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# Dissecting the Daily Feeding Pattern: Peripheral CLOCK/CYCLE Generate the Feeding/Fasting Episodes and Neuronal Molecular Clocks Synchronize Them

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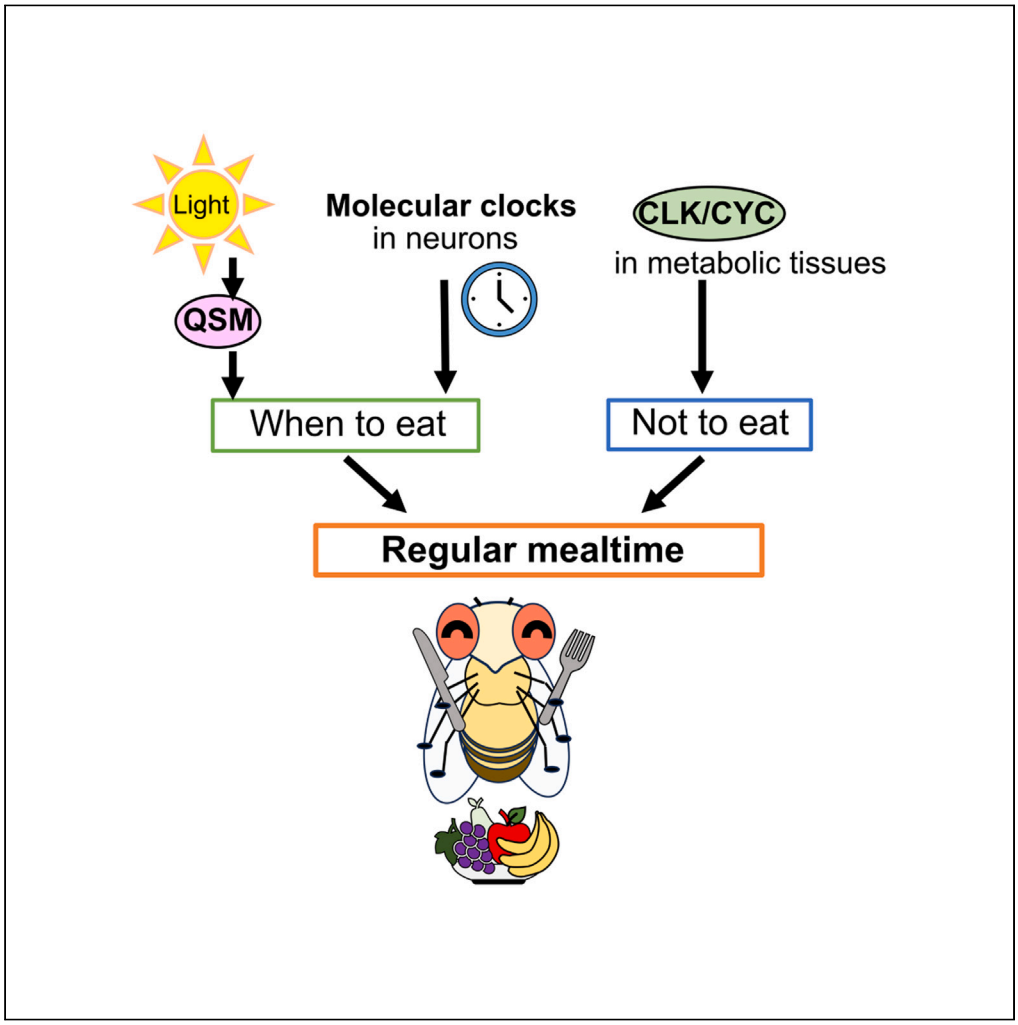


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## Highlights

QSM mediates the light-induced synchronization of feeding episodes

CLK/CYC in metabolic tissues generate feeding/fasting episodes

PER/TIM in neurons synchronize feeding/fasting episodes under constant darkness

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# Dissecting the daily feeding pattern: Peripheral CLOCK/CYCLE generate the feeding/fasting episodes and neuronal molecular clocks synchronize them

Akiko Maruko,[1,5] Koichi M. Iijima,[2,3] and Kanae Ando[4,6,*]

## SUMMARY

**A 24-h rhythm of feeding behavior, or synchronized feeding/fasting episodes during the day, is crucial for survival. Internal clocks and light input regulate rhythmic behaviors, but how they generate feeding rhythms is not fully understood. Here we aimed to dissect the molecular pathways that generate daily feeding patterns. By measuring the semidiurnal amount of food ingested by single flies, we demonstrate that the generation of feeding rhythms under light:dark conditions requires *quasimodo* (*qsm*) but not molecular clocks. Under constant darkness, rhythmic feeding patterns consist of two components: CLOCK (CLK) in digestive/metabolic tissues generating feeding/fasting episodes, and the molecular clock in neurons synchronizing them to subjective daytime. Although CLK is a part of the molecular clock, the generation of feeding/fasting episodes by CLK in metabolic tissues was independent of molecular clock machinery. Our results revealed novel functions of *qsm* and CLK in feeding rhythms in *Drosophila*.**

## INTRODUCTION

Feeding behavior is critical for the survival of every living organism and the basis of physiology and behavior. It is driven by homeostatic needs, a sense of hunger-satiety, and circadian rhythms.[1] Rhythmic feeding patterns, or synchronized feeding/fasting episodes during the day, coincide with the environmental conditions, such as light cycle, temperature, risk of predation, and food availability, maximizing the chance of survival. Rhythmic feeding is also critical for efficient digestion and metabolism,[2,3] and it can affect all rhythmic behavior via resetting circadian clocks in the peripheral tissues.[3–7]

The molecular clocks maintain circadian rhythmicity under constant darkness and function in central and peripheral tissues.[8] In *Drosophila*, the transcription factors CLOCK (CLK) and CYCLE (CYC) promote the expression of repressor genes, *period* (*per*) and *timeless* (*tim*), and accumulated repressor proteins feedback to inhibit repressor gene transcription.[9] Many cells contain this molecular oscillator, which confers the circadian rhythmicity of tissue-specific functions.[10,11] While these peripheral clocks can be entrained independently, peripheral clocks are under the control of the master clock in the brain.[12] About 150 neurons in the lateral and dorsal brain are known as the central clock and synchronize the peripheral clocks, thus acting as the master regulator for rhythmic behavior, including feeding.[13,14] Central clocks regulate peripheral clocks via hormones such as neuropeptide F/Y and insulin-like peptides.[15–17] On the other hand, food availability is a potent environmental cue that resets molecular clocks and directs circadian locomotor activity.[18,19] Expression of molecular clock genes in digestive/metabolic tissues, such as the liver in mammals and the fat body in *Drosophila*, are synchronized by feeding, and its disruption causes changes in metabolic processes and feeding behavior.[3,4,6,7] However, the precise roles of each molecular clock gene in the generation of feeding patterns still need to be fully understood.

Light-dark cycles are another major potent environmental cue for setting circadian rhythms. Clock mutant flies lose rhythmic patterns of locomotion and feeding under constant darkness (DD), while they show normal feeding patterns under light-dark conditions.[20] CRYPTOCHROME (CRY) and the visual system are the primary mediators of the light signal to reset central clocks in *Drosophila*.[21–24] Interestingly, flies lacking CRY lose the early morning bouts of food intake and show an additional peak in the evening, while they still have 24h feeding rhythms,[20] suggesting that a CRY- and clock-independent light input pathway generates daytime feeding rhythm under light: dark (LD) conditions. *Quasimodo* was identified as a clock-controlled gene that encodes a light-responsive protein, QUASIMODO (QSM), which degrades TIM independently of CRY[25] and affects clock neuron excitability in response to light.[26] The role of QSM in light-responsible feeding behavior has not been studied.

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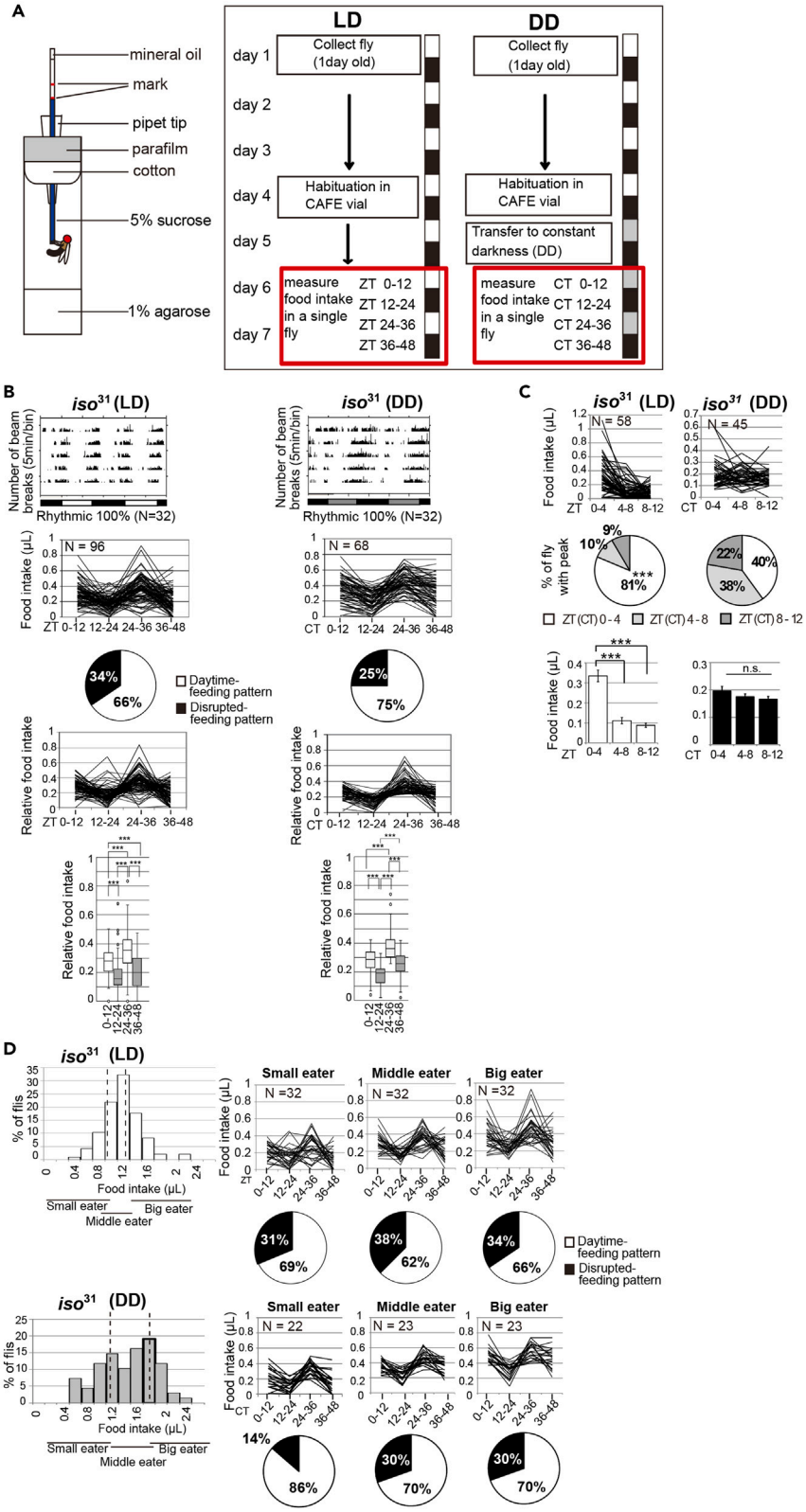
A
mineral oil
mark
pipet tip
parafilm
cotton
5% sucrose
1% agarose
LD
DD
day 1
day 2
day 3
day 4
day 5
day 6
day 7
Collect fly (1day old)
Habituation in CAFE vial
Transfer to constant darkness (DD)
measure food intake in a single fly
ZT 0-12
ZT 12-24
ZT 24-36
ZT 36-48
CT 0-12
CT 12-24
CT 24-36
CT 36-48
B
iso31 (LD)
iso31 (DD)
Number of beam breaks (5min/bin)
Rhythmic 100% (N=32)
Food intake (μL)
N = 96
N = 68
34%
66%
25%
75%
Daytime-feeding pattern
Disrupted-feeding pattern
Relative food intake
C
N = 58
N = 45
% of fly with peak
9%
10%
81%
22%
40%
38%
ZT (CT) 0-4
ZT (CT) 4-8
ZT (CT) 8-12
***
n.s.
D
% of flis
Food intake (μL)
Small eater
Middle eater
Big eater
N =32
N = 22
N = 23
31%
69%
38%
62%
34%
66%
14%
86%
30%
70%

**Figure 1. Flies show a daytime feeding pattern with more food intake during the subjective day**

(A) (left) Modified version of CAFE assay. A single fly was placed in a vial with a glass capillary filled with 5% sucrose solution. The amount of sucrose solution ingested was measured by marking the capillary. 1% agarose was supplied as water source. (Right) Design of experiments. Flies were trained under LD conditions for 2 days before being transferred to CAFE vial. After 24 h of habituation, the amount of food ingested was measured every 12 h for 2 days.

(B) Wild-type flies showed daytime feeding rhythms under LD and DD conditions. (Top) Locomotor rhythms of wild-type fly (*iso*$^{31}$) in LD and DD. Representative actogram and percentages of flies with locomotor rhythms are shown. (Middle) Line graphs show the amount of food ingested by individual flies for every 12 h measured by the modified CAFE assay plotted over 48 h. Pie charts show the ratio of flies showing daytime feeding patterns (more food ingestion during daytime or subjective daytime than nighttime for both days (white)) and disrupted feeding patterns (more food ingestion during nighttime or subjective nighttime, inconsistent patterns between the first and second day, or showed steady increases or decreases (black)). (Bottom) The amount of food ingested was normalized by the total amount of food consumed during the entire assay (48 h) and plotted over time. The same data are also shown in boxplots. Boxplot centerline: median; box limits: first and third quartile; whiskers: min/max values. Outliers are displayed as points. ***$p < 0.001$, TukeyHSD test.

(C) Daytime feeding peak in the morning is enhanced by light. (Top) the amount of food ingested every 4 h during daytime by individual flies under LD (left) and DD (right) conditions. (Middle) % of the flies with peaks at each point. ***$p < 0.001$; Chi-square test. (Bottom) the average amount of food intake. The error bars represent mean ± SEM. ***$p < 0.001$, TukeyHSD test.

(D) The wild flies (*iso*$^{31}$) were divided into three groups depending on total food consumption under LD (Top) and DD (Bottom) conditions. All groups (small, middle, and big eaters) of *iso*$^{31}$ flies display robust diurnal feeding patterns. No significant differences in feeding patterns between small, middle, and big eaters were detected ($p > 0.05$, Fisher's extract test). See also Figure S1.

In this study, we set out to investigate how daily feeding patterns are generated under LD and DD in *Drosophila*. We found two components of feeding rhythm, the feeding/fasting episodes and their synchronization, are separately regulated under DD: CLK/CYC digestive/metabolic tissues generate feeding/fasting episodes, and molecular clocks in neurons synchronize them. We also found that LD cycles generate rhythmic daytime feeding via the QSM independently from molecular clocks.

## RESULTS

### Flies show a daily feeding pattern with more food intake during the subjective day in light/dark cycles and constant darkness

To analyze the daily feeding patterns of individual flies, we used a modified version of CAFE assay,[27] in which flies consume liquid food from a calibrated glass microcapillary (Figure 1A). Flies were entrained under 12-h:12-h light/dark (LD) cycles, then kept under this cycle or transferred to constant darkness (DD) (Figure 1A). The amount of food consumed during 12 h by individual flies was measured and plotted for consecutive 48 h (Figure 1B, *iso*$^{31}$ (LD) and *iso*$^{31}$ (DD)). We found that the majority of flies (66% in LD and 75% in DD) ingested more food during the day, or subjective day, on both the first day and second day (daytime feeding pattern). The others (34% in LD and 25% in DD) showed inconsistent patterns between the first and second day or show steady increases or decreases (disrupted feeding pattern). When the food intake amount in each time period was normalized with the total food intake amount, the pattern was more prominently observed (Figure 1B, relative food intake). Another strain, *y w*, also showed a similar result (69% in LD and 90% in DD showed the daytime feeding pattern, Figure S1A).

### Light enhances the feeding peak in the morning

It has been reported that flies eat primarily in the early morning.[17,20] To identify the period in which an individual fly eats the most, we analyzed the amount of food intake in the subjective morning (ZT/CT 0–4), mid-day (ZT/CT 4–8), and late afternoon (ZT/CT 8–12) by using the modified CAFE assay. Under LD conditions, we found that 81% of the flies ingested food the most during ZT 0–4 ($p < 0.001$) (Figure 1C, LD), which is in line with the previous report from Seay & Thummel.[20] In DD, although 40% of the flies ingested food the most in the subjective morning, the number of those flies was not significantly different from that expected from random chance alone ($p > 0.05$) (Figure 1C, DD). These results suggest that the light signal directly stimulates feeding.

### Total food consumption is not associated with a particular feeding pattern

We noticed a large variation in total food consumption among individuals. Total food consumption during 48 h varied as much as 6-fold (Figure 1D, minimum 0.35 μL and maximum 2.15 μL for LD and minimum 0.41 μL and maximum 2.26 μL for DD). Similarly, a large variation was observed with another fly strain, *y w* (minimum 0.26 μL and maximum 1.87 μL for LD and minimum 0.51 μL and maximum 2.99 μL for DD) (Figure S1B). To ask whether the amount of total food consumption is associated with a particular feeding pattern, the food intake patterns of the flies with different amounts of food consumption were compared. We categorized feeding patterns into "daytime feeding pattern" and "disrupted feeding pattern": "daytime feeding pattern" in which the flies ingest more food during the day or subjective day in both the first day and second day. As for "disrupted feeding pattern," the flies ingest more food during the night or subjective night, or the flies show inconsistent patterns between the first and second day, or showed steady increases or decreases. The flies were divided into three groups depending on total food consumption: small eater, middle eater, and big eater. We found that percentages of flies with the daytime feeding pattern and those with the disrupted feeding pattern were similar between the group of big eaters and that of small eaters in LD (Figure 1D, $p > 0.05$, Fisher's exact test). Similar results were obtained with flies in DD, while the total food consumption was increased in DD (Figures 1D and S1C, $p > 0.05$, Fisher's exact test)). These results indicate that total food consumption and diurnal feeding patterns are not associated.

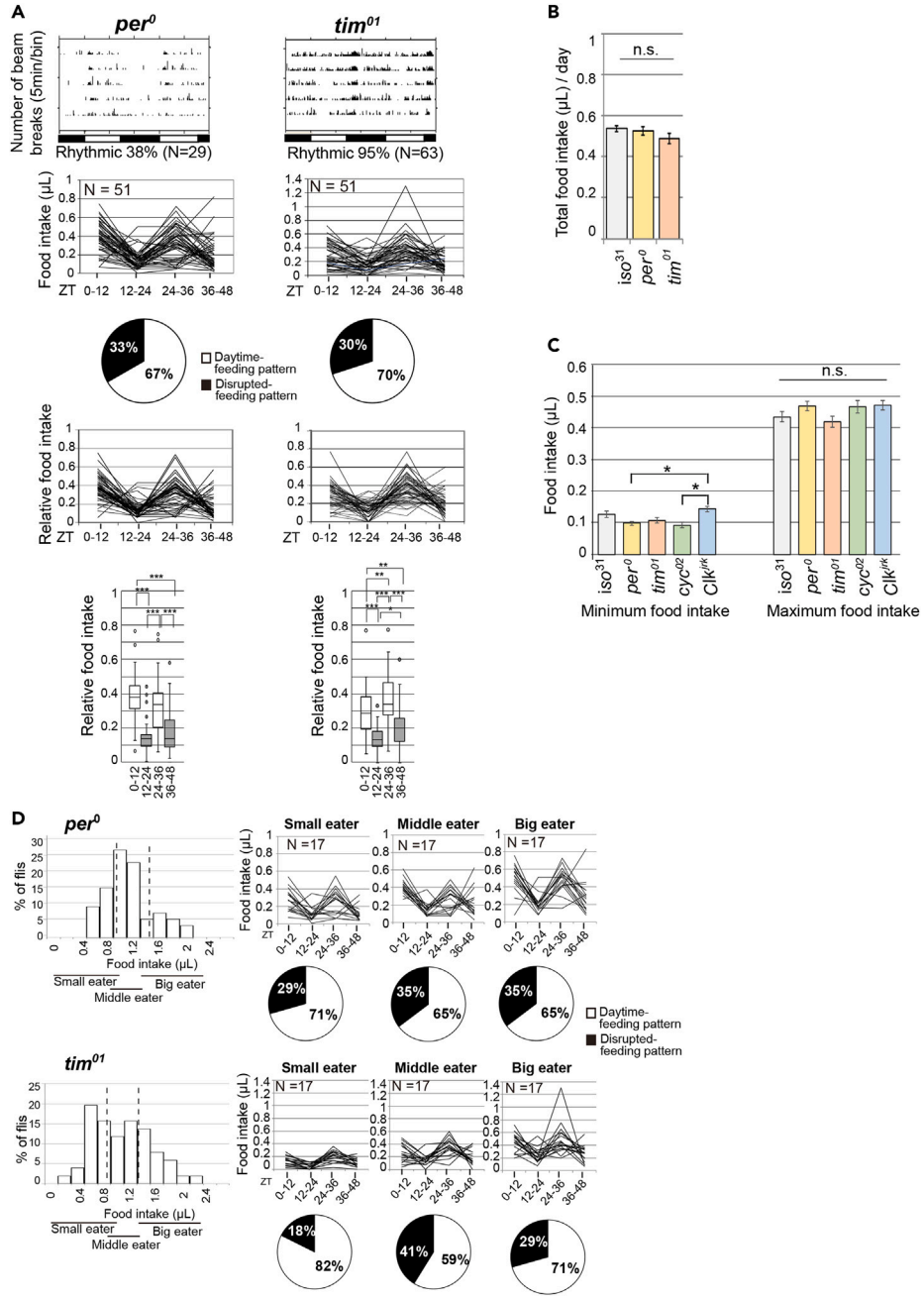


**Figure 2. LD cycle synchronizes the daytime feeding pattern independent of molecular clocks**

(A) (Top) Locomotor rhythms of clock-mutant flies ($per^0$ and $tim^{01}$) in LD. Representative actogram and percentages of flies with locomotor rhythms are shown. (Middle) Line graphs show the amount of food ingested by individual flies for every 12 h measured by the modified CAFE assay plotted over 48 h. Pie chart show the ratio of flies with daytime feeding patterns (more food ingestion during daytime than nighttime for both days (white)) and disrupted feeding patterns (more food ingestion during nighttime, inconsistent patterns between the first and second day, or showed steady increases or decreases (black)). (Bottom) The amount of food ingested was normalized by the total amount of food consumed during the entire assay and plotted over time. The same data are also shown in boxplots (Relative food intake). *p < 0.05,**p < 0.01,***p < 0.001, TukeyHSD test. The centerline of the boxplots indicates the median, the box displays the first and third quartile, and the whiskers shows min/max values. Outliers are displayed as points.

(B) The total amount of food consumed in 24 h.

(C) The minimum or maximum amounts of food ingested for 12 h over a 2-day period. (B and C) The error bars represent mean ± SEM. *, p < 0.05, TukeyHSD test.

(D) The clock-mutant flies ($per^0$, $tim^{01}$) were divided into three groups depending on total food consumption under LD condition (Left). All groups (small, middle, big eater) of $per^0$ and $tim^{01}$ display robust diurnal feeding patterns. No significant differences in feeding patterns between small, middle, and big eaters were detected (p > 0.05, Fisher's extract test).

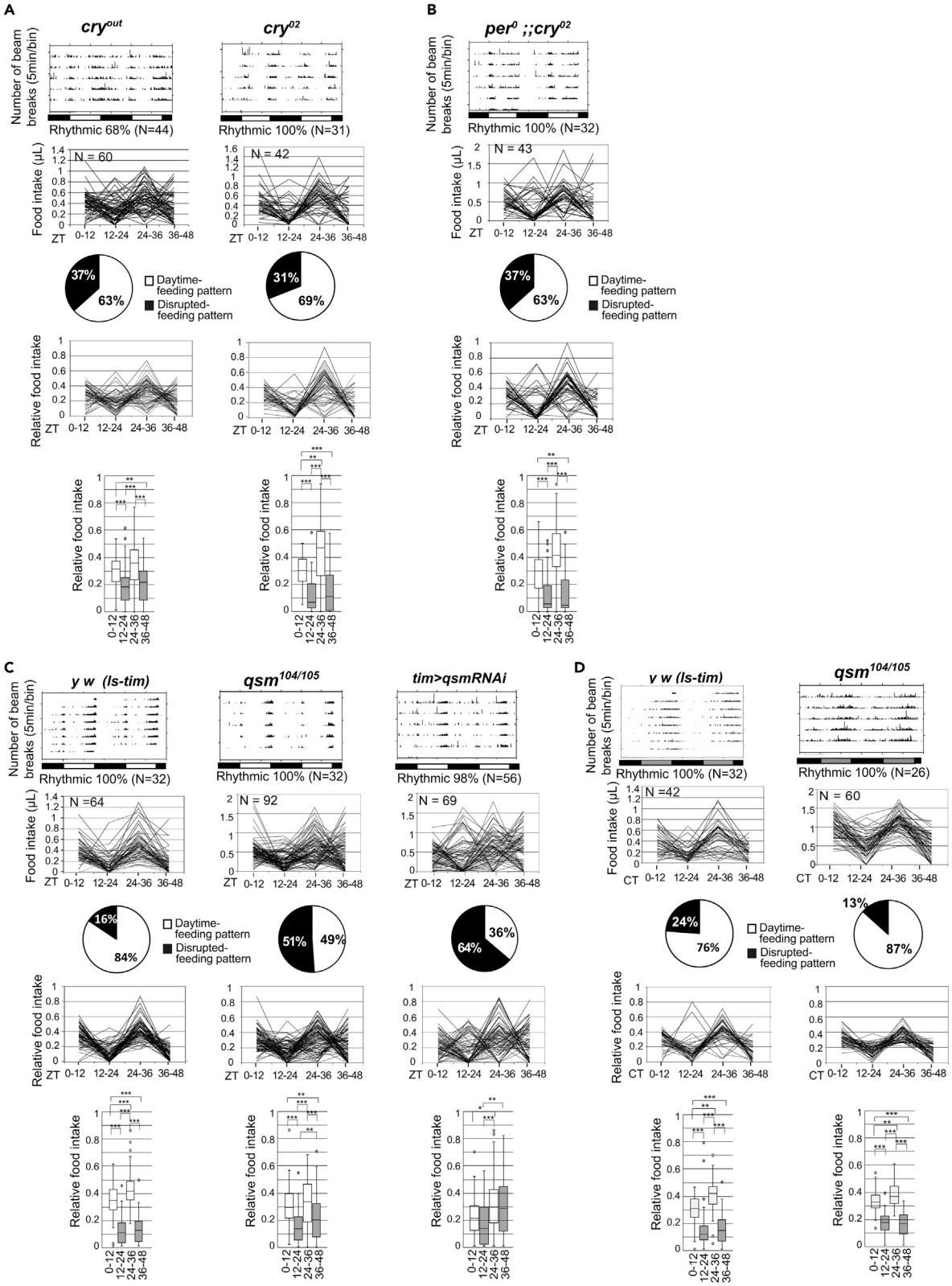
A
cry^out
cry^02
Number of beam breaks (5min/bin)
Rhythmic 68% (N=44)
Rhythmic 100% (N=31)
N = 60
N = 42
Food intake (μL)
ZT 0-12 12-24 24-36 36-48
37%
63%
31%
69%
Daytime-feeding pattern
Disrupted-feeding pattern
Relative food intake
B
per^0 ;;cry^02
Rhythmic 100% (N=32)
N = 43
C
y w (ls-tim)
qsm^104/105
tim>qsmRNAi
Rhythmic 100% (N=32)
Rhythmic 100% (N=32)
Rhythmic 98% (N=56)
N =64
N = 92
N = 69
16%
84%
51%
49%
36%
64%
D
v w (ls-tim)
qsm^104/105
Rhythmic 100% (N=32)
Rhythmic 100% (N=26)
N =42
N = 60
CT 0-12 12-24 24-36 36-48
24%
76%
13%
87%

**Figure 3. *qsm* regulates daytime feeding rhythms in LD**

(A and B) *cry*-null ($cry^{out}$ and $cry^{02}$) (A) and $per^{0}$;; $cry^{02}$ double mutant (B) showed daytime feeding rhythms under LD conditions. (Top) Representative actogram of locomotor activity. (Middle) Line graphs show the amount of food ingested every 12 h (Food intake). Pie charts show percentage of flies with daytime- and disrupted feeding patterns. (Bottom) The amount of food ingested was normalized by total food consumption and plotted over 48 h. The same data are and also shown in boxplots.

(C) The percentage of flies with disrupted feeding patterns was increased by *qsm*-null ($qsm^{104/105}$) and knockdown of *qsm* with *qsm RNAi* driven by tim-Gal4 (*tim > qsm RNAi*) compared to the control (*y w* (*ls-tim*)) under LD conditions. (Top) Representative actogram of locomotor activity. (Middle) The amount of food ingested every 12 h (Food intake) and percentage of flies with daytime- and disrupted feeding patterns (pie chart). (Bottom) The amount of food ingested was normalized by total food consumption and plotted over 48 h and in boxplots (Relative food intake).

(D) *qsm*-null flies showed robust diurnal feeding under DD condition. (Top) Representative actogram of locomotor activity. (Middle) The amount of food ingested every 12 h (Food intake) and percentage of flies with daytime- and disrupted feeding patterns (pie chart). (Bottom) The amount of food ingested normalized by total food consumption plotted over 48 h and in boxplots (Relative food intake). (A–D) The centerline of the boxplots indicates the median, the box displays the first and third quartile, and the whiskers showed min/max values. Outliers are displayed as points. *p < 0.05, **p < 0.01, ***p < 0.001, TukeyHSD test.

## Light/dark cycle synchronizes the daytime feeding pattern independent of molecular clocks

We analyzed daily feeding patterns in flies with null mutations in core circadian clock genes *period* ($per^{0}$) and *timeless* ($tim^{01}$). As previously reported,[20] under LD conditions, *per*- and *tim*-null flies showed the daytime feeding pattern similar to wild-type flies (Figure 2A, $per^{0}$ and $tim^{01}$), indicating that the molecular clock functions are not required to express the daily feeding pattern in LD.[20]

Total food consumption during 48 h (Figure 2B) and minimum and maximum food intake (Figure 2C) in circadian mutant flies were not significantly different from control flies. The food intake patterns of $per^{0}$ and $tim^{01}$ with different amounts of food consumption were also compared (Figure 2D). There was no significant difference in percentages of flies with the disrupted feeding pattern among the groups of big eaters, middle eaters, and small eaters in $per^{0}$ or $tim^{01}$ in LD ($p > 0.05$, Fisher's exact test).

## A light-responsive protein QSM regulates daytime feeding rhythms in light/dark

To elucidate the mechanisms that regulate the daily feeding pattern in LD, we investigated the roles of *cryptochrome* (*cry*).[28] CRY mediates light input to molecular clocks to regulate locomotor rhythms and contributes to a morning feeding peak by suppressing feeding in the evening in LD.[20] We analyzed daily feeding patterns in two strains of *cry* null ($cry^{02}$ and $cry^{out}$),[23,29] and found that the percentages of flies showing the daytime feeding pattern in LD were similar to wild-type flies (63% and 69% ($cry^{out}$ and $cry^{02}$, respectively, Figure 3A), 66% in $iso^{31}$ (Figure 1B) and 69% in *y w* (Figure S1A)). Since *cry* resets the molecular clock by mediating the light-dependent degradation of TIM,[30] the daily feeding pattern of *cry* null flies in LD might be driven by molecular clocks as if they were in DD. To examine this possibility, we tested the daily feeding pattern in the flies lacking both *cry* and *per* ($per^{0}$;;$cry^{02}$). $per^{0}$;;$cry^{02}$ flies showed a daytime feeding pattern similar to *cry* null flies (Figure 3B). These results suggest that *cry* is not critical for daytime feeding patterns in LD.

*quasimodo* (*qsm*) codes a light-responsive factor and controls the rhythmic firing of clock neurons.[25,26] QSM regulates the circadian clock in a light-responsive manner similar to, but independently of, CRY.[25] We analyzed feeding patterns of $qsm^{104/105}$, two intragenic *gal4* insertion line that reduces *qsm* expression,[25] and flies with RNAi-mediated knockdown of *qsm* by *tim*-GAL4, which drives expression in all *timeless*-expressing cells, including clock cells and metabolic tissues (*tim>qsm* RNAi).[25] $qsm^{104/105}$ and *qsm* knockdown flies (*tim>qsm* RNAi) showed regular locomotor rhythms as previously reported in LD ([25]; Figure 3C). $qsm^{104/105}$ caused a significant reduction in the number of flies with daytime feeding patterns compared to the control (Figure 3C, $qsm^{104/105}$), and *qsm* knockdown also disrupted the daytime feeding pattern in LD (Figure 3C, *tim>qsm* RNAi). If *qsm* mediated light-mediated feeding pattern in LD, those flies would show normal feeding patterns in DD. We found that $qsm^{104/105}$ showed the daytime feeding pattern similar to the control in DD (Figure 3D, $qsm^{104/105}$). These results suggest that *qsm* is critical for the light-mediated formation of the daytime feeding pattern in LD.

## Period/timeless and clock/cycle play different roles in the expression of feeding patterns in darkness

Next, we compared daily feeding patterns in flies with null mutations in core circadian clock genes, *period* ($per^{0}$), *timeless* ($tim^{01}$), *cycle* ($cyc^{02}$), and *clock* ($Clk^{jrk}$) in DD. As previously reported, these clock mutant flies did not show a bimodal pattern of locomotor activity (Figure 4A top). Most of these flies did not show daytime feeding patterns (Figure 4A, $per^{0}$: 31%, $tim^{01}$: 32%, $cyc^{02}$: 13%, $Clk^{jrk}$: 4%). Loss of *cyc* or *Clk* caused more severe disruption in daily feeding patterns than *per*- or *tim*-null flies.

When the food intake amount in each period was normalized with the total food intake amount (Figure 4A, relative food intake), the differences between the feeding pattern of *cyc*- or *Clk*-null flies and those in *per*- or *tim*-null flies were prominent. *per*- or *tim*-null flies show bimodal feeding with a more-feeding phase (feeding period) and a less-feeding phase (fasting period); however, these phases were not synchronized and observed either during the subjective day or night (Figure 4A). In contrast, *cyc*- or *Clk*-null flies ingested food constantly; thus, the shift between feeding/fasting episodes was less prominent compared to wild type or *per*- or *tim*-null flies (Figure 4A). To express the strength of the shift between feeding/fasting episodes, we compared the deviation of the normalized food intake amount in each period from the average. These values in *per*- or *tim*-null flies were higher or similar to control flies, respectively, while they were significantly smaller in *cyc*- or *Clk*-null flies (Figure 4B).

The total amount of food ingested during the entire assay varies among circadian mutants (Figure 4C). *per*-null flies tend to ingest more food as previously reported[31] (Figure 4C), and *tim*-null flies ingested less food compared to the control: however, unlike the feeding patterns,

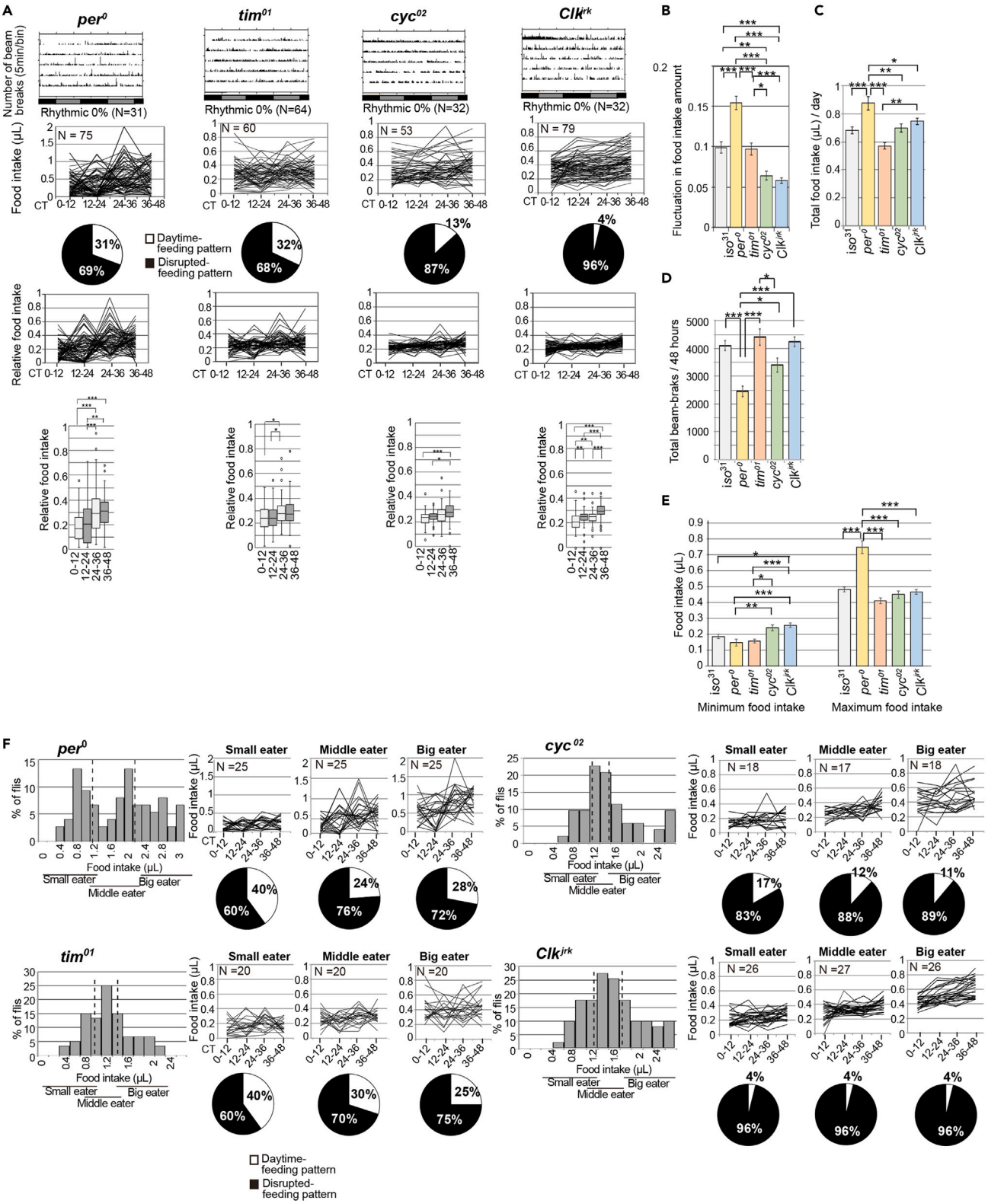


**Figure 4. Feeding/fasting episodes are desynchronized in period- or timeless-null mutants and lost in cycle- or clock-null mutants in DD**

(A) (Top) Locomotor rhythms of clock-mutant flies ($per^0$, $tim^{01}$, $cyc^{02}$, $Clk^{jrk}$) in DD. Representative actogram and percentages of flies with locomotor rhythms are shown. (Middle) Line graphs show the amount of food ingested by individual flies for every 12 h plotted over 48 h. Pie charts show the ratio of flies with daytime

**Figure 4. *Continued***

feeding patterns (white) and disrupted feeding patterns (black)). (Bottom) The amount of food ingested normalized by total food consumption and plotted over time. The same data are also shown in boxplots (Relative food intake). *p < 0.05,**p < 0.01,***p < 0.001, TukeyHSD test.

(B–E) (B) The deviation of the normalized food intake amount from the average. (C) The total amount of food consumed in 24 h. (D) Total numbers of beam-break over 48 h. (E) The minimum or maximum amounts of food ingested for 12 h over 48 h. Data expressed as mean ± SEM. *, p < 0.05. **, p < 0.01. ***, p < 0.001, TukeyHSD test.

(F) Percentages of small, middle, big eaters in $per^{0}$, $tim^{01}$, $cyc^{02}$, $Clk^{jrk}$ under DD condition. No significant differences in feeding patterns between small, middle, and big eaters were detected (p > 0.05, Fisher's extract test).

*per*- and *tim*-null and *cyc*- and *Clk*-null did not divide into two groups (Figure 4C). We also compared the numbers of total beam breaks among these genotypes to see if they are correlated with feeding patterns. The total numbers of beam breaks differed among the genotypes (Figure 4D), but *per*- and *tim*-null and *cyc*- and *Clk*-null did not divide into two groups either. These results suggest that the difference in the feeding pattern between *per*- or *tim*-null flies and that of *cyc*- or *Clk*-null flies is not likely explained by dramatic changes in metabolism or energy demands. The increase or decrease in the total food intake amount of these mutants and the total number of beam breaks were not correlated (Figure 4D), suggesting that the feeding patterns are not determined by their locomotor activity.

Reduction in the feeding rhythm strength in *cyc*- or *Clk*-null flies may be due to more food ingestion during the less-feeding episode or less food ingestion during the more-feeding episode. We calculated the minimum or maximum amounts of food ingested for any 12 h during the measurement periods for each fly, and compared if they were different from that of control flies. Loss of *cyc* or *Clk* increased the average of minimum food intake amount compared to $iso^{31}$, especially in *Clk* (p = 0.01), while the maximum amount of food intake was not increased in clock null flies (Figure 4E). The minimum food intakes were not increased in $per^{0}$ and $tim^{01}$ (Figure 4E). This result suggests that *cyc* and *Clk* mediate suppression of food intake during the fasting period.

We also compared the feeding patterns of these circadian mutant flies with different amounts of food intake, and no difference in the percentages of rhythmic eaters and arrhythmic eaters between the group of big and small eaters in each genotype (p > 0.05, Figure 4F).

These results suggest that, in the generation of bimodal feeding patterns, *Clk/cyc* is required for the generation of the fasting episode in addition to its synchronization.

## Molecular clocks in neurons synchronize the feeding/fasting episodes in darkness

To understand the differences between the roles of *per/tim* and *Clk/cyc* in bimodal feeding patterns, we aimed to determine their functions in neurons and the fat body. To analyze the role of per in neurons, PERIOD was expressed to the neurons of $per^{01}$ flies by UAS-*per* driven by the pan-neuronal *elav*-GAL4 driver[32] ($per^{01}$, *elav* > *per*). We used two independent lines carrying UAS-per, UAS-per #2-1 and UAS-per #3-1.[32] As previously reported, these flies showed restored locomotor activity rhythms,[32] indicating successful expression of PERIOD protein in neurons. The rescue *of* $per^{01}$ locomotor behavior with the line UAS-per #2-1 was poorer than that with #3-1 as reported previously (Figure 5A; [32]). We found that the supplementation of PERIOD in neurons of $per^{01}$ flies with UAS-per#3-1 significantly increased the number of individuals with the daytime feeding pattern (Figure 5A, p < 0.005). UAS-per #2-1 line also increased the number of individuals with the daytime feeding pattern, although the difference was not significant (p > 0.05). The fluctuation in food intake amount was not reduced in $per^{01}$ background (Figure 4), and the supplementation of PERIOD in neurons with either UAS-per#3-1 or UAS-per#2-1 did not affect it (Figure 5A).

The disruption of molecular clock functions by expressing a dominant negative form of CLK (dnCLK)[4] in PDF neurons lowered the number of individuals with the daytime feeding pattern, and the fluctuation in food intake amount was not significantly affected (Figure 5B). These results indicate that molecular clocks in neurons are sufficient to synchronize feeding/fasting episodes and do not affect the fluctuation in food intake amount.

## Clock/cycle in the metabolic tissues generates feeding/fasting episodes in darkness

The results with *Clk* null and *cyc* null flies suggest that *Clk/cyc* is required for the generation of the fasting episode in addition to its synchronization (Figure 4). We hypothesized that CLK/CYC functions in the fat body to generate the fasting episode.

To test this hypothesis, we examined the effects of blocking CLK functions in the fat body on feeding rhythms by expressing a dominant negative form of CLK (dnCLK) in these tissues. We found that expression of dnCLK in the fat bodydisrupted the feeding pattern in DD conditions (Figure 6A, *to* > dnCLK). The amount of food intake show a trend to increase over time (Figure 6A, *to* > dnCLK). As observed in *cyc* or *Clk* null mutants, the degree of feeding amount fluctuation was significantly reduced by the expression of dnCLK in the fat body (Figure 6A). We also analyzed the feeding pattern of the flies expressing CYC only in the fat body of $cyc^{02}$ flies ($cyc^{02}$, *to* > *cyc*).[33] When CYC was expressed in the fat body of $cyc^{02}$ flies by using the fat-body specific driver, *takeout*-GAL4 (*to*-GAL4),[34] feeding patterns remained desynchronized, and the degree of fluctuation in food intake amount was increased (Figure 6B, *to* > *cyc*; $cyc^{02}$, p < 0.001). These results suggest that CLK/CYC in the fat body mediates the generation of feeding/fasting episodes.

To further test this model, we asked whether blocking CLK functions in the fat body further disrupts the daytime feeding pattern in period null flies. $per^{01}$ shows bimodal feeding without synchronization, and expression of dnCLK in the fat body in $per^{01}$ increased the percentage of flies with disrupted feeding patterns, although the difference was not significant (p = 0.053, Figure 6C). The degree of fluctuation in food

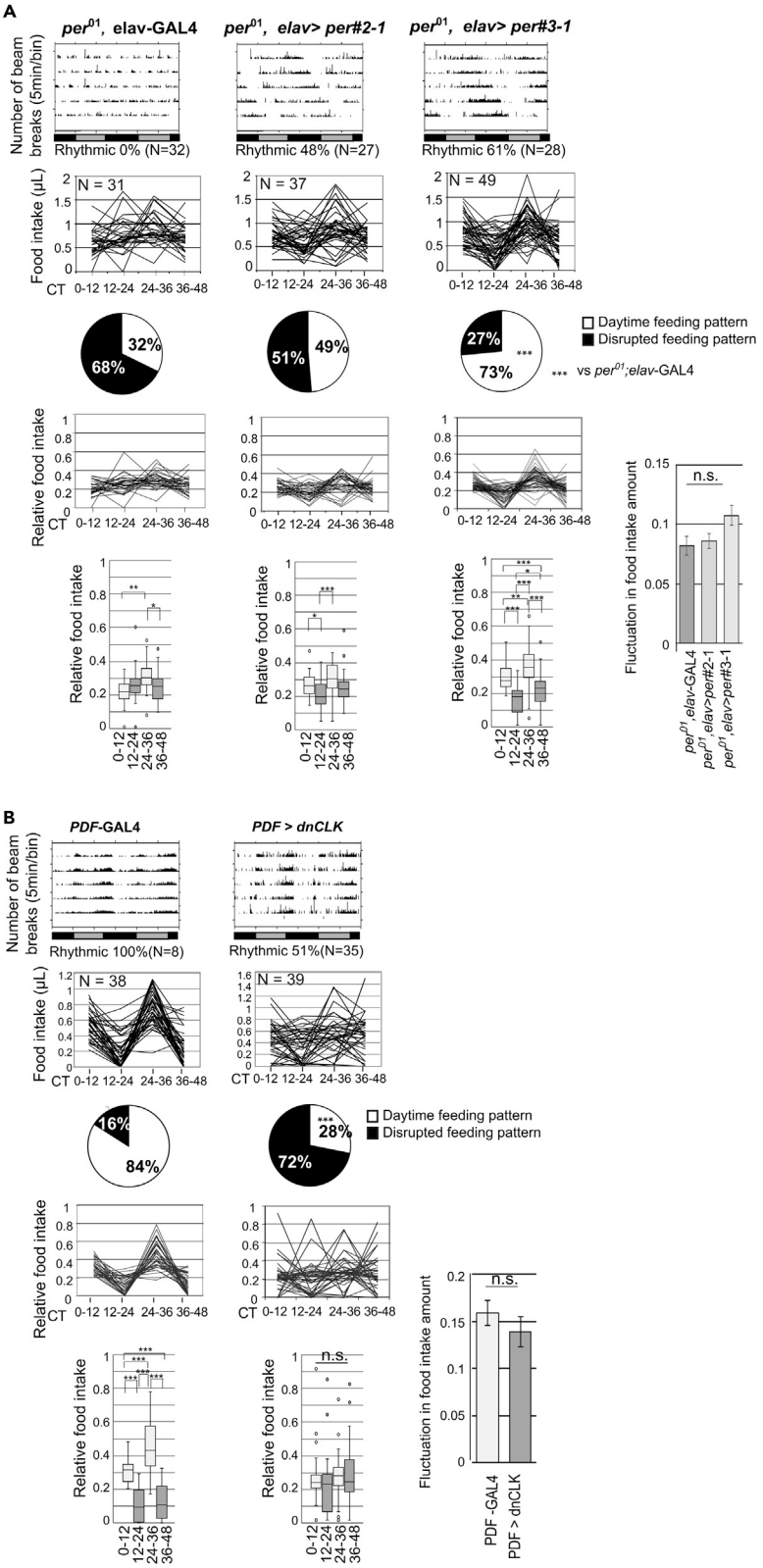
A
per01, elav-GAL4
per01, elav> per#2-1
per01, elav> per#3-1
Number of beam breaks (5min/bin)
Rhythmic 0% (N=32)
Rhythmic 48% (N=27)
Rhythmic 61% (N=28)
Food intake (μL)
N = 31
N = 37
N = 49
CT 0-12 12-24 24-36 36-48
32%
68%
51%
49%
27%
73%
***
*** vs per01;elav-GAL4
Daytime feeding pattern
Disrupted feeding pattern
Relative food intake
Fluctuation in food intake amount
n.s.
per01,elav-GAL4
per01,elav>per#2-1
per01,elav>per#3-1
B
PDF-GAL4
PDF > dnCLK
Rhythmic 100%(N=8)
Rhythmic 51%(N=35)
N = 38
N = 39
16%
84%
28%
72%
n.s.
PDF -GAL4
PDF > dnCLK

**Figure 5. The molecular clocks in neurons synchronize the feeding/fasting episodes in DD**

(A) Per expression in neurons in *per*-null background restored locomotor rhythms and daytime feeding patterns in DD. (Top) Representative actograms of locomotor activity under DD condition. (Middle) Line graphs show the amount of food ingested every 12 h plotted over time (Food intake). Pie charts show the ratio of flies with daytime- and disrupted feeding patterns (pie chart). *per*$^{0}$*; elav > per #3-1* showed significantly higher percentage of daytime-feeding pattern than *per*$^{0}$*;elav* (***p < 0.001; Fisher's extract test). (Bottom) The amount of food ingested normalized by total food consumption plotted over time. The same data are also shown in boxplots (Relative food intake). Bar plots show the deviation of the normalized food intake amount from the average (Fluctuation in food intake amount).

(B) Expression of dnCLK in PDF neuron (*PDF>dnCLK*) disrupted daytime feeding patterns in DD. (Top) Representative actograms of locomotor activity under DD condition. (Middle) The amount of food ingested every 12 h (Food intake) and percentage of flies with daytime- and disrupted feeding patterns (pie chart). *PDF > dnCLK* showed significantly lower percentage of daytime-feeding pattern than *PDF-GAL4* (***p < 0.001; Fisher's extract test). (Bottom) Relative food intake (boxplot) and fluctuation in food intake amount (bar plot). (A-B) The centerline of the boxplots indicates the median, the box displays the first and third quartile, and the whiskers show min/max values. Outliers are displayed as points. Bar plot data expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant; TukeyHSD test.

intake amount was reduced by the expression of dnCLK in the fat body (p < 0.05, Figure 6C). This result suggests that the generation of feeding/fasting episodes by CLK in metabolic tissues is additive to the functions of molecular clock machinery.

### Clock in the fat body contributes to the strength of feeding rhythms under light/dark

Finally, we asked whether the attenuation of the strength of the feeding rhythm due to CLK/CYC deficiency is also observed under LD. As for *cyc*- or *Clk*-null flies, their locomotion is suppressed under the light as previously reported[35] (Figure S2), which caused the majority of *cyc*$^{02}$ and *Clk*$^{Jrk}$ flies to show a nocturnal feeding pattern in LD (Figure S2). In contrast, blocking CLK function in the fat body by dnCLK expression did not affect locomotor activity[4] (Figure 7), allowing us to analyze the effects of light on feeding patterns without locomotor impairment. Most of these flies showed the daytime feeding pattern in LD as previously reported[4] (Figure 7), while the fluctuation of feeding amount in these flies was reduced compared to the control (Figure 7), as observed in DD (Figure 6C). These results indicate that light input can synchronize the bimodal daily feeding pattern without molecular clocks, yet CLK/CYC in the fat body contributes to the strength of feeding rhythms under LD conditions.

## DISCUSSION

Many organisms, including insects, fish, birds, rodents, and primates, show particular feeding patterns during the day.[19,36] In this study, we investigated the roles of molecular clock genes in food intake in *Drosophila*. We found that, in LD, light stimuli were sufficient to induce feeding via the QSM-mediated pathway (Figures 1, 2, and 3), while feeding rhythms in DD can be dissected into two components: generation of feeding/fasting episodes by *Clk/cyc* in metabolic tissues and their synchronization by molecular clocks in neurons (Figures 4, 5, 6, and 7). These results suggest novel roles of *qsm* and clock molecules regulating feeding behavior.

There has been a significant advance in understanding the circuits and neurotransmitters that mediate central clocks in the brain that control feeding behavior.[37,38] As for the peripheral clocks, their functions in feeding behavior seem more diverse than those in the central clocks. In addition to contributing to the regulation of 24-h feeding rhythms,[4,39] it was also reported that peripheral clocks negatively regulate food intake amount[4] and feeding rhythm strength.[39] However, it was elucidated whether the increase in food intake amount is caused by the disruption of the molecular clock machinery or other functions of CLK/CYC. Our results suggest that fat body CLK regulates food intake amount independent of molecular clocks. Since flies with Clk/cyc disruption with various genotypes consistently show a reduction in fluctuation in food intake compared to their control (i.e., *Clk*$^{Jrk}$, *cyc*$^{out}$, *cyc*$^{02}$, *to>dnClk*, *cyc*$^{02}$, *to > cyc*), it is less likely that fluctuation in food intake is due to differences in the body size or other background effects. The suppression of CLK function in the fat body reduced fluctuations of feeding amount in *per*$^{01}$ background (Figure 6C), indicating that CLK/CYC in the metabolic tissues contributes to rhythmic feeding behavior in addition to its function as components of the molecular clock (Figure 5B). It explains the different effects of clock genes on patterns of feeding rhythms: *per*- and *tim*-null mutants showed a shift in the peak of the feeding timing, and *Clk*- and *cyc*-null mutants ingest food constantly without peak of the feeding timing (Figure 4).

Our results suggest that the effect of CLK/CYC in the metabolic tissues to reduce feeding rhythm strength is likely to be mediated by suppression of feeding during the fasting period (Figure 4E). Several factors that act on suppression of food intake in peripheral tissues have been reported,[40,41] and expression of some of these genes, such as allatostatin A and its receptor, are regulated by peripheral CLK/CYC.[41] In addition, rhythmic expression of the *allatostatin A receptor -2* gene is affected by the disruption of the fat body clock.[4] CLK is also associated with cAMP-responsive element binding protein (CREB), which is involved in the energy homeostasis of insects and mammals. Blocking CREB activity in the fat body increases food intake in flies,[42] and Nejire, a homolog of CREB-binding protein (CBP)/p300, has been reported as a regulator of CLK/CYC-dependent transcription.[43,44] Further studies on these pathways may reveal a novel signaling axis that constitute the feeding/fasting cycle.

We found the novel role of *qsm* in feeding behavior in LD (Figure 3). It has been reported that *cry*-null mutants do not display the morning peak in LD.[20] We also observed that *cry*-null mutants do not display the early morning peak in LD, while these flies still showed the synchronized feeding/fasting episodes in LD (Figure 3A). In addition, the feeding/fasting episodes in DD were observed without the morning peak. Thus, the morning peak is not associated with the daytime feeding pattern we focused on in this study. Our results revealed that *qsm* is indispensable in the daytime feeding pattern (Figure 3). *qsm* encodes a ZP (Zona Pellucida) domain and constitutes part of CRY-independent light input to the circadian clock.[25] QSM is expressed in many cells in the immediate proximity of clock neurons,[25] and it is not clear in which cells

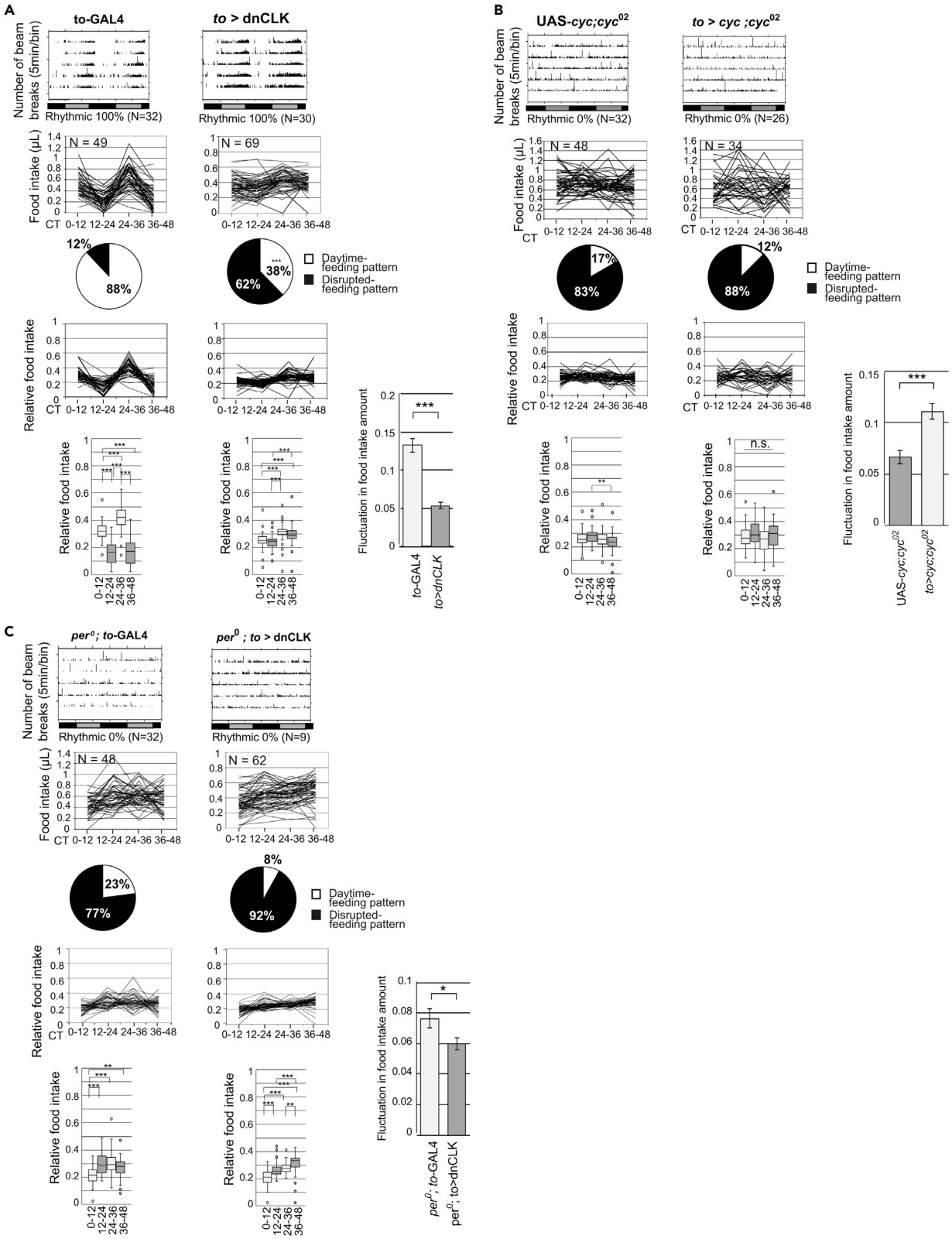
A
to-GAL4
to > dnCLK
Number of beam breaks (5min/bin)
Rhythmic 100% (N=32)
Rhythmic 100% (N=30)
Food intake (μL)
N = 49
N = 69
CT 0-12 12-24 24-36 36-48
12%
88%
62%
38%
***
Daytime-feeding pattern
Disrupted-feeding pattern
Relative food intake
Fluctuation in food intake amount
to-GAL4
to>dnCLK
B
UAS-cyc;cyc02
to > cyc ;cyc02
Rhythmic 0% (N=32)
Rhythmic 0% (N=26)
N = 48
N = 34
17%
83%
12%
88%
n.s.
UAS-cyc;cyc02
to>cyc;cyc02
C
per0; to-GAL4
per0 ; to > dnCLK
Rhythmic 0% (N=32)
Rhythmic 0% (N=9)
N = 48
N = 62
23%
77%
8%
92%
per0; to-GAL4
per0; to>dnCLK

**Figure 6. CLK/CYC in the fat body generates feeding/fasting episodes in DD**

(A) Flies expressing dominant-negative CLK in the fat body (*to > dnCLK*) showed normal locomotor activity rhythms and disrupted diurnal feeding rhythms in DD. (Top) Representative actograms of locomotor activity. (Middle) Line graphs show the amount of food ingested every 12 h plotted over time (Food intake). Pie charts show the ratio of flies with daytime- and disrupted feeding patterns. *to > dnCLK* showed significantly lower percentage of daytime-feeding pattern compared to *to-GAL4* (***p < 0.001; Fisher's extract test). (Bottom) Relative food intake (boxplot) and fluctuation in food intake amount (bar plot: mean ± SEM, ***, p < 0.001; Student's *t* test).

(B) Expression of cyc in the fat body in *cyc* null flies did not rescue the daytime feeding patterns but restored the fluctuation of food intake amount. (Top) Representative actograms of locomotor activity. (Middle) The amount of food ingested every 12 h (Food intake) and percentage of flies with daytime- and disrupted feeding patterns (pie chart). No significant difference between *UAS-cyc; $cyc^{02}$* and *to > cyc;$cyc^{02}$* (p > 0.05; Fisher's extract test). (Bottom) The amount of food ingested normalized by total food consumption plotted over time. The same data are also shown in boxplots (Relative food intake). Bar plots show the deviation of the normalized food intake amount from the average (Fluctuation in food intake amount). Expression of cyc in the fat body of *$cyc^{02}$* flies (*to > cyc;$cyc^{02}$*) increased the fluctuation of feeding amount. Mean ± SEM. ***, p < 0.005; Student's *t* test.

(C) Expression of dnCLK in the fat body of *$per^0$* flies (*$per^0$; to > dnCLK*) further reduced the percentage of flies with the daytime feeding pattern. (Top) Representative actograms of locomotor activity. (Middle) Food intake patterns and percentages of flies with daytime- and disrupted feeding patterns. *$per^0$; to > dnCLK* flies show lower percentage of daytime-feeding pattern compared to *$per^0$; to* (p = 0.053; Fisher's extract test). (Bottom) Relative food intake and fluctuation in food intake amount. Expression of dnCLK in the fat body of *$per^0$* flies (*$per^0$; to > dnCLK*) reduced the fluctuation of feeding amount. Mean ± SEM. *, p < 0.05; Student's *t* test. For A-C, the centerline of the boxplots indicates the median, the box displays the first and third quartile, and the whiskers show min/max values. Outliers are displayed as points. *p < 0.05, ***p < 0.001; TukeyHSD test.

QSM influences the feeding pattern for now. Further study to understand where and how the QSM regulates feeding rhythms in LD would help us understand light-induced regulation of feeding behavior.

In summary, our results revealed novel pathways that regulate the formation of feeding rhythms in *Drosophila*. Feeding/fasting rhythms coordinate metabolism and affect aging and life span.[45] Further studies of these axes may contribute to human health.

## Limitation of the study

In this study, we successfully dissected the molecular pathways that regulate feeding patterns. However, our analyses are limited to several key components, and their upstream and downstream molecules remain to be elucidated. We found that *qsm* regulates feeding rhythms under light:dark conditions, while its downstream signaling is unknown. We also found that, under constant darkness conditions, CLK/CYC in digestive/metabolic tissues generates feeding/fasting episodes, and the molecular clock in neurons synchronizes them. However, molecular mechanisms that link those two components remain to be elucidated. Further studies are needed to understand the entire picture of the molecular pathways that generate feeding patterns.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
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  - CAFE assay
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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108164.

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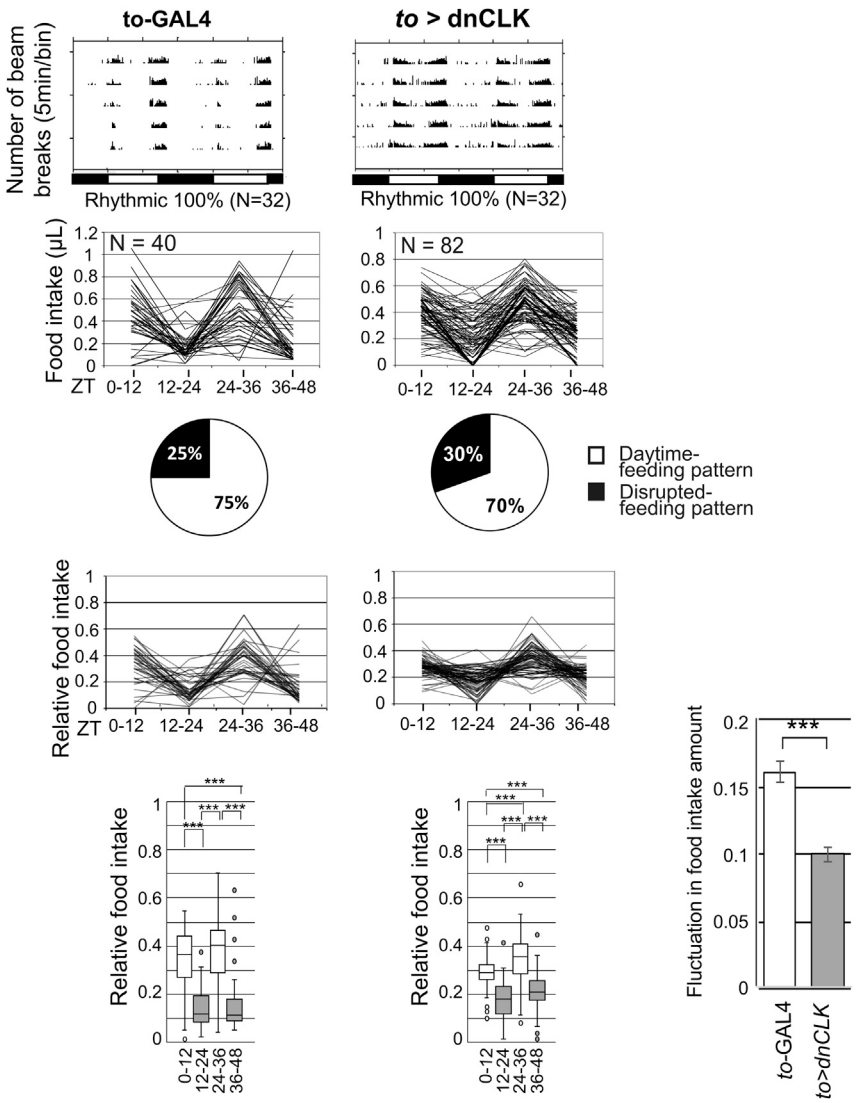


**Figure 7. CLK in the fat body contributes to the strength of feeding rhythms under LD**

(Top) Flies expressing dominant-negative CLK in the fat body (*to > dnCLK*) showed normal locomotor activity rhythms in LD. Representative actograms of locomotor activity. (Middle) Line graphs show the amount of food ingested every 12 h plotted over time (Food intake). Pie charts show the ratio of flies with daytime- and disrupted feeding patterns are shown in pie charts. No significant difference between *to-GAL4* and *to > dnCLK* ($p > 0.05$; Fisher's extract test). (Bottom) The amount of food ingested normalized by total food consumption plotted over time. The same data are also shown in boxplots (Relative food intake) (***$p < 0.001$; TukeyHSD test). Bar plots show the deviation of the normalized food intake amount from the average (Fluctuation in food intake amount). *to > dnCLK* showed significantly reduced fluctuation in feeding amount. mean ± SEM, ***, $p < 0.001$; Student's *t* test. See also Figure S2.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AM, KMI, and KA. Performed the experiments: AM. Analyzed the data: AM and KA. Wrote the article: AM and KA.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| glass micropipettes (5 μL) | VWR | catalog no. 53432-706 |
| FD&C Blue No.1 | Sigma Aldrich | 80717 |
| Deposited data | | |
| CAFE assay data (amount of food intake) | This paper; Mendeley Data | https://doi.org/10.17632/2h63jfn9pw.1 |
| Experimental models: Organisms/strains | | |
| D.melanogaster. *iso*$^{31}$ | Ryder et al.[46] | N/A |
| D.melanogaster. *per*$^{0}$ | Sehgal et al.[47,48] | RRID:BDSC_80917<br>FlyBase ID: FBal0013649 |
| D.melanogaster. *tim*$^{01}$ | Sehgal et al.[47,48] | RRID:BDSC_80930<br>FlyBase ID: FBgn0014396 |
| D.melanogaster. *cyc*$^{02}$ | Tanoue et al.[33] | FlyBase ID: FBal0105033 |
| D.melanogaster. *Clk*$^{jrk}$*/TM6Sb* | Bloomington Drosophila Stock Center | RRID:BDSC_80927<br>FlyBase ID: FBst0080927 |
| D.melanogaster. *cry*$^{out}$ | Chen et al.[25] | RRID:BDSC_19331<br>FlyBase ID: FBti0042954 |
| D.melanogaster. *cry*$^{02}$ | Chen et al.[25] | RRID:BDSC_86267<br>FlyBase ID: FBst0086267 |
| D.melanogaster. *y w(ls-tim)* | Chen et al.[25] | FlyBase ID: FBal0211083 |
| D.melanogaster. *qsm* $^{104}$ | Chen et al.[25] | N/A |
| D.melanogaster. *qsm* $^{105}$ | Chen et al.[25] | N/A |
| D.melanogaster. *tim-qsmRNAi* | Chen et al.[25] | N/A |
| D.melanogaster. *UAS-per2-1* | Sehgal et al.[32] | FlyBase ID: FBti0017419 |
| D.melanogaster. *UAS-per3-1* | Sehgal et al.[32] | RRID:BDSC_80685<br>FlyBase ID: FBst0080685 |
| D melanogaster. *elav*$^{C155}$*-GAL4* | Sehgal et al.[32] | RRID:BDSC_6920 |
| D.melanogaster. *PDF-GAL4* | Bloomington Drosophila Stock Center | RRID:BDSC_6899<br>FlyBase ID: FBst0006899 |
| D.melanogaster. *UAS-dnCLK* | Sehgal et al.[4] | FlyBase ID: FBal0241182 |
| D.melanogaster. *UAS-cyc* | Tanoue et a.[33] | N/A |
| D melanogaster. to-GAL4 | Bloomington Drosophila Stock Center | RRID:BDSC_80938<br>FlyBase ID: FBst0080938 |
| D.melanogaster. *y w* | Bloomington Drosophila Stock Center | RRID:DGGR_101254<br>FlyBase ID: FBst0300266 |
| Software and algorithms | | |
| MATLAB | The MathWorks, Inc. | https://matlab.mathworks.com/ |
| R v.4.2.3 | R Core Tram | https://www.r-project.org/ |
| Other | | |
| DAMSystem | TriKinetics Inc. | https://trikinetics.com/ |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kanae Ando (k_ando@tmu.ac.jp).

### Materials availability

This study did not generate new/unique *Drosophila* lines.

### Data and code availability

- CAFE assay data have been deposited on Mendeley and are publicly available as of the data of publication. DOI is listed in the key resources table.
- All original code has been deposited at Mendeley and is publicly available as of the date of publication. DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

### Fly stocks and dietary conditions

*Drosophila melanogaster* were reared on standard cornmeal media and entrained for three days in 12-h: 12-h light: dark cycles at 25°C. Isogenic w1118 (*iso31*)[46] and *y w* (RRID:DGGR_101254) were used as wild-type strains. $per^{0}$ (RRID:BDSC_80917),[47,48] $tim^{01}$ (RRID:BDSC_80930),[47,48] $cyc^{02}$,[33] *Clkjrk* (RRID:BDSC_80927),[32] $cry^{0225}$ (RRID:BDSC_86267) were kind gifts from Dr. Kyunghee Koh. UAS-*cyc* flies were a kind gift from Dr. Paul E. Hardin.[33] $elav^{C155}$-*GAL4* (RRID:BDSC_6899), UAS-*P(per)2-1*,[32] UAS-*P(per)3-1* (RRID:BDSC_80685),[30] and UAS-*dnCLK*,[4] *takeout (to)-GAL4* (RRID:BDSC_80938) flies were a kind gifts from Dr. Amita Sehgal. $cry^{out}$ (RRID:BDSC_19331), *qsm104/+*, *qsm105/+*, *tim-qsmRNAi*, and *y w (ls-tim)* used as this control were a kind gifts from Dr. Ralf Stanewsky.[25] We used male flies aged 1–3 days after eclosion for all experiments.

## METHOD DETAILS

### CAFE assay

CAFE assay[27] was carried out with the following modification. Male flies aged 1–3 days were individually transferred to CAFE vials with 1% agar at the bottom and fed 5% sucrose solution containing 1% FD&C Blue No.1 in calibrated glass micropipettes (5 μL, catalog no. 53432-706; VWR, West Chester, PA). A mineral oil (0.1 μL) was layered on top to minimize evaporation. For the light: dark cycle (LD) measurements, flies were habituated in the vials for two days, then the amount of food ingested was measured (Figure 1A). For measurement in the constant darkness (DD), flies were entrained in LD for three days, habituated in the CAFE vials for one day in LD, then shifted to DD. Measurement starts after another day of habituation. The amount of food ingested was measured every 12 h over two days. Food was refilled during the training period, which allows measurement without a refill during the measurement period.

We added several modifications to minimize experiment variations. First, to reduce the evaporation of food, the vials were wrapped tightly with parafilm and kept in an incubator with 70% humidity. Second, to reduce the disturbance caused by moving microcapillaries during measurement, food volume was recorded by marking capillaries with fine marker pens without removing them from the vial.

Non-absorbable dye was added to the sucrose solution to confirm that the fly ingested food from the capillary. Flies that ingested food show blue in the gut, visible through the cuticle in the abdomen. Some flies did not ingest food even after the habituation period, which may be due to insufficient physical ability. These flies were removed from analyses. Flies with food ingestion survived in CAFE vials until the end of the measurement.

After the recording period, the distance between marks at two consecutive time points was determined by using a microscale under a magnifying glass. The amount of the liquid evaporated was measured with a microcapillary placed in a CAFE vial without a fly in the same incubator.

### Analyses of feeding patterns

Food intake amount was recorded every 12 h for two days for each fly (ZT/CT0-12, 12–24, 24–36, 36–48). Feeding patterns were categorized into 'daytime feeding pattern, and 'disrupted feeding pattern': 'daytime feeding pattern' in which the flies ingest more food during the day or subjective day in both the first day and second day, and 'disrupted feeding pattern,' in which the flies show more food ingestion during the night or subjective night, inconsistent patterns between the first and second day, or steady increases or decreases. 'Relative food intake' was calculated by dividing the food intake amount in each time period by the total food intake of the same individual. The degree of fluctuation in food intake amount was evaluated with the standard deviation values of relative food intake. The minimum and maximum food intakes were the lowest and highest for 48 h, respectively. The average of day 1 (ZT0-24) and day 2 (ZT24-48) was used as the daily food intake.

### Locomotor behavior

Circadian locomotor analyses were carried out using Drosophila Activity Monitoring System (DAMS) (Trikinetics). Male flies (1–3 days after eclosion) were entrained to a 12-h: 12-h LD for three days and loaded into locomotor assay tubes containing 5% sucrose and 1% agarose. Their activity was monitored for at least six days in LD and DD conditions. Data were analyzed using the tau (Minimitter) and MATLAB (MathWorks, Natic, MA).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics were carried out with the R v.4.2.3 (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.) The number of replicates, n, precision measurements, and the definition of error bars are indicated in Figure Legends. Data are shown as mean ± SEM. For pairwise comparisons, Student's *t* test was performed. For multiple comparisons, data were analyzed with Tukey's HSD multiple-comparisons test. A chi-square test was used to determine if there were statistically significant differences in daytime feeding peak time within groups. Fisher's exact test was used to determine if there were statistically significant differences in daytime feeding patterns between groups. A p value of less than 0.05 was considered to be statistically significant.