of energy to produce milk. To meet these increased energy demands, it is thought that secretion of leptin is decreased, as a result feeding time is increased [33, 56]. Because feeding behavior is decreased soon after removal of pups, it is considered that the existence of pups is cue for increasing feeding (Figure 24).

In the previous reports, when food is available ad libitum, feeding behavior in lactating rats is more frequent at night, and maternal behavior is concentrated during the daytime [28, 57]. Rats can be conducted under restricted feeding only during the daytime, then maternal behavior is observed at night, and the relationship between feeding time and the timing of maternal behavior is antiphase [57]. In mice, when we performed restricted

feeding during the dark phase, cannibalism occurred, and we couldn't carry out the experiments. From these results, mice, especially the BALB/c strain, are thought not to be able to engage in prolonged feeding behavior in the light or dark phase like rats; they need to eat throughout the day. These differences can be explained the difference in the amount of food that can be ingested at one time between rats and mice. The size of an animal's internal organs and metabolic rate are generally proportional to its body weight [58, 59]. The weight of rats is 10 times that of mice, rats have stomachs that are about 10 times larger, and metabolic rates about 10 times higher than those of mice [60]. Since rats can ingest throughout the night, and the contents of their stomachs serve as a reservoir of nutritional needs during the resting period [61], they can adapt to increased food demand during lactation by continuous nocturnal feeding. On the other hand, mice are not capable of prolonged feeding behavior because their stomachs are smaller than rats. Therefore, the BALB/c mice in this study took food intake during the first half of the night and immediately gave the digested and absorbed nutrients to the pups during the second half of the night.

Both maternal and feeding behaviors, which are prominent in lactating dams, are motivated behaviors for their survival and their pups [48, 62-64]. Maternal behavior is regulated by hormone such as prolactin and oxytocin [65-67] in brain regions such as the medial preoptic area (MPOA) [48, 68], the anteroventral periventricular nucleus (AVPV) [69], the paraventricular thalamus (PVT) [65], and the hypothalamic paraventricular nucleus (PVH) [69]. The lateral hypothalamic area (LHA) and the arcuate nucleus (ARC) are essential brain regions in feeding behavior [64, 70, 71]. These brain regions may be controlled by the central clock in the SCN via direct or indirect neural contacts, and control maternal and feeding behaviors in conjunction with the reward system [64, 72-

76]. In other words, the circadian clock may regulate both of physiological and metabolic phenomena, so that each of the multiple motivated behaviors, which cannot be occur simultaneously, can be performed in the appropriate time frame.

The body temperature rises after delivery as a byproduct of increased metabolism accompanied by an increase of food intake and milk production[35]. In this study, we confirmed the body temperature of lactating mice is increased as well as in previous reports (Figure 26). In addition, the diurnal rhythm of body temperature, which reaches peak value at the beginning of the dark period and decreases largely from the end of the dark period, is not changed compared to virgin mice, there is crouching-dominant time at the timing of decreasing body temperature (Figure 27). In lactating rats, it is known that they perform lactating behavior longer under conditions of good heat dissipation and not too high body temperature, nursing behavior and body temperature are closely related. Shaving the back hair of lactating rats increases nursing behavior, while cutting their tail, which supports heat dissipation, reduces it. [31, 32]. Therefore, during the lactation period, when the basal body temperature is higher than before delivery, the time when the body temperature decreases is appropriate for continuous crouching behavior to be carried out during the day. Comparing the onset of crouching-dominant time and the onset of temperature drop, the onset of temperature drop preceded that of crouching-dominant time (Figure 28, right). The temperature drop in the second half of the night can be seen in virgin and pregnant mice as well, suggesting that the crouching-dominant time appeared late at nighttime because the body temperature dropped preceding and rapidly at that time, and not vice versa. Since rats have higher body temperatures during the night, and the temperature drop begins at the onset of daytime and is sustained throughout the daytime [77-79]. The difference in the time crouching frequently performed between rats

and BALB/c mice may be reflected in the difference in body temperature rhythm.

Therefore, it is considered that the behavior of dams during the lactation period is controlled by the circadian clock in accordance with the physiological state of dams. Then what is the merit for pups of rhythmic maternal behavior? When both of wild-type and *Per*-null dams raised their own *Per*-hetero pups, there is no difference in the increase of pups' body weight, suggesting that the presence or absence of rhythmicity of crouching behavior wouldn't affect the physical growth of pups (Figure 14). There is a report that wild-type pups reared by *Clock* mutant mice increased anxiety behavior [80], to clarify the influence on pups' behavior is next challenge.

ii. Metabolic gene change of lactating mice

We observed metabolic changes such as feeding time and increase of body temperature. Next, we also examined the changes over time of liver which is basis of metabolism. The peripheral clock in liver is not only regulated by SCN but also feeding behavior [10], so it is not surprising that clock gene expression rhythm of liver in lactating mice which changes the pattern of feeding behavior. However, in the liver of lactating mice in DD, the core clock genes, including *Per1*, *Per2*, and *Bmal1*, exhibited the same expression profiles as those of virgin suggesting that core clock machinery in the liver remained unaffected (Figure 29). In contrast, the expression amplitudes of *Per1* and *Per2* were slightly suppressed under LD. This result is consistent with Casey et al.'s report [81] that the amplitude of the expression rhythm of *Per2* and *Cry1* in the liver sampled 3 days after delivery is diminished, and Greenwell et al.'s report [82] that *Per2* also flattened slightly when the mice were force-fed continuously under LD conditions. Therefore, continuous feeding and the light–dark cycles of LD conditions may interfere with each other.

Although there are some differences in liver clock oscillations between LD and DD, the expression rhythm of core clock genes was not largely altered during lactating period. The central clock in the SCN remained unaffected (see Figure 28). Additionally, the evidence showing that the corticosterone secretory rhythm, an essential factor in mediating the coupling between central and peripheral clocks, is maintained during lactation [81], suggests that the oscillations of both the central and peripheral clocks, as well as their coupling, are largely unchanged by behavioral modifications that occur during lactation. Although the effects on core clock genes are small, the amplitude of *Rev-erb* α, *Dbp*, and *Hlf,* genes of the interlock accessory loop, became flattened (Figure 29). These genes may be affected by physiological state or lactating behavior.

However, various genes of metabolic factors located downstream of the oscillating machinery of the peripheral clock were significantly altered during lactation (Figure 30). Nutrient metabolism, including nutrient distribution among organs (gastrointestinal tract, liver, mammary gland, and adipose tissue), changed during lactation. Additionally, hormone secretion, such as prolactin, growth hormone, and insulin also altered [83]. Interfering with these multiple signal transduction pathways downstream of the peripheral clock oscillators may occur. One possible interference pathway is regulation via the D-box. D-box-mediated regulation is involved in the acceleration of TTFL during circadian clock oscillations [82]. There are three PAR-domain basic leucine zipper (PAR bZip) proteins, D-site-binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukemia factor (HLF), which are examples of D-box mediators [84, 85] . In this study, these PAR bZip proteins, especially the expression level of *Hlf*, were strongly suppressed during the lactation period. Since PAR bZip proteins regulate the rhythmic expression profiles of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) [85],

the altered expression profiles of *Ppar* α in lactating mice may be a result of changed expression of PAR bZip proteins.

PPAR α functions as a molecular sensor for endogenous fatty acids, and regulates the transcription of genes involved in lipid uptake and catabolism [86, 87]. Recently, it has also been reported that PPAR α plays a role in balancing glucose homeostasis [88]. In non-lactating virgin mice, PPAR α mRNA expression peaked just before the beginning of feeding, but in lactating mice, PPAR α expression remained low throughout the day. It is suggested that the rhythmicity of PPAR-regulated genes such as *Gys2* and *Cpt1a* was lost, reflecting these changes.

These changes in metabolism-related genes make sense considering the physiological state of dams. In rats during lactation, an increase in the uptake of fatty acids by the mammary gland [49], an elevated expression of glucose transporter in the mammary gland [89], and a decrease in blood glucose level [90] have been reported. Dams perform lactation day and night, they need to produce nutrients in the liver for breast milk throughout the day.

Since dams use glucose for milk production without storage as glycogen, the expression level of Gys2 may decrease. High expression levels of Fasn (Fatty acid synthase) and Elovl6 (ELOVL fatty acid elongase 6) throughout the day may contribute to the demand for long-chain fatty acids in breast milk. Long-chain fatty acids are essential for the development of the brain and eyes of pups [91, 92]. Since these are supplied from the liver [50], fatty acids must continue to be synthesized in the liver. Fatty acids are needed for nutrients of breast milk, so β-oxidation, a stage in the decomposition of fatty acids, may be suppressed. Enzyme activity is regulated not only by transcription level but also by translation and its metabolites. For example, the expression level of

Cpt1a decreased, however, its activity is inhibited by Malonyl CoA [93]. It is needed to analyze metabolites to reveal changes in enzyme activity at the protein level.

Therefore, gene expression rhythm in peripheral organs is changed according to physiological state of lactating mice, clock gene oscillation in SCN was not affected (Figure 28). It may not need to shift oscillation of SCN to adapt physiological state of the individual [72]. The connections between the oscillations of the peripheral clock and the various downstream factors are weak, and peripheral organs may be adapted depending on the individual's state flexibly. During lactation period, hormone secretion and its receptors are thought to change. To clarify which signal pathway cause the downstream factors to dissociate from their control by the peripheral clock is a future challenge.

iii. Conclusion

In this study, clock genes expression rhythm of the central clock in the SCN was maintained, suggesting that the central clock is not affected by physiological state or behavioral alteration. Under physiological conditions in which multiple behavioral demands are altered, this strong oscillatory signal of the SCN may act neuronally or endocrinologically in various brain regions and contribute to rhythmic behavioral expression in a suitable time frame. Therefore, observation of behaviors during lactation is an interesting research subject that is ideal for investigating the mechanisms that allow multiple behaviors to be compatible under natural physiological conditions. Although we use primiparous female mice in this research, further research using multiparous mice will reveal the plasticity of change in physiological state accompanied by parturition and maternal behavior.

VI. References

1. Bass, J., *Circadian topology of metabolism.* Nature, 2012. **491**(7424): p. 348-56.
2. Takahashi, J.S., *Transcriptional architecture of the mammalian circadian clock.* Nat Rev Genet, 2017. **18**(3): p. 164-179.
3. Patke, A., M.W. Young, and S. Axelrod, *Molecular mechanisms and physiological importance of circadian rhythms.* Nat Rev Mol Cell Biol, 2020. **21**(2): p. 67-84.
4. Koronowski, K.B. and P. Sassone-Corsi, *Communicating clocks shape circadian homeostasis.* Science, 2021. **371**(6530).
5. Roenneberg, T. and M. Merrow, *The Circadian Clock and Human Health.* Curr Biol, 2016. **26**(10): p. R432-43.
6. Herzog, E.D., et al., *Regulating the Suprachiasmatic Nucleus (SCN) Circadian Clockwork: Interplay between Cell-Autonomous and Circuit-Level Mechanisms.* Cold Spring Harb Perspect Biol, 2017. **9**(1).
7. Pittendrigh, C.S. and S. Daan, *Circadian oscillations in rodents: a systematic increase of their frequency with age.* Science, 1974. **186**(4163): p. 548-50.
8. Chao, H.W., et al., *Circadian clock regulates hepatic polyploidy by modulating Mkp1-Erk1/2 signaling pathway.* Nat Commun, 2017. **8**(1): p. 2238.
9. Sawaki, Y., I. Nihonmatsu, and H. Kawamura, *Transplantation of the neonatal suprachiasmatic nuclei into rats with complete bilateral suprachiasmatic lesions.* Neurosci Res, 1984. **1**(1): p. 67-72.
10. Hara, R., et al., *Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus.* Genes Cells, 2001. **6**(3): p. 269-78.
11. Damiola, F., et al., *Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus.* Genes Dev, 2000. **14**(23): p. 2950-61.
12. Hrdy and S., *Mothers and others.* 2009, Cambridge, MA: Harvard University Press.
13. Numan, M. and B. Woodside, *Maternity: neural mechanisms, motivational processes, and physiological adaptations.* Behav Neurosci, 2010. **124**(6): p. 715-41.
14. Gómez-Robles, A., et al., *The evolution of human altriciality and brain development in comparative context.* Nat Ecol Evol, 2024. **8**(1): p. 133-146.
15. Kuroda, K.O., et al., *Neuromolecular basis of parental behavior in laboratory mice and rats: with special emphasis on technical issues of using mouse genetics.* Prog Neuropsychopharmacol Biol Psychiatry, 2011. **35**(5): p. 1205-31.
16. Caldji, C., et al., *Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5335-40.

17. Agrati, D. and N. Uriarte, *What can challenging reproductive contexts tell us about the rat's maternal behavior?* Front Behav Neurosci, 2023. **17**: p. 1239681.
18. Rilling, J.K. and L.J. Young, *The biology of mammalian parenting and its effect on offspring social development.* Science, 2014. **345**(6198): p. 771-6.
19. Lévy, F. and M. Keller, *Olfactory mediation of maternal behavior in selected mammalian species.* Behav Brain Res, 2009. **200**(2): p. 336-45.
20. D'Amato, F.R., et al., *Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups?* Behav Genet, 2005. **35**(1): p. 103-12.
21. Bakowska, J.C. and J.I. Morrell, *Atlas of the neurons that express mRNA for the long form of the prolactin receptor in the forebrain of the female rat.* J Comp Neurol, 1997. **386**(2): p. 161-77.
22. Brown, R.S.E., et al., *Prolactin action in the medial preoptic area is necessary for postpartum maternal nursing behavior.* Proc Natl Acad Sci U S A, 2017. **114**(40): p. 10779-10784.
23. Koch, M. and G. Ehret, *Immunocytochemical localization and quantitation of estrogen-binding cells in the male and female (virgin, pregnant, lactating) mouse brain.* Brain Res, 1989. **489**(1): p. 101-12.
24. Tsuneoka, Y., et al., *Functional, anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to maternal behavior in the mouse.* J Comp Neurol, 2013. **521**(7): p. 1633-63.
25. Terkel, J., R.S. Bridges, and C.H. Sawyer, *Effects of transecting lateral neural connections of the medial preoptic area on maternal behavior in the rat: nest building, pup retrieval and prolactin secretion.* Brain Res, 1979. **169**(2): p. 369-80.
26. KAISER, I.H. and F. HALBERG, *Circadian periodic aspects of birth.* Ann N Y Acad Sci, 1962. **98**: p. 1056-68.
27. Ader, R. and L.J. Grota, *Rhythmicity in the maternal behaviour of Rattus norvegicus.* Anim Behav, 1970. **18**(1): p. 144-50.
28. Grota, L.J. and R. Ader, *Behavior of lactating rats in a dual-chambered maternity cage.* Horm Behav, 1974. **5**(4): p. 275-82.
29. Hoshino, K., et al., *Circadian Clock mutation in dams disrupts nursing behavior and growth of pups.* Endocrinology, 2006. **147**(4): p. 1916-23.
30. Croskerry, P.G., G.K. Smith, and M. Leon, *Thermoregulation and the maternal behaviour of the rat.* Nature, 1978. **273**(5660): p. 299-300.
31. Król, E., M. Murphy, and J.R. Speakman, *Limits to sustained energy intake. X. Effects of fur removal on reproductive performance in laboratory mice.* J Exp Biol, 2007. **210**(Pt 23): p. 4233-43.

32. Leon, M., P.G. Croskerry, and G.K. Smith, *Thermal control of mother-young contact in rats.* Physiol Behav, 1978. **21**(5): p. 79O-811.
33. Asakuma, S., et al., *Diurnal rhythm of cerebrospinal fluid and plasma leptin levels related to feeding in non-lactating and lactating rats.* J Endocrinol, 2004. **180**(2): p. 283-6.
34. Nelson, W., L. Cadotte, and F. Halberg, *Circadian timing of single daily "meal" affects survival of mice.* Proc Soc Exp Biol Med, 1973. **144**(3): p. 766-9.
35. Gamo, Y., et al., *Limits to sustained energy intake. XX. Body temperatures and physical activity of female mice during lactation.* J Exp Biol, 2013. **216**(Pt 19): p. 3751-61.
36. Gamo, Y., et al., *Limits to sustained energy intake XXIV: impact of suckling behaviour on the body temperatures of lactating female mice.* Sci Rep, 2016. **6**: p. 25665.
37. Cermakian, N., et al., *Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene.* Embo j, 2001. **20**(15): p. 3967-74.
38. Zheng, B., et al., *The mPer2 gene encodes a functional component of the mammalian circadian clock.* Nature, 1999. **400**(6740): p. 169-73.
39. Shearman, L.P., et al., *Targeted disruption of the mPer3 gene: subtle effects on circadian clock function.* Mol Cell Biol, 2000. **20**(17): p. 6269-75.
40. Doi, M., et al., *Non-coding cis-element of Period2 is essential for maintaining organismal circadian behaviour and body temperature rhythmicity.* Nat Commun, 2019. **10**(1): p. 2563.
41. Murakami, A., et al., *Prolonged Light Exposure Induces Circadian Impairment in Aquaporin-4-Knockout Mice.* J Biol Rhythms, 2023. **38**(2): p. 208-214.
42. Sokolove, P.G. and W.N. Bushell, *The chi square periodogram: its utility for analysis of circadian rhythms.* J Theor Biol, 1978. **72**(1): p. 131-60.
43. Koike, N., et al., *Transcriptional architecture and chromatin landscape of the core circadian clock in mammals.* Science, 2012. **338**(6105): p. 349-54.
44. Doi, M., et al., *Gpr176 is a Gz-linked orphan G-protein-coupled receptor that sets the pace of circadian behaviour.* Nat Commun, 2016. **7**: p. 10583.
45. Doi, R., K. Oishi, and N. Ishida, *CLOCK regulates circadian rhythms of hepatic glycogen synthesis through transcriptional activation of Gys2.* J Biol Chem, 2010. **285**(29): p. 22114-21.
46. Yamaguchi, Y., et al., *Mice genetically deficient in vasopressin V1a and V1b receptors are resistant to jet lag.* Science, 2013. **342**(6154): p. 85-90.
47. Pelikan, A., et al., *Venn diagram analysis overestimates the extent of circadian rhythm reprogramming.* FEBS J, 2022. **289**(21): p. 6605-6621.
48. Numan, M., *Motivational systems and the neural circuitry of maternal behavior in the rat.* Dev Psychobiol, 2007. **49**(1): p. 12-21.

49. Makarova, E.N., E.D. Kochubei, and N.M. Bazhan, *Regulation of food consumption during pregnancy and lactation in mice.* Neurosci Behav Physiol, 2010. **40**(3): p. 263-7.
50. Gutgesell, A., et al., *Downregulation of peroxisome proliferator-activated receptor alpha and its coactivators in liver and skeletal muscle mediates the metabolic adaptations during lactation in mice.* J Mol Endocrinol, 2009. **43**(6): p. 241-50.
51. Trayhurn, P., J.B. Douglas, and M.M. McGuckin, *Brown adipose tissue thermogenesis is 'suppressed' during lactation in mice.* Nature, 1982. **298**(5869): p. 59-60.
52. Pedraza, N., et al., *Impaired expression of the uncoupling protein-3 gene in skeletal muscle during lactation: fibrates and troglitazone reverse lactation-induced downregulation of the uncoupling protein-3 gene.* Diabetes, 2000. **49**(7): p. 1224-30.
53. Song, S., et al., *Peroxisome proliferator activated receptor alpha (PPARalpha) and PPAR gamma coactivator (PGC-1alpha) induce carnitine palmitoyltransferase IA (CPT-1A) via independent gene elements.* Mol Cell Endocrinol, 2010. **325**(1-2): p. 54-63.
54. Choi, W.I., et al., *Proto-oncogene FBI-1 (Pokemon) and SREBP-1 synergistically activate transcription of fatty-acid synthase gene (FASN).* J Biol Chem, 2008. **283**(43): p. 29341-54.
55. Kumadaki, S., et al., *Mouse Elovl-6 promoter is an SREBP target.* Biochem Biophys Res Commun, 2008. **368**(2): p. 261-6.
56. Johnstone, L.E. and T. Higuchi, *Food intake and leptin during pregnancy and lactation.* Prog Brain Res, 2001. **133**: p. 215-27.
57. Stern, J.M. and R. Levin, *Food availability as a determinant of the rats' circadian rhythm in maternal behavior.* Dev Psychobiol, 1976. **9**(2): p. 137-48.
58. Stahl, W.R., *Organ weights in primates and other mammals.* Science, 1965. **150**(3699): p. 1039-42.
59. KLEIBER, M., *Body size and metabolic rate.* Physiol Rev, 1947. **27**(4): p. 511-41.
60. McConnell, E.L., A.W. Basit, and S. Murdan, *Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments.* J Pharm Pharmacol, 2008. **60**(1): p. 63-70.
61. Armstrong, S., J. Clarke, and G. Coleman, *Light-dark variation in laboratory rat stomach and small intestine content.* Physiol Behav, 1978. **21**(5): p. 785-8.
62. Antle, M.C. and R. Silver, *Circadian Insights into Motivated Behavior.* Curr Top Behav Neurosci, 2016. **27**: p. 137-69.
63. Gammie, S.C., *Current models and future directions for understanding the neural circuitries of maternal behaviors in rodents.* Behav Cogn Neurosci Rev, 2005. **4**(2): p. 119-35.
64. la Fleur, S.E., et al., *Circadian influences on feeding behavior.* Neuropharmacology, 2024.

256: p. 110007.

65. Watarai, A., et al., *The blockade of oxytocin receptors in the paraventricular thalamus reduces maternal crouching behavior over pups in lactating mice.* Neurosci Lett, 2020. **720**: p. 134761.
66. Salais-López, H., et al., *Maternal Motivation: Exploring the Roles of Prolactin and Pup Stimuli.* Neuroendocrinology, 2021. **111**(9): p. 805-830.
67. Bosch, O.J. and I.D. Neumann, *Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action.* Horm Behav, 2012. **61**(3): p. 293-303.
68. Kohl, J., et al., *Functional circuit architecture underlying parental behaviour.* Nature, 2018. **556**(7701): p. 326-331.
69. Scott, N., et al., *A sexually dimorphic hypothalamic circuit controls maternal care and oxytocin secretion.* Nature, 2015. **525**(7570): p. 519-22.
70. Padilla, S.L., et al., *Kisspeptin Neurons in the Arcuate Nucleus of the Hypothalamus Orchestrate Circadian Rhythms and Metabolism.* Curr Biol, 2019. **29**(4): p. 592-604.e4.
71. Sayar-Atasoy, N., et al., *AgRP neurons encode circadian feeding time.* Nat Neurosci, 2024. **27**(1): p. 102-115.
72. Saper, C.B., et al., *The hypothalamic integrator for circadian rhythms.* Trends Neurosci, 2005. **28**(3): p. 152-7.
73. Bailey, M. and R. Silver, *Sex differences in circadian timing systems: implications for disease.* Front Neuroendocrinol, 2014. **35**(1): p. 111-39.
74. Abrahamson, E.E. and R.Y. Moore, *Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections.* Brain Res, 2001. **916**(1-2): p. 172-91.
75. Vujovic, N., et al., *Projections from the subparaventricular zone define four channels of output from the circadian timing system.* J Comp Neurol, 2015. **523**(18): p. 2714-37.
76. Colavito, V., et al., *Limbic thalamus and state-dependent behavior: The paraventricular nucleus of the thalamic midline as a node in circadian timing and sleep/wake-regulatory networks.* Neurosci Biobehav Rev, 2015. **54**: p. 3-17.
77. Fioretti, M.C., et al., *Control of the circadian rhythm of the body temperature in the rat.* Life Sci, 1974. **14**(11): p. 2111-9.
78. Refinetti, R., *Comparison of the body temperature rhythms of diurnal and nocturnal rodents.* J Exp Zool, 1996. **275**(1): p. 67-70.
79. Friedman, A.H. and C.A. Walker, *Circadian rhythms in rat mid-brain and caudate nucleus biogenic amine levels.* J Physiol, 1968. **197**(1): p. 77-85.
80. Koizumi, H., et al., *Increased anxiety in offspring reared by circadian Clock mutant mice.*

PLoS One, 2013. **8**(6): p. e66021.

81. Casey, T.M., et al., *Tissue-specific changes in molecular clocks during the transition from pregnancy to lactation in mice.* Biol Reprod, 2014. **90**(6): p. 127.
82. Greenwell, B.J., et al., *Rhythmic Food Intake Drives Rhythmic Gene Expression More Potently than the Hepatic Circadian Clock in Mice.* Cell Rep, 2019. **27**(3): p. 649-657.e5.
83. Wade, G.N. and J.E. Schneider, *Metabolic fuels and reproduction in female mammals.* Neurosci Biobehav Rev, 1992. **16**(2): p. 235-72.
84. Mitsui, S., et al., *Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism.* Genes Dev, 2001. **15**(8): p. 995-1006.
85. Gachon, F., et al., *Proline- and acidic amino acid-rich basic leucine zipper proteins modulate peroxisome proliferator-activated receptor alpha (PPARalpha) activity.* Proc Natl Acad Sci U S A, 2011. **108**(12): p. 4794-9.
86. Napal, L., P.F. Marrero, and D. Haro, *An intronic peroxisome proliferator-activated receptor-binding sequence mediates fatty acid induction of the human carnitine palmitoyltransferase 1A.* J Mol Biol, 2005. **354**(4): p. 751-9.
87. Hunt, M.C., et al., *The peroxisome proliferator-activated receptor alpha (PPARalpha) regulates bile acid biosynthesis.* J Biol Chem, 2000. **275**(37): p. 28947-53.
88. Peeters, A. and M. Baes, *Role of PPARα in Hepatic Carbohydrate Metabolism.* PPAR Res, 2010. **2010**.
89. Nemeth, B.A., et al., *Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland.* Pediatr Res, 2000. **47**(4 Pt 1): p. 444-50.
90. Robinson, A.M., J.R. Girard, and D.H. Williamson, *Evidence for a role of insulin in the regulation of lipogenesis in lactating rat mammary gland. Measurements of lipogenesis in vivo and plasma hormone concentrations in response to starvation and refeeding.* Biochem J, 1978. **176**(1): p. 343-6.
91. Belkind-Gerson, J., et al., *Fatty acids and neurodevelopment.* J Pediatr Gastroenterol Nutr, 2008. **47 Suppl 1**: p. S7-9.
92. Moriguchi, T., R.S. Greiner, and N. Salem, *Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration.* J Neurochem, 2000. **75**(6): p. 2563-73.
93. McGarry, J.D., G.F. Leatherman, and D.W. Foster, *Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA.* J Biol Chem, 1978. **253**(12): p. 4128-36.

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