Supplementary Material

Systemic oscillator-driven and nutrient-responsive hormonal regulation of daily expression rhythms for gluconeogenic enzyme genes in the mouse liver

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Supplementary methods

Quantitative real-time PCR

One hundred ng of total RNA was reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Specific primer pairs were as follows: *Bmal1* (140 bp product), 5'-AATCCTGTGGACCTTTGAGC-3' (forward) and 5'-TGAAGTCGCTGATGGTTGAG-3' (reverse:); *18S rRNA* (124 bp product), 5'-GACTCAACACGGGAAACCTC-3' (forward) and 5'-AACCAGACAAATCGCTCCAC-3' (reverse). Quantitative real-time PCR analysis was performed with Brilliant II Fast SYBR Green QPCR Master Mix reagents (Stratagene, La Jolla, CA, USA) and an Mx3000P instrument (Stratagene). Relative product levels of *Bmal1* were normalized to those of *18S rRNA*.

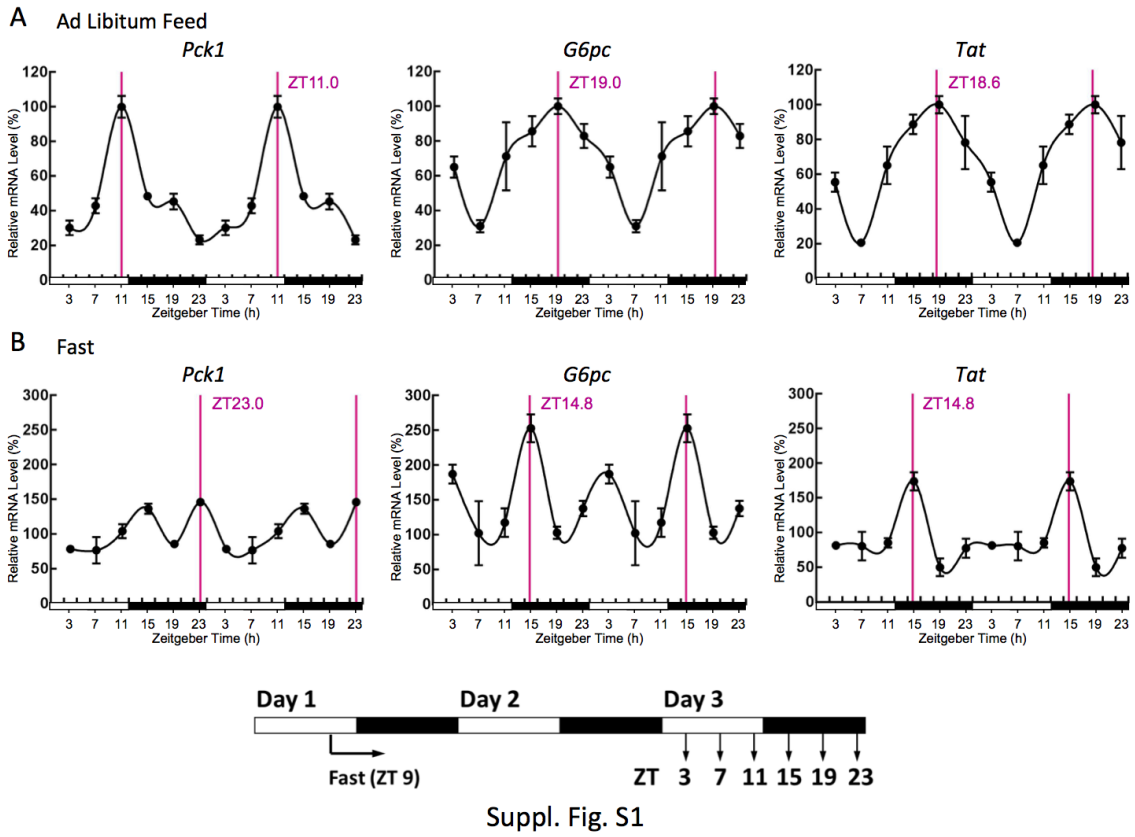
Spline curve fitting analysis for peak and bottom phases of mRNA rhythms

Since daily rhythmicity or fluctuation detected by one-way ANOVA in the present study does not necessarily show significant cosine curve fitting with the program Acro of Circadian Rhythm Laboratory (http://www.circadian.org/softwar.html) nor JTK_CYCLE (Hughes et al. 2010) (data not shown), peak and bottom phases of mRNA rhythms were calculated by cubic spline analysis using the software GraphPad Prism version 7.0.3 (GraphPad Software, La Jolla, CA, USA) with segments of 0.2 h over the 44 h period of double-plotted data for mRNA levels.

Supplementary reference

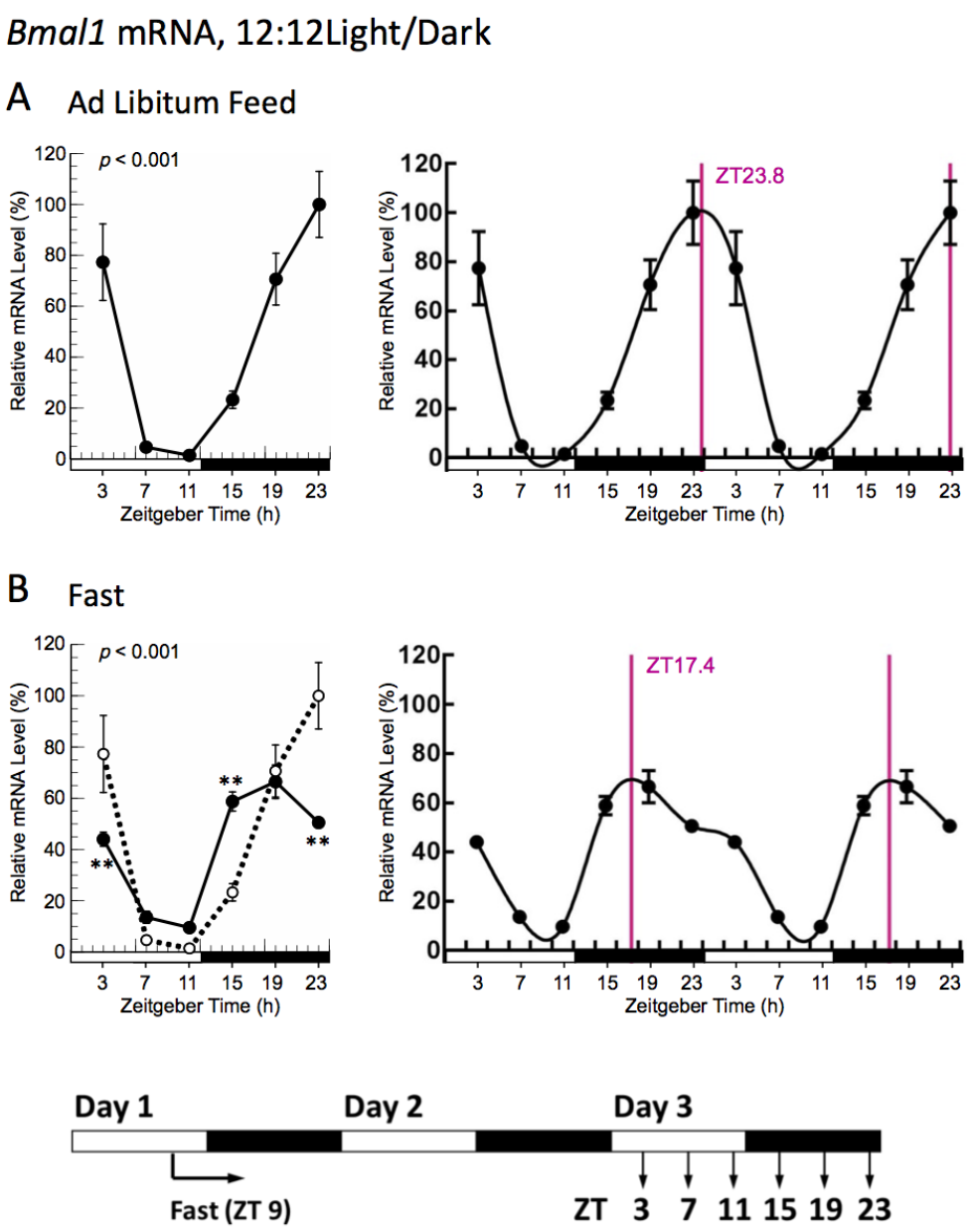
Hughes ME, Hogenesch JB, Kornacker K. 2010. JTK_CYCLE: An efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J Biol Rhythms. 25:372-80.

Supplementary figure legends



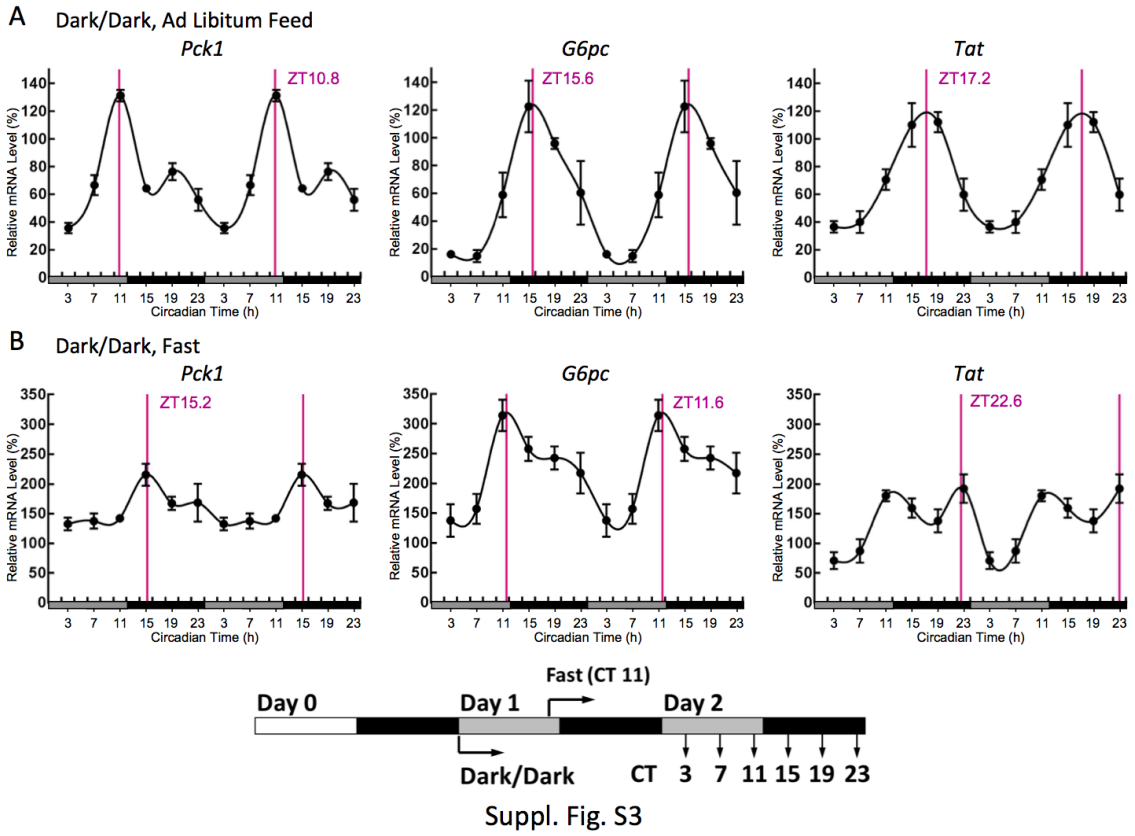
Suppl. Fig. S1

Supplementary Figure S1. Spline curve fitting analysis to calculate peak phases for daily rhythms of mRNA levels for *Pck1, G6pc,* and *Tat* in the mouse liver on feeding *ad libitum* (A) and prolonged fasting (B), related to Figure 1. Cubic spline analysis was performed with the double-plotted data of Figure 1. Red lines indicate peak phases labeled with Zeitgeber time (ZT).



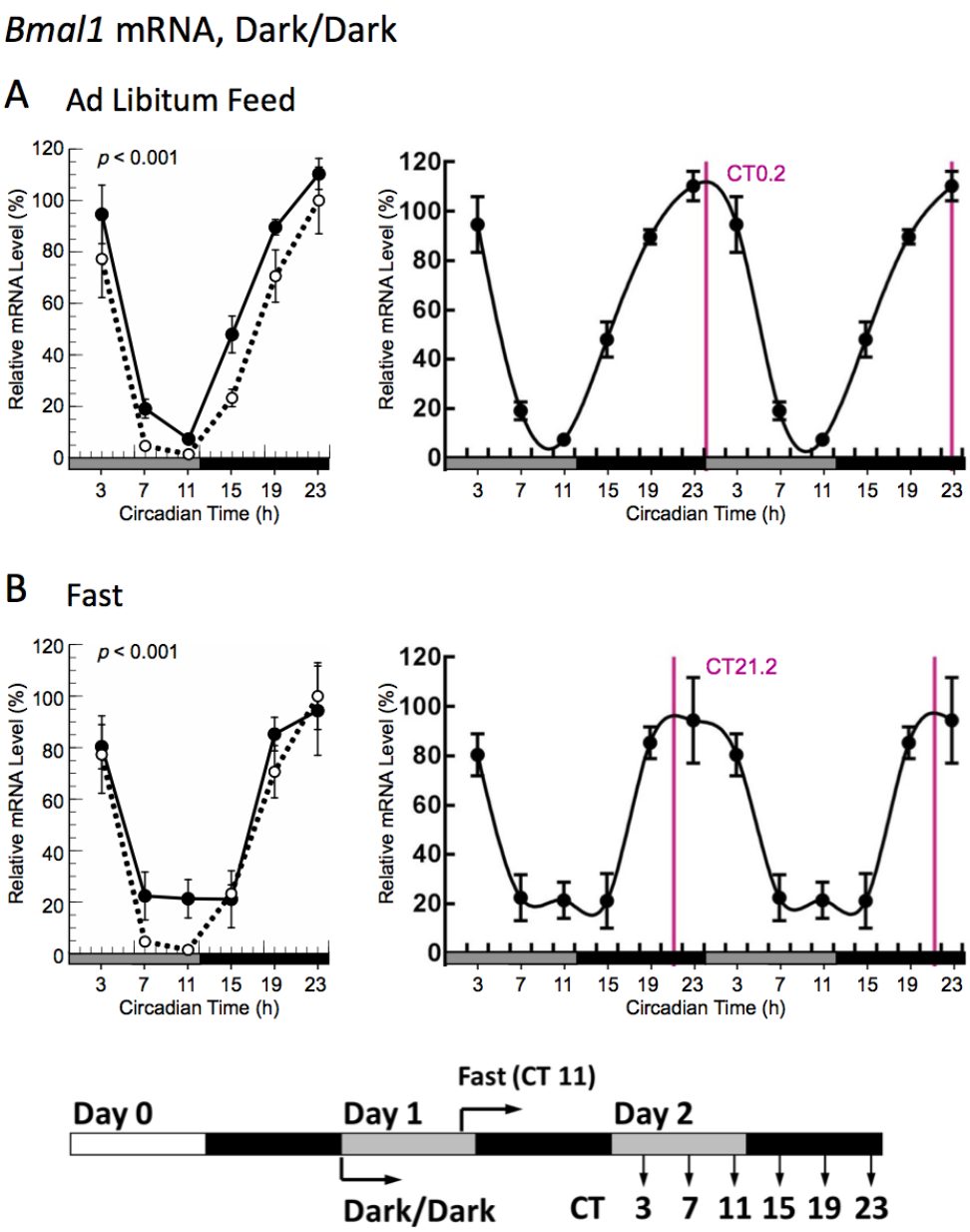
Suppl. Fig. S2

Supplementary Figure S2. Daily rhythms of mRNA levels for *Bmal1* in the mouse liver on feeding *ad libitum* (A) and prolonged fasting (B), related to Figure 1. (Left panels) Total RNA prepared as in Figure 1 was subjected to quantitative real-time PCR analysis. Results for *Bmal1* mRNA levels relative to the maximum value (100%) in A are represented as mean ± SEM. *p* values for rhythmicity were assessed by one-way ANOVA. The broken lines in B represent the results reproduced from A. $^{**}p < 0.01$ in comparison with A in each time point (two-way ANOVA followed by pairwise comparisons with Bonferroni correction). (Right panels) Cubic spline analysis to calculate peak phases was performed with the double-plotted data of left panels. Red lines indicate peak phases labeled with Zeitgeber time (ZT).



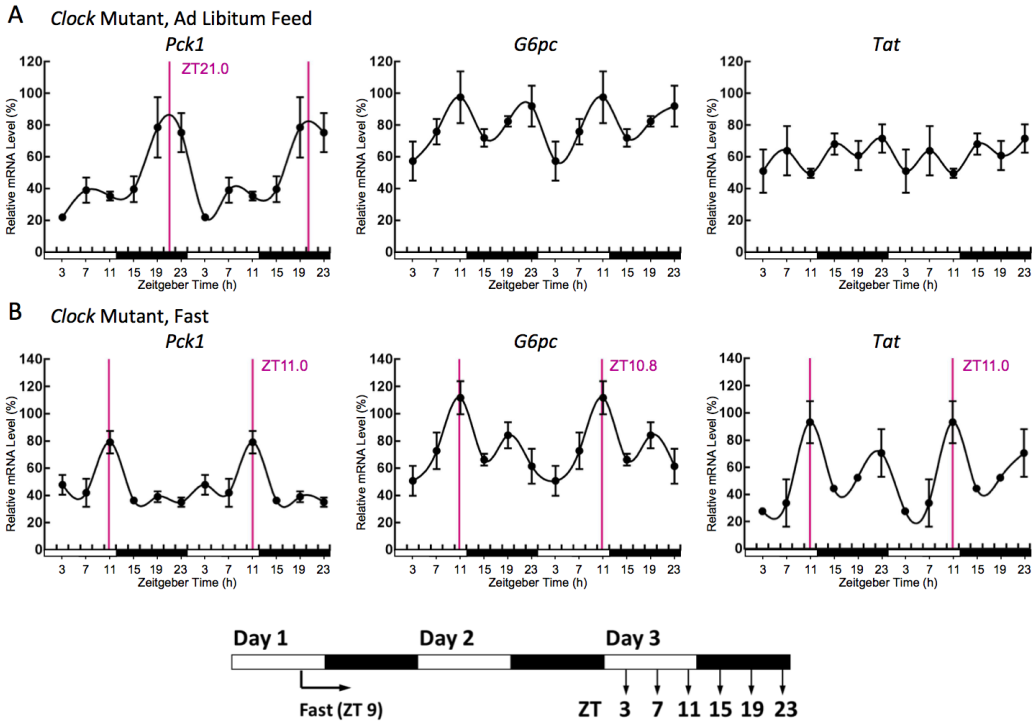
Suppl. Fig. S3

Supplementary Figure S3. Spline curve fitting analysis to calculate peak phases for circadian rhythms of mRNA levels for *Pck1, G6pc,* and *Tat* in constant darkness on feeding *ad libitum* (A) and prolonged fasting (B), related to Figure 2. Cubic spline analysis was performed with the double-plotted data of Figure 2. Red lines indicate peak phases labeled with circadian time (CT).



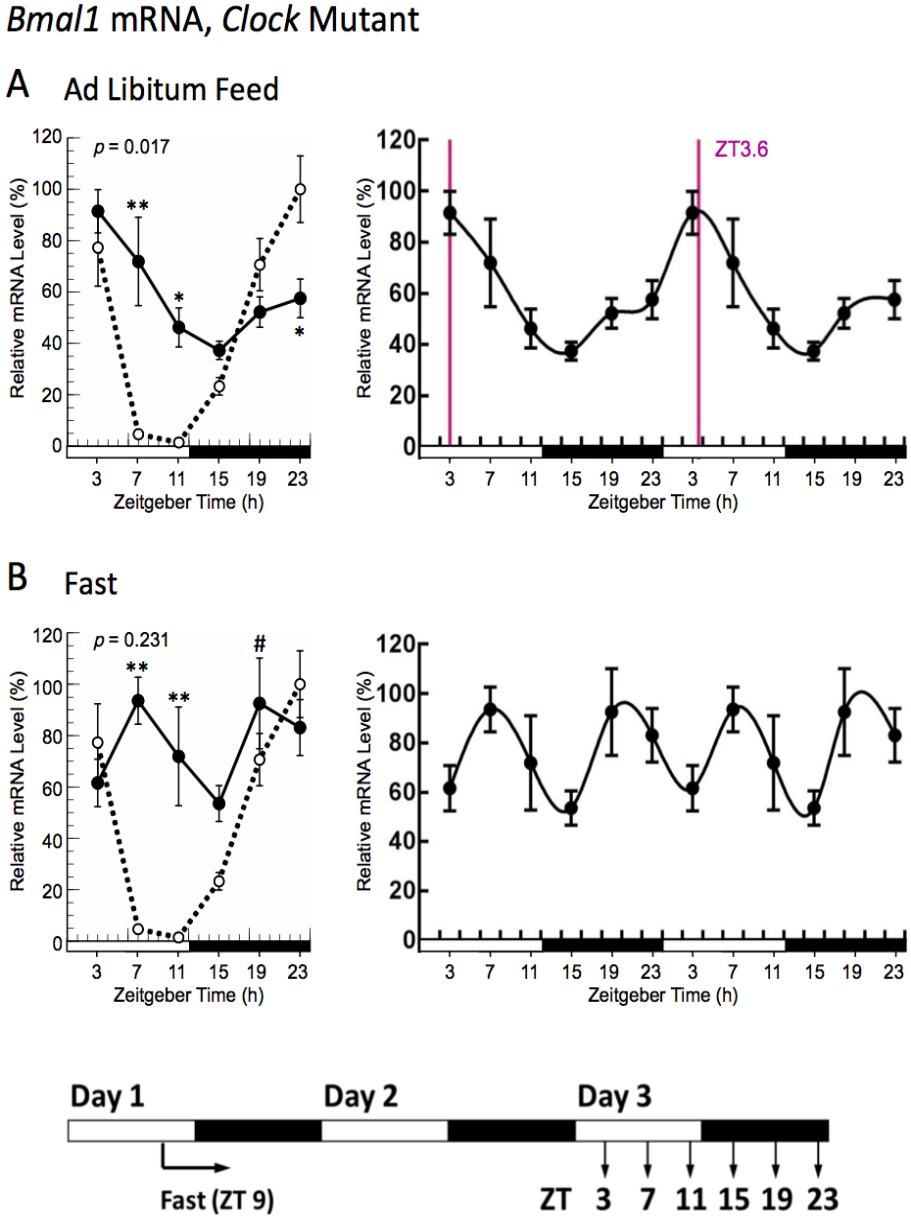
Suppl. Fig. S4

Supplementary Figure S4. Circadian rhythms of mRNA levels for *Bmal1* in constant darkness on feeding *ad libitum* (A) and prolonged fasting (B), related to Figure 2. (Left panels) Total RNA prepared as in Figure 2 was subjected to quantitative real-time PCR analysis to examine the rhythmicity of *Bmal1* mRNA levels, as described in the legend of Supplementary Figure S2. The broken lines represent the results reproduced from Supplementary Figure S2A. No significant difference among varied lighting and dietary groups was detected by two-way ANOVA. (Right panels) Cubic spline analysis to calculate peak phases was performed with the double-plotted data of left panels. Red lines indicate peak phases labeled with circadian time (CT).



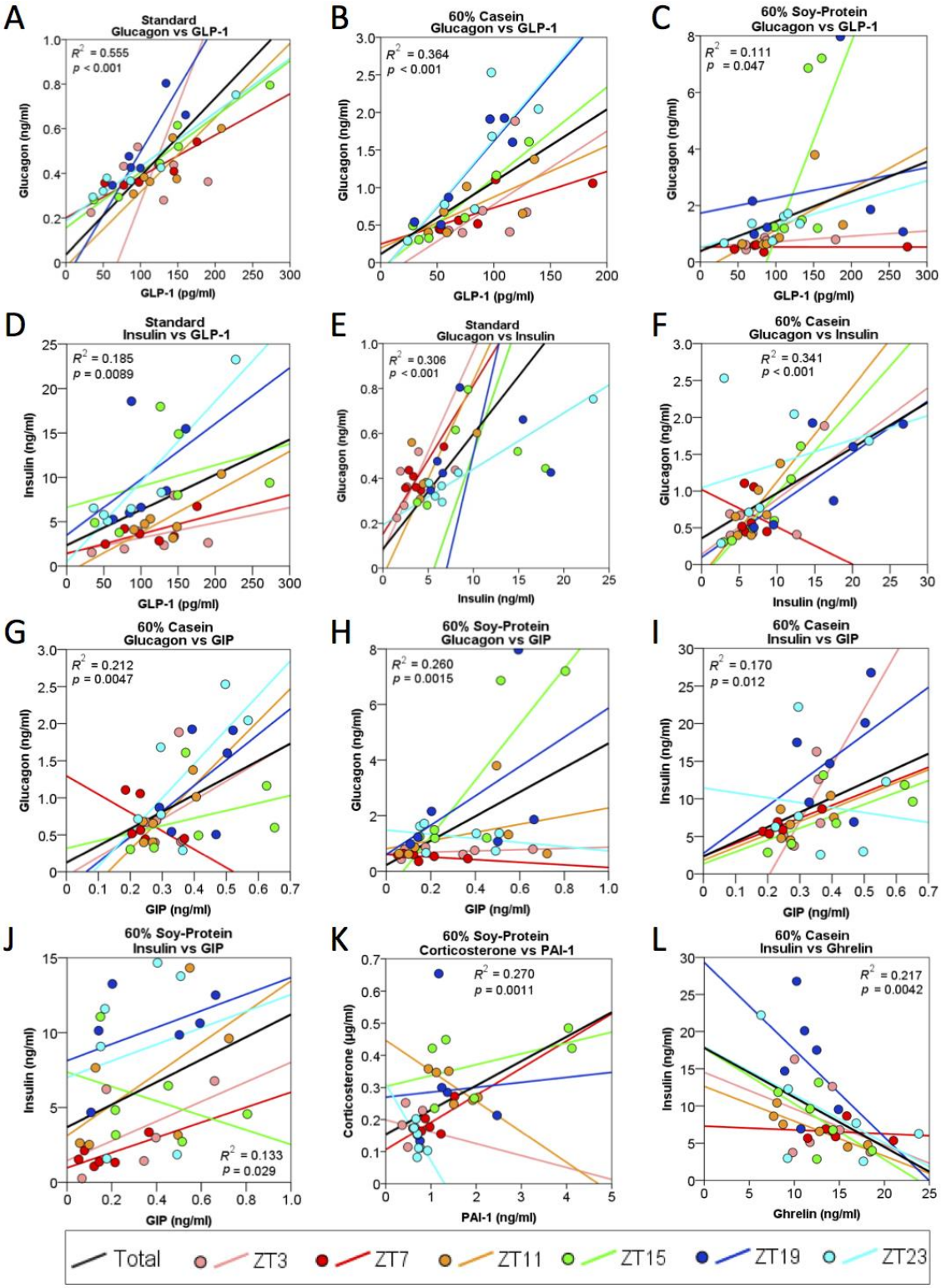
Suppl. Fig. S5

Supplementary Figure S5. Spline curve fitting analysis to calculate peak phases for daily rhythms of mRNA levels for *Pck1, G6pc,* and *Tat* in *Clock* mutant mice on feeding *ad libitum* (A) and prolonged fasting (B), related to Figure 3. Cubic spline analysis was performed with the double-plotted data of Figure 3. Red lines indicate peak phases labeled with Zeitgeber time (ZT), but are not shown when significant rhythmicity was not detected by one-way ANOVA in Figure 3.



Suppl. Fig. S6

Supplementary Figure S6. Daily rhythms of mRNA levels for *Bmal1* in *Clock* mutant mice on feeding *ad libitum* (A) and prolonged fasting (B), related to Figure 3. (Left panels) Male *Clock* mutant (mutation-homozygous) mice were examined for the rhythmicity of *Bmal1* mRNA levels, as described in the legend of Supplementary Figure S2. The broken lines represent the results reproduced from Figure S1A. *$p < 0.05$, **$p < 0.01$ in comparison with Figure S1A in each time point; $^{\#}p < 0.05$ for prolonged fasting (B) versus *ad libitum* feeding (A) in each time point (two-way ANOVA followed by pairwise comparisons with Bonferroni correction). (Right panels) Cubic spline analysis to calculate peak phases was performed with the double-plotted data of left panels. Red lines indicate peak phases labeled with Zeitgeber time (ZT), but are not shown when significant rhythmicity was not detected by one-way ANOVA in left panels.


A
Standard
Glucagon vs GLP-1
R^2 = 0.555
p < 0.001
Glucagon (ng/ml)
GLP-1 (pg/ml)
B
60% Casein
Glucagon vs GLP-1
R^2 = 0.364
p < 0.001
Glucagon (ng/ml)
GLP-1 (pg/ml)
C
60% Soy-Protein
Glucagon vs GLP-1
R^2 = 0.111
p = 0.047
Glucagon (ng/ml)
GLP-1 (pg/ml)
D
Standard
Insulin vs GLP-1
R^2 = 0.185
p = 0.0089
Insulin (ng/ml)
GLP-1 (pg/ml)
E
Standard
Glucagon vs Insulin
R^2 = 0.306
p < 0.001
Glucagon (ng/ml)
Insulin (ng/ml)
F
60% Casein
Glucagon vs Insulin
R^2 = 0.341
p < 0.001
Glucagon (ng/ml)
Insulin (ng/ml)
G
60% Casein
Glucagon vs GIP
R^2 = 0.212
p = 0.0047
Glucagon (ng/ml)
GIP (ng/ml)
H
60% Soy-Protein
Glucagon vs GIP
R^2 = 0.260
p = 0.0015
Glucagon (ng/ml)
GIP (ng/ml)
I
60% Casein
Insulin vs GIP
R^2 = 0.170
p = 0.012
Insulin (ng/ml)
GIP (ng/ml)
J
60% Soy-Protein
Insulin vs GIP
R^2 = 0.133
p = 0.029
Insulin (ng/ml)
GIP (ng/ml)
K
60% Soy-Protein
Corticosterone vs PAI-1
R^2 = 0.270
p = 0.0011
Corticosterone (µg/ml)
PAI-1 (ng/ml)
L
60% Casein
Insulin vs Ghrelin
R^2 = 0.217
p = 0.0042
Insulin (ng/ml)
Ghrelin (ng/ml)
Total
ZT3
ZT7
ZT11
ZT15
ZT19
ZT23

Suppl. Fig. S7

Supplementary Figure S7. Examples of correlations between blood humoral factors. Scatter plots between a pair of plasma concentrations of humoral factors on a nutritional condition are shown: between glucagon and GLP-1 (A-C) on the standard (A), 60% casein (B), and 60% soy-protein diets (C); between insulin and GLP-1 on the standard diet (D); between glucagon and insulin (E and F) on the standard (E) and 60% casein diets (F); between glucagon and GIP (G and H) on the 60% casein (G) and 60% soy-protein diets (H), between insulin and GIP (I and J) on the 60% casein (I) and 60% soy-protein diets (J); between corticosterone and PAI-1 on the 60% soy-protein diet (K); and between insulin and ghrelin on the 60% casein diet (L). The line for total samples is shown in black, and the line and circles for each time point are colored as indicated in the bottom box.

Supplementary Table S1. Summary of spline curve fitting analysis for peak and bottom phases of daily mRNA rhythms for gluconeogenic enzyme genes.

			*Pck*1				*G6pc*				*Tat*			
			Peak		Bottom		Peak		Bottom		Peak		Bottom	
		Group	Phase (h)	Level (%)	Phase (h)	Level (%)	Phase (h)	Level (%)	Phase (h)	Level (%)	Phase (h)	Level (%)	Phase (h)	Level (%)
Figure 1	A	Ad Libitum Feed	11.0	100.0	23.6	22.4	19.0	100.0	6.8	31.0	18.6	100.4	6.8	20.5
	B	Fast	23.0	146.0	4.8	67.0	14.8	252.9	8.8	82.7	14.8	174.6	20.0	41.5
Figure 2	A	Dark/Dark, Ad Libitum Feed	10.8	131.4	3.2	35.6	15.6	124.1	5.0	9.2	17.2	119.2	4.6	35.0
	B	Dark/Dark, Fast	15.2	215.8	4.0	130.6	11.6	318.3	4.8	122.6	22.6	194.2	4.6	54.1
Figure 3	A	*Clock* Mutant, Ad Libitum Feed	21.0	86.5	3.6	20.5	[10.6]	[98.0]	[3.6]	[56.0]	[22.6]	[71.9]	[11.0]	[49.6]
	B	*Clock* Mutant, Fast	11.0	79.1	16.2	32.0	10.8	112.1	2.4	50.4	11.0	93.2	4.8	18.6
Figure 4	A	6-h Light/18-h Dark	5.6	79.4	12.2	13.3	16.6	124.3	2.4	37.3	10.0	119.3	23.2	60.2
	B	18-h Light/6-h Dark	[14.2]	[44.4]	[3.2]	[16.5]	23.0	104.5	11.0	14.6	22.8	113.9	11.2	29.6
Figure 5	A	Day Feed	2.6	84.6	8.4	1.73	11.0	93.9	1.4	16.8	12.6	90.8	22.6	22.4
	B	Night Feed	14.8	87.9	20.2	7.2	23.4	125.9	9.6	0.1	15.0	93.9	9.8	30.3
Figure 6	A	High Fat	8.4	114.5	0.6	28.5	20.6	160.6	3.4	46.0	19.8	82.7	3.2	34.3
	B	High Carbohydrate (No Protein)	23.2	29.0	6.2	13.2	19.4	172.0	4.6	24.5	14.8	78.9	2.6	25.0
	C	60% Casein	[14.0]	[110.3]	[2.8]	[55.4]	17.4	148.4	7.2	49.1	15.2	142.0	6.4	52.3
	D	15% Casein	[14.4]	[64.4]	[3.4]	[30.6]	15.0	134.2	5.4	57.6	15.0	65.3	8.8	22.0
	E	60% Soy Protein	14.8	102.1	6.0	39.0	15.6	153.1	6.2	29.9	15.6	152.5	6.6	38.9
	F	15% Soy Protein	10.0	72.5	17.0	28.4	19.8	138.8	5.8	17.6	21.8	73.7	5.0	8.5

Phase (h): ZT, except for CT in Figure 2. Values in brackets are for cases that rhythmicity/fluctuation was not significant ($p > 0.05$, one-way ANOVA).