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RESEARCH PAPER

Sweet treats before sleep disrupt the clock system and increase metabolic risk markers in healthy rats

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Abstract

Aim: Biological rhythms are endogenously generated natural cycles that act as pacemakers of different physiological mechanisms and homeostasis in the organism, and whose disruption increases metabolic risk. The circadian rhythm is not only reset by light but it is also regulated by behavioral cues such as timing of food intake. This study investigates whether the chronic consumption of a sweet treat before sleeping can disrupt diurnal rhythmicity and metabolism in healthy rats.

Methods: For this, 32 Fischer rats were administered daily a low dose of sugar (160 mg/kg, equivalent to 2.5 g in humans) as a sweet treat at 8:00 a.m. or 8:00 p.m. (ZT0 and ZT12, respectively) for 4 weeks. To elucidate diurnal rhythmicity of clock gene expression and metabolic parameters, animals were sacrificed at different times, including 1, 7, 13, and 19 h after the last sugar dose (ZT1, ZT7, ZT13, and ZT19).

Results: Increased body weight gain and higher cardiometabolic risk were observed when sweet treat was administered at the beginning of the resting period. Moreover, central clock and food intake signaling genes varied depending on snack time. Specifically, the hypothalamic expression of *Nampt*, *Bmal1*, *Rev-erbα*, and *Cart* showed prominent changes in their diurnal expression pattern, highlighting that sweet treat before bedtime disrupts hypothalamic control of energy homeostasis.

Conclusions: These results show that central clock genes and metabolic effects following a low dose of sugar are strongly time-dependent, causing higher circadian metabolic disruption when it is consumed at the beginning of the resting period, that is, with the late-night snack.

KEYWORDS
chrononutrition, circadian rhythm, energy balance, metabolism, snacking, zeitgebers

See related editorial: K Eckel-Mahan, 2023. Treat time matters: Untimely sugar consumption implicated in long-term energy imbalance. *Acta Physiol.* (Oxf). e14051.

1 | INTRODUCTION

Many physiological and metabolic processes are aligned to specific biological rhythms. These rhythms contribute to the adaptation of the organism to external environmental changes such as food availability or light–dark cycles, resulting in an optimization of metabolism and energy management. Processes like cardiovascular activity, endocrine system, blood pressure, body temperature, sleep–wake cycle, kidney activity, gastrointestinal function, and liver metabolism are regulated to a greater or lesser extent by the circadian rhythms.[1,2] The circadian clock and metabolism are connected through multiple reciprocal mechanisms.[3,4] Because of the great impact of circadian rhythm on physiology and metabolism, its disruption due to modern lifestyles (shift work, artificial light, etc.) has been associated with the development of metabolic disorders that in the long term could lead to type 2 diabetes, cardiovascular diseases, overweight, and obesity.[5–7] These metabolic disorders typical of the metabolic syndrome (MetS), together with other comorbidities such as depression, sleep disturbances, cognitive dysfunction, and steatohepatitis, form part of the recently defined "Circadian Syndrome"[8] and, in the case of obesity, many researchers are also discussing whether obesity can be considered as a "chronobiological disease."[9,10]

The control center of these endogenous oscillations is in the hypothalamic suprachiasmatic nucleus (SCN), which maintains the rhythmicity of processes autonomously. At molecular level, the central clock is regulated by self-regulatory clock genes transcriptional–translation feedback loops.[11] Within these feedback loops, the heterodimers of CLOCK (circadian locomotor kaput protein production cycles) and BMAL1 (brain and muscle ARNT 1, also known as ARNTL) act as positive regulators to promote the transcription of period (PER1 and PER2) and cryptochrome (CRY1 and CRY2) proteins. PER and CRY proteins form a complex in the nucleus that acts as a negative regulator during the day, interacting with CLOCK/BMAL1 and inhibiting their own transcription. The subsequent degradation of PER and CRY proteins leads to the restart of the cycle with a period of approximately 24 h. CLOCK/BMAL1 also activate the circadian expression of the nuclear receptors RORα, RORβ (RAR-related orphan receptor α and β), ERV-ERBα, and REV-ERBβ (encoded by a separate gene known as nuclear receptor subfamily 1 group D member 1 and 2), which have an important role in stabilizing the central clock since their expression drives the transcription of BMAL1 and CLOCK.[11] In addition, the CLOCK/BMAL1 heterodimer enhances the transcription of key metabolic genes, such as nicotinamide phosphoribosyltransferase (*Nampt*) and the aforementioned *Rev-erbα*.[12] Recently, both *Nampt* and *Rev-erbα* have been shown to be involved in the modulation of food intake and hypothalamic neuroinflammation.[13–16] The homeostatic regulation of energy homeostasis is regulated in the arcuate nucleus (Arc) of the hypothalamus, a region that consists of two main neuronal populations: neuropeptide Y–agouti-related protein (NPY-AGRP) neurons and pro-opiomelanocortin–cocaine–amphetamine-regulated transcript (POMC-CART) neurons. NPY-AGRP orexigenic neurons respond to ghrelin signals promoting food intake and decreasing energy expenditure. On the other hand, POMC-CART anorexigenic neurons are activated by leptin promoting satiety and increasing energy expenditure in response to feeding.[13,17] In this context, Brunetti et al. reported that NAMPT administration in the Arc of rats promoted increased food intake and decreased *Cart* expression.[18] Additionally, Adlanmerini et al. observed that the absence of *Rev-erbα* gene expression in the tuberal hypothalamic nuclei, which includes the Arc, promoted body weight (BW) gain and changes in insulin sensitivity in male mice.[16] The importance of the hypothalamic circadian biology and meal timing (i.e., day- or night-time-restricted feeding) was recently demonstrated using a transcriptomic profiling in female mice.[19]

External cues called zeitgebers, such as light or food, play a crucial role in reprogramming or altering the diurnal rhythmicity.[20,21] Light is perceived by the retina via the retinohypothalamic tract (RHT) and reset of the central clock, but the expression of clock genes in the other peripheral tissues such as liver[22,23] are also regulated by diet composition. Hence, mice fed a high-fat diet showed alterations in the expression of clock genes, nuclear receptors that regulate circadian transcription factors, and clock-controlled genes involved in fuel utilization in the hypothalamus, liver, and adipose tissue. Moreover, changes in the rhythmicity of locomotor activity have also been observed, when mice were fed a high-fat diet.[24] In addition, the frequency and timing of meals regulated diurnal rhythms and energy metabolism in liver and muscle of rats.[23] Furthermore, the intake of sugars, lipids, or other nutrients at different times of the day is known to have a different impact on the body.[25,26] In this regard, a study has reported the importance of the timing pattern of food intake on liver gene transcription in wild-type mice.[26] Changes in brown adipose tissue and skeletal muscle metabolism dependent on feeding time have also been reported in Wistar rats.[27] In addition, it was observed in humans that late-night food consumption is associated with an increase in body fat.[28,29] Thus, not only the composition of our food can modulate the peripheral clocks, but the timing of our food intake is now gaining importance. Indeed, late-night food intake is a topic of recent interest because of its contribution to food intake dysregulation and the development of obesity and other metabolic

disorders.[25,29] Interestingly, although the feeding cycle peaks during the activity phase, the craving for high-caloric foods and the appetite for sweet treats increase to a maximum at the end of the active phase or beginning of the resting phase.[30,31] In fact, an increase in late snacking was found to be associated with shift work and may have an impact on the desynchrony of the clock system and the development of metabolic disorders.[32]

Therefore, the aim of the present study was to know whether long-term late intake of a low dose of sugar, equivalent to 2.5 g in humans (half teaspoon), is a determining factor in expression of central clock genes, the diurnal rhythm of markers of metabolism, and hypothalamic expression of genes involved in energy balance in healthy rats. For this purpose, 32 rats were fed standard (STD) chow diet and given a low dose of sugar as a sweet treat at 8:00 a.m. or 8:00 p.m. (ZT0 and ZT12). The central clock genes expression and the main biochemical parameters and hormones in serum were studied by obtaining samples at four different times in a 24-h period. Expression of hypothalamic genes involved in the control of energy homeostasis and food intake was also analyzed.

2 | RESULTS

2.1 | Time of sugar administration significantly affected body weight gain, leptin levels, and energy balance

BW gain increased unequally depending on the time of sugar administration (Figure 1A). An increased BW gain and corresponding area under the curve (AUC) were observed in animals administered at ZT0 compared to those supplemented at ZT12. This difference in BW gain was significant during the last 3 weeks of the experiment. No significant changes in food intake were observed, although there was a slight tendency toward higher cumulative food intake in rats supplemented with sugar at ZT0 compared to ZT2 (Figure 1A).

Serum leptin levels and hypothalamic appetite gene expression were studied and their diurnal rhythm was evaluated by the cosinor method. A tendency to show diurnal rhythmicity was detected ($p = 0.054$) for serum leptin concentration in rats supplemented at ZT12, showing the acrophase 3 h before turning off the light (Figure 1B) (Tables S1 and S2). However, leptin serum levels in rats supplemented at ZT0 were similar throughout the four time points and no diurnal rhythmicity was detected. Furthermore, higher leptin serum levels were observed at ZT1 in rats administered at the beginning of their resting phase compared to those administered before their active phase.

Regarding homeostatic appetite signaling genes, the expression of the anorexigenic gene *Cart* (Figure 1C) presented diurnal rhythmicity in both experimental groups ($p = 0.009$ and $p = 0.022$ for ZT0 and ZT12, respectively). However, both groups displayed opposite diurnal rhythmicity, exhibiting reverse acrophase at ZT18 in rats supplemented at ZT0 and ZT7 in rats administered at ZT12 ($p < 0.001$) (Tables S3 and S4). In addition, there was a decrease in *Cart* gene expression at ZT7 in rats administered at ZT0 compared to those supplemented at ZT12. On the contrary, during the dark phase, a significant increase was observed at ZT13 and ZT19 in the ZT0 group compared to ZT12. No differences were found between groups neither for the anorexigenic gene expression of *Pomc* nor for the orexigenic gene expression of *Npy* and *Argp* (Figure S2 and Table S4).

2.2 | Differential effects of sugar administration time on biochemical parameters and insulin levels

No differences were found in serum glucose levels at any time point between rats supplemented at ZT0 and ZT12 by Mann–Whitney test. Nevertheless, diurnal rhythm of serum glucose levels was only detected by cosinor method ($p = 0.032$) in the group that received the sugar dose at ZT12 (Figure 2A and Table S1). Additionally, in the case of insulin levels, the diurnal rhythmicity was detected in both ZT0 and ZT12 groups ($p = 0.031$ and $p = 0.048$, respectively, for ZT0 and ZT12) (Figure 2B). Nonetheless, this parameter displayed an opposite rhythm depending on the sugar administration time, with the acrophase appearing always 6 h after receiving the sugar dose (at ZT18 and ZT6 for the ZT12 and ZT0 groups, respectively) (Table S1). According to this, higher insulin levels were found at ZT7 when sugar was given at ZT0, while decreased levels were observed in this same group at ZT13 ($p = 0.083$, tendency) and ZT19 ($p = 0.021$) compared to the groups administered at ZT12 by Mann–Whitney test.

Other biochemical parameters such as cholesterol, triglycerides, and NEFAs (Figure 2C–E) were also studied. No rhythms were detected by cosinor method for serum cholesterol and NEFAs in any experimental group (Table S1). However, both a decrease in cholesterol and a tendency towards decreased NEFAs serum levels were observed at ZT7 and ZT19, respectively, in rats supplemented at ZT0 by Mann–Whitney test. In the case of triglycerides, diurnal rhythmicity was found in both groups, with a tendency to have a lower amplitude in the case of the ZT0-supplemented group (25.79 vs. 52.06). In the time points ZT7 and ZT19, a tendency to decrease serum triglyceride

concentration in the ZT0 group compared to the ZT12 animals was observed by Mann–Whitney test.

2.3 | Differential effects of sugar administration time on serum hormones

Serum corticosterone levels showed a clear diurnal rhythmicity in both experimental groups ($p=0.02$ and $p=0.001$ for ZT0 and ZT12, respectively) (Figure 3A). However, although no differences were found between the groups at any time point, a tendency for acrophase to show up earlier was observed when the sugar was administered after the active phase before sleep, which may suggest a dysregulation of the stress response in terms of diurnal corticosterone oscillation. While the corticosterone diurnal rhythm of the group receiving the sugar dose before bedtime peaked around ZT11, just before lighting off, in rats supplemented at ZT12, this peak appeared 2 h after lighting off, around ZT14 ($p=0.089$) (Table S1). Regarding glucocorticoid receptors, no diurnal rhythmicity was detected in either group for *Nr3c1* or *Nr3c2* (nuclear receptor subfamily 3 group C member 1 and 2, respectively) expression in hypothalamus (Table S3). Despite this, the diurnal rhythm models of these receptors showed reverse acrophases between groups. In addition, we could observe a lower expression of *Nr3c1* during the dark phase at ZT13 ($p=0.021$) and a tendency to decrease its expression at ZT19 ($p=0.083$) in animals supplemented at ZT0. In addition, in the case of *Nr3c2*, a significant decrease in its expression was observed at ZT7 ($p=0.02$) when rats were administered at ZT0 (before bedtime).

Serum melatonin levels displayed diurnal rhythmicity for both groups ($p=0.037$ and $p=0.011$ for ZT0 and ZT12 groups, respectively) (Figure 3B and Table S1). The observed melatonin diurnal rhythmicity was in both groups as expected, showing the highest melatonin levels during the dark phase and the lowest during the light phase. However, serum testosterone levels and its diurnal rhythmicity were different depending on the sugar administration time (Figure 3C). A significant testosterone rhythm was detected in rats that received sugar at ZT12 by cosinor method ($p=0.039$) while no rhythm was observed in those supplemented at ZT0 (Table S1). In addition, the rats receiving sugar at ZT0 showed a marked testosterone acrophase shift ($p=0.024$) for the prediction model by the cosinor method, being close to ZT14 for this group and around ZT21 for the group receiving sugar at night (Tables S1 and S2). However, no significant differences were found in serum testosterone levels between both experimental groups at different time points using Mann–Whitney test. Nonetheless, a slight increase in testosterone levels was observed at ZT13 when the rats received sugar at ZT0 compared to ZT12 group.

The two main thyroid hormones were also studied (Figure S3). Regarding serum T3 levels, no significant diurnal rhythm was detected for both supplementation conditions (Table S1). However, modeling showed a higher T3 concentration in rats that received sugar at ZT0. Furthermore, serum T3 showed significantly different levels at different time points depending on the time of administration. A significantly higher concentration of this hormone was observed at ZT1 and ZT7 and a tendency to increase at ZT19 when rats received sugar at ZT0 (Figure S3A). In the case of serum T4 (Figure S3B), no significant diurnal rhythms were found for any condition, although a tendency to display rhythm was observed in animals administered with sugar before bedtime. Nevertheless, the models showed an opposite behavior between groups in T4 concentration in the 24-h period, peaking in the dark for ZT0 group and in the light period for ZT12 group (Tables S1 and S2). In addition, a tendency to decrease at ZT13 and increase at ZT19 were observed in the animals administered at ZT0 compared to ZT12 group. With respect to the T3-to-T4 ratio, diurnal rhythm was detected ($p=0.013$) only for the group supplemented with sugar at ZT12. The ZT0 group did not display diurnal rhythm, but the model showed a significant acrophase shift compared to ZT12-supplemented rats ($p<0.001$) (Tables S1 and S2). Furthermore, an increased T3-to-T4 ratio was observed in ZT7 and ZT13 for rats supplemented with sugar at ZT0 (Figure 3D).

2.4 | Diurnal rhythm patterns of hypothalamic clock genes were altered differently according to sugar administration time

Hypothalamic clock genes were analyzed in order to evaluate the central clock rhythmicity of the animals administered sugar in different conditions. These genes control the central clock synchronizing light signals from the environment with the peripheral clocks of the organism. The results showed important differences in the diurnal expression patterns of hypothalamic clock genes in the groups supplemented at different times (Figure 4). The supplementation at ZT0 resulted in a significant decrease in *Bmal1* expression ($p=0.043$) at ZT19 and a tendency ($p=0.083$) to decrease at ZT13 (Figure 4A). Regarding the diurnal rhythm of this gene, a rhythmicity was detected for both groups ($p<0.01$) by cosinor analysis. Nevertheless, differences in the acrophases ($p<0.001$) were observed between groups (Tables S3 and S4). In ZT0 group, the expression of *Bmal1* peaked at the beginning of the light phase

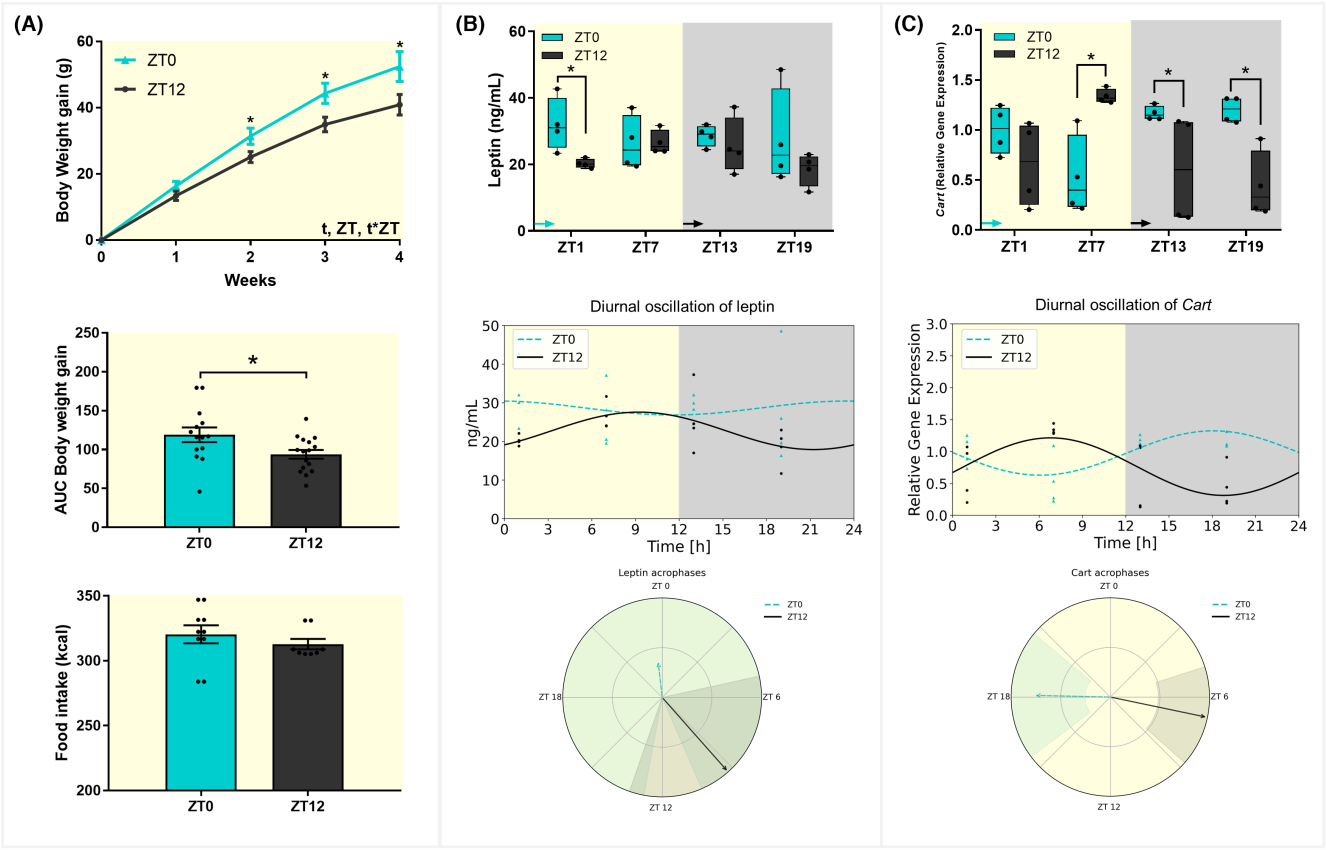


FIGURE 1 Body weight gain (g), cumulative food intake (kcal), serum leptin (ng/mL), and hypothalamic *Cart* expression (relative gene expression). (A) In the top figure, body weight gain throughout the 4 weeks of the experiment. In the middle figure, AUC (area under curve) of body weight gain, and in the bottom figure, cumulative food intake throughout the 4 weeks of the experiment. ZT, time of supplementation effect; t, time effect; t*ZT, interaction between ZT and t. Repeated-measure ANOVA was used followed by Student's *t* test between ZT0 and ZT12 groups ($p \leq 0.05$). (B) In the top figure, data shown as median and interquartile range of serum leptin. In the middle figure, diurnal rhythmicity estimated by cosinor method, and in the bottom figure, acrophases with their amplitude represented by serum leptin for ZT0 and ZT12 groups. (C) In the top figure, data shown as median and interquartile range of hypothalamic *Cart* expression. In the middle figure, diurnal rhythmicity estimated by cosinor method, and in the bottom figure, acrophases with their amplitude represent by *Cart* expression for ZT0 and ZT12 groups. *Indicates significant differences between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.05$).

(around ZT2), while the group supplemented at ZT12 showed the acrophase before the end of the dark phase (around ZT22). Significant changes were also observed in *Rev-erbα* gene expression (Figure 4B). In this case, a significant decrease in expression was observed both at ZT13 ($p = 0.021$) and ZT19 ($p = 0.021$) in the ZT0-supplemented group compared to ZT12-supplemented group. These differences translated into a change in the diurnal pattern of the expression of this gene. Although the expression of this gene displayed diurnal rhythm in the animals administered at ZT0 and ZT12, both acrophase and oscillation amplitude showed differences between the groups ($p = 0.008$ and $p = 0.010$, respectively) (Table S4).

Nevertheless, the genes that showed more differences in their expression depending on the sugar administration time were *Nampt* and *Rorα*. Regarding *Nampt* gene expression, no significant diurnal rhythm was detected in either group (Figure 4C). However, the peak of expression of this gene was different depending on the sugar administration time ($p = 0.024$). While for rats administered at ZT0, the acrophase was observed at ZT23, for rats supplemented at ZT12, it was found at ZT15 (Table S3). In addition, a decrease in the expression of this gene was observed at ZT13 when rats were supplemented with sugar at ZT0 by Mann–Whitney test. Another finding related to this gene was an increased variability of its expression in the hours following supplementation, at ZT1 and ZT7 and at ZT13 and ZT19 in rats supplemented at ZT0 and ZT12, respectively. For *Rorα* expression, diurnal rhythm was only detected for the group supplemented at ZT12 ($p = 0.018$) (Figure 4D and Table S3). The diurnal rhythm model of *Rorα* in rats that received sugar at ZT0 showed a trend to have a lower expression of the gene over 24 h. In addition, a significantly lower expression was observed

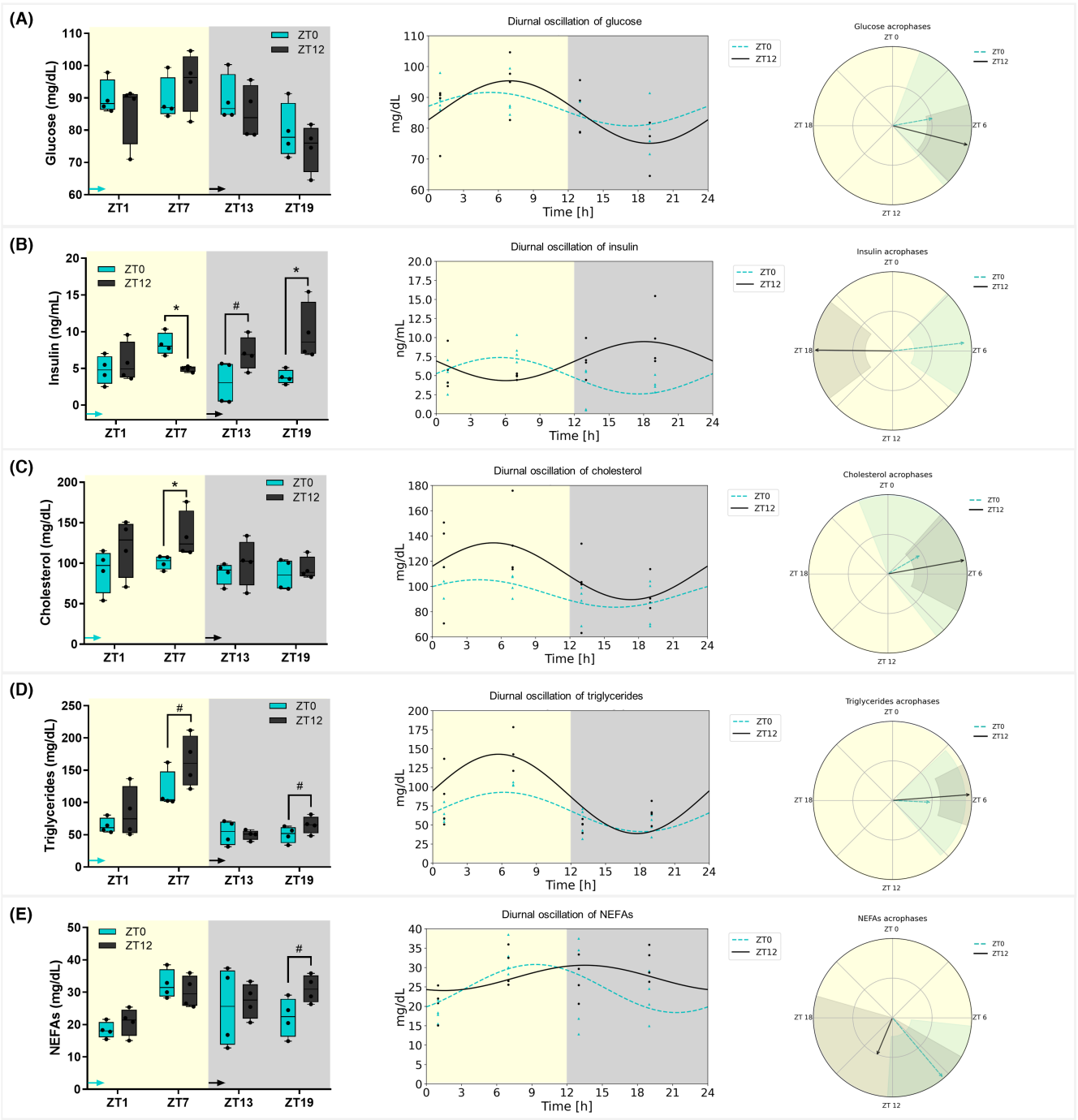


FIGURE 2 Serum glucose (mg/dL), insulin (ng/mL), cholesterol (mg/dL), triglycerides (mg/dL), and NEFAs (mg/dL). (A) On the left of the figure, data shown as median and interquartile range of serum glucose. In the middle figure, diurnal rhythmicity was estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by serum glucose for ZT0 and ZT12 groups. (B) On the left of the figure, data shown as median and interquartile range of serum insulin. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by insulin for ZT0 and ZT12 groups. (C) On the left of the figure, data shown as median and interquartile range of serum cholesterol. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by serum cholesterol for ZT0 and ZT12 groups. (D) On the left of the figure, data shown as median and interquartile range of serum triglycerides. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by serum triglycerides for ZT0 and ZT12 groups. (E) On the left of the figure, data shown as median and interquartile range of serum NEFAs. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by serum NEFAs for ZT0 and ZT12 groups. *Indicates significant differences between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.05$). # Indicates tendency between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.1$).

at ZT1 ($p = 0.043$) and ZT13 ($p = 0.021$) when the dose was administered at ZT0 compared to ZT12-supplemented rats (Figure 4D).

2.5 | Sugar administration time changed both metabolomics profile and the number of metabolites with diurnal rhythm

A total of 66 metabolites were identified and integrated into the serum (Table S5). Overall effects of sugar supplementation time on the serum metabolomic profile were analyzed by principal component analysis (PCA) and the sparse partial least squares discriminant analysis (sPLS-DA). No clustering by dose time was observed using PCA (Figure 5A). However, samples did cluster by the time of dose when analyzed by sPLS-DA (Figure 5B). The heatmap analysis revealed a change in the serum metabolomic profile according to the sugar administration time (Figure 5C), differentiating animals that received the sugar before bedtime from animals that were supplemented in the dark period. In addition, the heatmap also showed that most of the metabolites displayed a diurnal oscillation, or at least differences between light and dark phases, especially in the ZT12-supplemented groups (Figure 5C). In this regard, metabolites were analyzed with the cosinor method to elucidate their diurnal oscillation (Table S6). A significant difference in the circadian profile of serum metabolomics was detected according to the time of sugar supplementation (Figure 5D). While diurnal rhythmicity was only detected in 6 metabolites ($p < 0.05$) when the sugar dose was administered before bedtime, for the ZT12-supplemented groups, diurnal rhythmicity was detected in 17 metabolites. Many metabolites with diurnal oscillation participate in important energy metabolism processes such as lipolysis (dodecanoic acid, palmitic acid, 2-hydroxy butyric acid, and oleic acid). All these metabolites showed diurnal oscillations when sugar was administered in ZT12, but no oscillations were detected in any of them when sugar was administered at ZT0. Other processes such as the Krebs cycle and glycolysis also showed or did not show rhythmicity according to the time of sugar administration, thus citric acid and oxalic acid presented diurnal rhythmicity only if the dose was administered at ZT0, while glycerol-1-phosphate, malic acid, and fumaric acid presented rhythm only when the animals were supplemented at ZT12. Diurnal oscillation was also observed in 12 amino acids, of which alanine showed rhythmicity in both groups. However, taurine displayed rhythmicity only in the ZT0 group and the remaining 10 amino acids displayed diurnal oscillation only when the sugar was supplemented at ZT12. Moreover, statistical analysis between the different groups by Kruskal–Wallis test found 22 metabolites whose concentration was significantly different between groups. For example, ZT0 sugar supplementation caused a strong significant increase in the concentration of heptanoic acid, 3-hydroxyisovaleric acid, glycolic acid, and dodecanoic acid (Table S5). Other metabolites involved in energy metabolism such as pyruvic acid and 3-phosphoglyceric acid in glycolysis and citric acid in the Krebs cycle were also affected (Figure 5E). Specifically, a decrease in pyruvic acid levels was observed 1 h after ZT12 supplementation compared to animals supplemented at ZT0. Furthermore, increased levels of citric acid and 3-phospho acid were observed at the night and ZT0 time points, respectively, when animals received sugar at ZT12 (Figure 5E).

3 | DISCUSSION

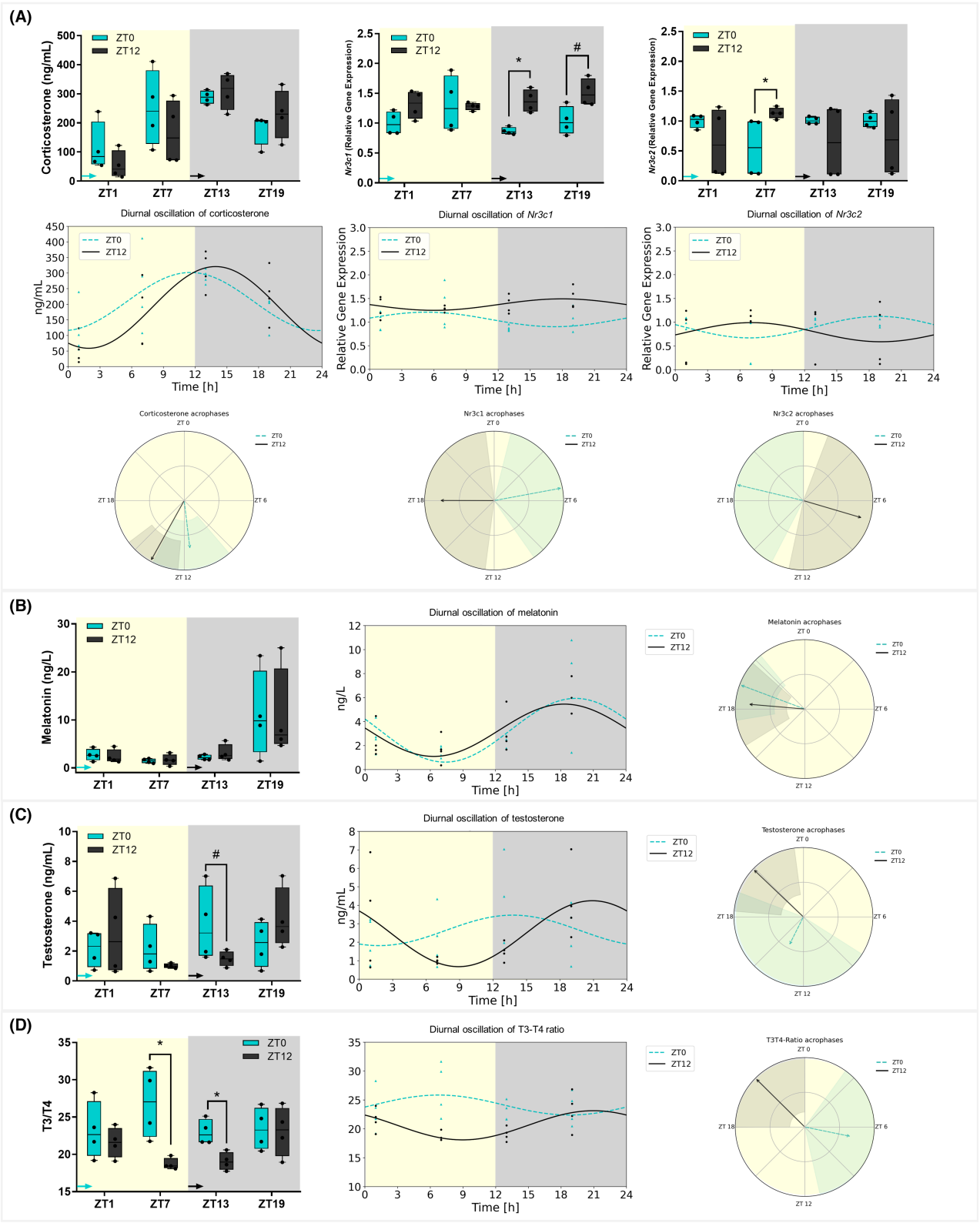
In recent years, the time of food intake has been reported to have an important impact on the diurnal rhythmicity of the organism, influencing behavior, metabolism, and some physiological processes.[10,25] These effects of food intake patterns on body diurnal oscillations have also been related to metabolic disorders.[25,26] Hence, the timing of food intake impacts the development of overweight or obesity, conditions associated with diabetes, cardiovascular diseases, dyslipidemia, among others,[29] increasing the risk of suffering from MetS.[8] This study aimed to evaluate for the first time the effects of long-term intake of amount of a low sugar treat at the beginning of the active phase or at the beginning of the resting phase on the diurnal rhythmicity of the central clock genes, metabolic markers, and hypothalamic expression of genes involved in energy balance and food intake. Several studies have demonstrated the importance of the time of caloric intake at the physiological and metabolic level,[26–28,38] pointing out that an abnormal food consumption at the beginning of active phase promotes metabolic disorders.[29] Specifically, a 12-week clinical trial showed a differential effect on BW gain depending on the timing of intake, with participants who consumed a high-calorie meal before the resting phase, dinner versus breakfast, gaining more BW during the intervention.[39] Interestingly, our results also showed an effect on BW gain by food consumption at the beginning of active phase since healthy rats that received a low dose of sugar during the resting phase showed a higher BW gain compared to rats that received sugar in the active phase. This amount of sugar, using a translation of animal to human doses[40] and estimating the daily intake for a 70 kg human, corresponds to an intake of 2.5 g/day (half teaspoon). However, the observed effects cannot be directly extrapolated to humans only in terms of the amount of

sugar in mass because the rats are on a controlled diet, and the impact of this amount of sugar could be higher in animals than in humans whose diets are rich in sugars. Nevertheless, although results obtained in our study should not be directly translatable to humans, the fact that only 160 mg/kg of sugar promotes differences in BW gain depending on the time of administration suggests that in humans, the daily nocturnal intake of snacks should not be underestimated, especially considering that in terms of kcal ingested, the intake of half a teaspoon of sugar is approximately 10 kcal, which is 10 to 15 times less than a commercial snack or a sugary drink common on the market. Nevertheless, further investigations are needed to clarify the real impact of chronic nocturnal snacks on metabolic human health.

Similar findings were reported in an experimental model of a simulated shift-work protocol, in which the animals were administered chocolate after the active phase/before sleeping or before the active phase (ZT0 or ZT12, respectively). In this study, it was described that chocolate, a hypercaloric and palatable food, ingested at the beginning of the light phase showed a greater BW gain than rats that ate chocolate at the beginning of the dark phase.[41] Furthermore, Escobar et al. not only observed changes in BW gain but also in biochemical parameters. Thus, when the rats received chocolate before the sleeping phase, the results showed loss in the glucose peak and a disrupted circadian oscillation of glucose levels compared to those that received chocolate before the active phase.[41] In our study, a differential effect of sugar administration time on the diurnal rhythmicity of serum glucose levels was observed with loss of diurnal oscillation in animals administered sugar at ZT0. In addition, a more pronounced drop in serum glucose oscillations was observed in ZT12 animals during the dark phase, coinciding with the highest expression of the core clock gene *Bmal1*. This gene plays a crucial role in glucose metabolism, as its deletion leads to decreased insulin secretion and altered glucose levels.[42] Interestingly, these changes in glucose oscillation and *Bmal1* expression depending on sugar administration time were accompanied by changes in insulin diurnal oscillations. In fact, while the insulin acrophase of ZT0 group appeared in the light phase, as did the acrophase of *Bmal1* expression, ZT12 group displayed the insulin and *Bmal1* acrophases in the dark phase. Regarding the lipid biochemical parameters, no major changes were found between groups, although a little increase in serum cholesterol and triglycerides at some time point when supplementation was at ZT12 led to a better circadian profile of these parameters in these rats compared to animals supplemented at ZT0, results that coincide with those obtained for triglyceride levels when rats received chocolate for breakfast compared to those that received chocolate for dinner.[41] Interestingly, another recent study observed a marked effect on glucose, insulin, and free fatty acid levels when animals received chocolate in the resting phase compared to animals that received chocolate in the active phase or those that did not receive chocolate in an acute study.[43] In addition, Begemann et al. observed that this acute consumption of chocolate during the resting phase promoted changes in the expression of genes such as *Bmal1* and *Rev-erbα* of peripheral clocks, locomotor activity, and body temperature.[43] All these results and evidence suggest a crucial effect of consuming high-calorie meals at night on the resting phase, increasing BW gain and altering biochemical parameters, which may be driven by the disruption of the body's diurnal oscillation and clock system caused by changes in eating behavior (eating in the resting phase).

The most important controller of the circadian rhythms is the central clock, this pacemaker is in hypothalamus, and maintains the synchronization of the peripheral clocks with the environment and its oscillators through hormone signals and neural connections.[3,4,6] *Nampt* is an important clock gene and its expression is related to energetic metabolism and food intake control through the regulation of the signaling intake genes, and genes related to mitochondrial function.[13–15] Indeed, NAMPT

FIGURE 3 Diurnal rhythmicity of serum corticosterone (ng/mL), hypothalamic *Nr3c1* and *Nr3c2* expression (relative gene expression), serum melatonin (ng/L), testosterone (ng/mL), and T3/T4 ratio. (A) In the top figure, data shown as median and interquartile range of serum corticosterone, and hypothalamic *Nr3c1* and *Nr3c2* expression. In the middle figure, diurnal rhythmicity estimated by cosinor method, and in the bottom figure, acrophases with their amplitude represented by serum corticosterone and hypothalamic *Nr3c1* and *Nr3c2* expression for ZT0 and ZT12 groups. (B) On the left of the figure, data shown as median and interquartile range of serum melatonin. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by serum melatonin for ZT0 and ZT12 groups. (C) On the left of the figure, data shown as median and interquartile range of serum testosterone. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by serum testosterone for ZT0 and ZT12 groups. (D) On the left of the figure, data shown as median and interquartile range of T3-to-T4 ratio. In the middle figure, diurnal rhythmicity estimated by cosinor method, and on the right of the figure, acrophases with their amplitude represented by T3-to-T4 ratio for ZT0 and ZT12 groups. *Indicates significant differences between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.05$). # Indicates tendency between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.1$).



is the rate-limiting enzyme in the biosynthesis of NAD+ through its salvage pathway.[44] NAD+ homeostasis is related to the free radicals-mediated production of reactive oxygen species, which has been linked to innumerable pathologies, including metabolic diseases.[45] Moreover, this metabolite activates several NAD-dependent deacetylases (SIRT), controlling the activity of many cellular proteins.[15] However, in this study, hypothalamic diurnal rhythmicity for this gene was not detected for any group. Nevertheless, an estimation of the rhythmicity with cosinor method showed clear differences between both groups, indicating that the timing of sugar administration would be also

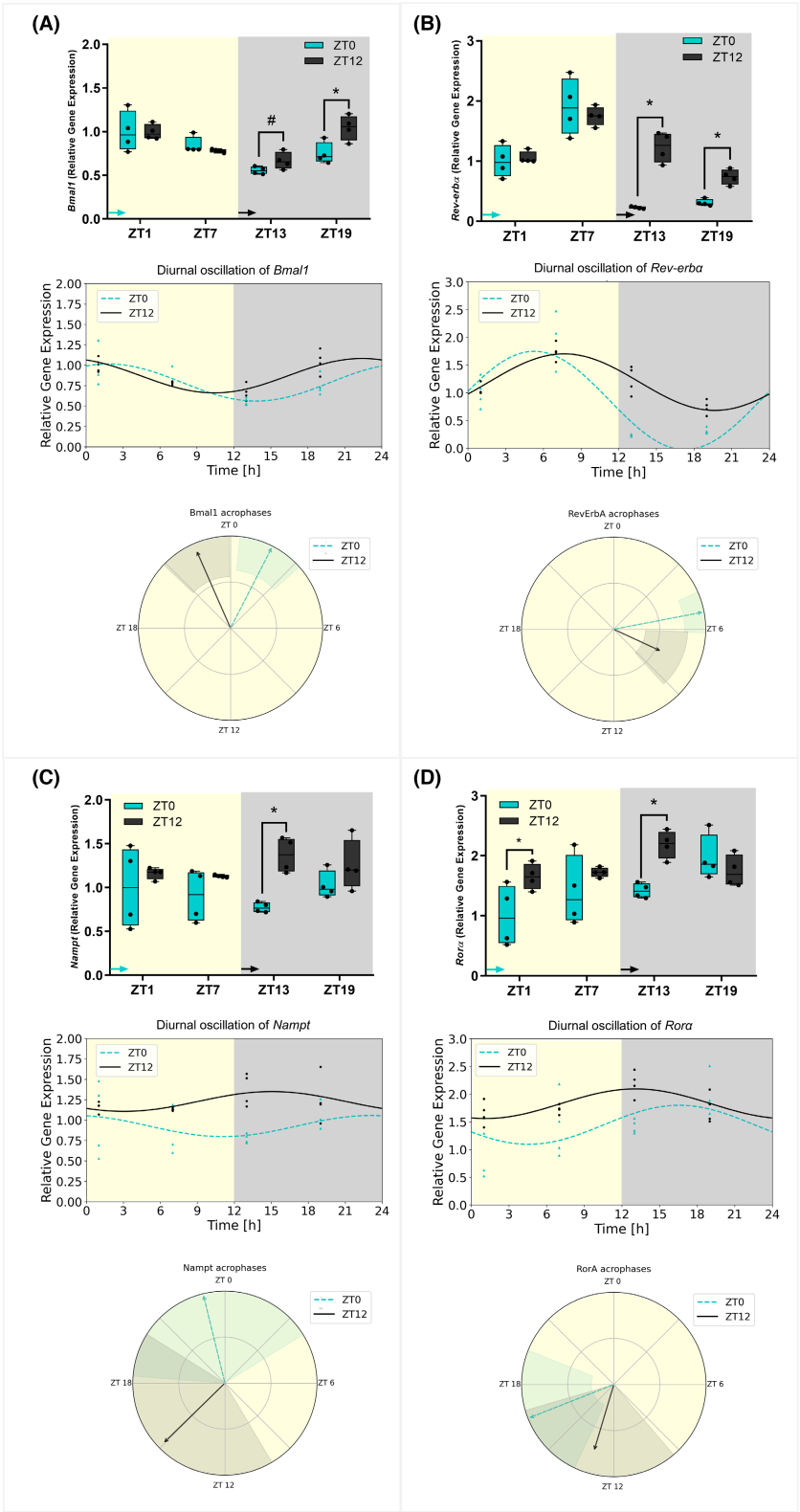


FIGURE 4 Diurnal rhythmicity of hypothalamic *Bmal1*, *Rev-erbα*, *Nampt*, and *Rorα* expression (relative gene expression). (A) In the top figure, data shown as median and interquartile range of hypothalamic *Bmal1* expression. In the middle figure, diurnal rhythmicity estimated by cosinor method, and in the bottom figure, acrophases with their amplitude represented by *Bmal1* expression for ZT0 and ZT12 groups. (B) In the top figure, data shown as median and interquartile range of hypothalamic *Rev-erbα* expression. In the middle figure, diurnal rhythmicity estimated by cosinor method, and in the bottom figure, acrophases with their amplitude represented by *Rev-erbα* expression for ZT0 and ZT12 groups. (C) In the top figure, data shown as median and interquartile range of hypothalamic *Nampt* expression. In the middle figure, diurnal rhythmicity estimated by cosinor method, and in the bottom figure, acrophases with their amplitude represented by *Nampt* expression for ZT0 and ZT12 groups. (D) In the top figure, data shown as median and interquartile range of hypothalamic *Rorα* expression. In the middle figure, diurnal rhythmicity estimated by cosinor method and in the bottom figure, acrophases with their amplitude represented by *Rorα* expression for ZT0 and ZT12 groups. *Indicates significant differences between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.05$). # Indicates tendency between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.1$).

determinant in the expression of this gene. Specifically, the results showed remarkably changes in the expression of *Nampt* between groups, peaking in the dark phase in the ZT12 group and before bedtime in the animals administered at ZT0. Regarding the absence of hypothalamic rhythmic expression of *Nampt*, it has been previously reported using hypothalamic neurons that presented a functional circadian clock, displaying circadian rhythmicity for core clock genes as *Bmal1*.[14] Accordingly, our results also showed a rhythmic expression *Bmal1* both in ZT0 and ZT12 animals, although a delay in the acrophase of ZT0 animals compared to ZT12 rats was observed.

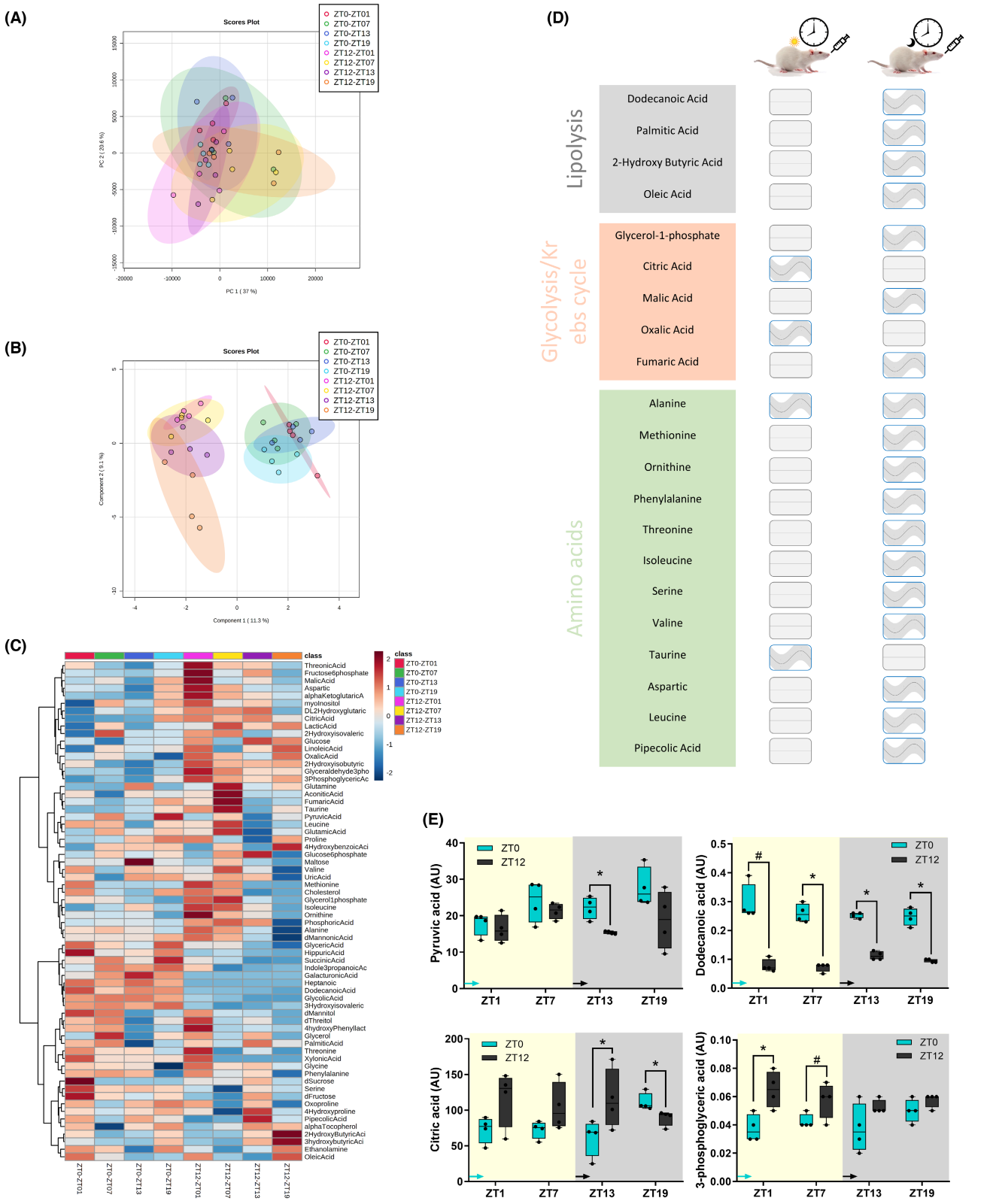


FIGURE 5 Metabolomics (AU; arbitrary units) analysis for groups supplemented at ZT0 and ZT12. Relative metabolomic composition throughout (A) principal component analysis (PCA) and (B) the sparse partial least squares discriminant analysis (sPLS-DA) of plasma samples of each group. (C) Heatmap of abundance of serum metabolites per experimental group. (D) General overview of metabolites and their diurnal rhythmicity for ZT0 and ZT12 groups. (E) In the top figure, data shown as median and interquartile range of serum pyruvic acid and dodecanoic acid levels, and in the bottom figure, data shown as median and interquartile range of serum citric acid and 3-phosphoglyceric acid levels for ZT0 and ZT12 groups. *Indicates significant differences between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.05$). # Indicates tendency between ZT0 and ZT12 groups at each time point by Mann–Whitney ($p < 0.1$).

The changes observed in *Bmal1* expression could explain the differences observed for *Nampt* expression between groups and could be promoting the changes in the rhythms of other metabolites and hormones, according to the defined role of *Bmal1* as a master regulator of molecular clock system.[46] In addition to the key role of NAD+ in the synchronization of metabolism, it has been reported that NAMPT administration promoted increased food intake and decreased *Cart* expression in the arcuate nucleus of rats.[18] Our results showed that *Cart* expression is lower during the dark phase in rats supplemented with sugar at ZT12, while *Nampt* showed higher expression in this phase than in the light period. On the contrary, for rats supplemented at ZT0, an upregulation of *Cart* was observed during the dark phase while *Nampt* was lower during most of this phase, and in any case, lower than rats supplemented with sugar at ZT12. In addition, although no significant diurnal oscillations were obtained, circadian oscillations models of *Pomc* expression showed an acrophase located in the dark phase of rats supplemented with sugar at ZT0 and a higher peak expression than rats supplemented at ZT12. Moreover, a significant change in leptin was observed despite no changes in food intake. This hormone, secreted by fat cells, exhibit important functions in the regulation of food intake and metabolism, and leptin resistance may promote the development of obesity.[47] In humans, leptin shows a marked diurnal rhythmicity with a peak 2 h before the onset of the activity phase, and an alteration in its secretion promotes the loss of the diurnal rhythmicity of metabolism and feeding.[48,49] In our study, diurnal rhythmicity was detected in serum leptin concentrations in the ZT12 group, with a peak 3 h before the activity phase, whereas no diurnal rhythmicity was detected in the rats that received sugar at ZT0. Thus, the change in the diurnal rhythms of the anorexigenic genes *Cart* and *Pomc* and their differences in the time of maximum expression depending on timing of sugar administration, together with the circadian changes of leptin, suggest differences in the energetic metabolism between groups.

In addition to *Nampt*, *Rev-erbα* is a gene which has also been related to the regulation of energy metabolism.[13–16] It has been observed that the absence of expression of this gene is associated in male mice with an increase in BW.[16] Our results showed a decrease in the expression of this gene during the dark phase in ZT0-supplemented rats, which gained more BW than ZT12-supplemented rats. These results could indicate a rhythmic disruption of metabolism via *Nampt* and *Rev-erbα* expression by sugar intake outside of the normal feeding cycle. In fact, it has been suggested that the hedonic center, which is associated with the activation of the neural reward system in response to a highly palatable food, could play an important role in the maintenance of the clock system by signaling the central clock with a snack at the beginning of the activity phase in a situation of disruption of the light/dark cycle.[50] In our case, we observed that the metabolic and circadian profile of the rats that received the dose at the beginning of the resting phase is worse than those that received the dose at the beginning of the activity phase, this could be due to a change in the feeding and activity behavior patterns, including the food anticipatory activity, promoted by the hedonic center.[30,43,51] According to this, ZT0-supplemented group also presented a decreased *Cart* expression in the light period, which could result in an increased food intake during its resting phase. In fact, these changes in feeding behavior could be involved in the found differences between both groups. Nevertheless, a limitatioin of the experiment was not being able to perform analyses that could provide further evidence of changes in the food anticipatory activity. In this context, in order to know the metabolic profile of these rats, their metabolic profile was evaluated by serum metabolomics. In addition to the direct involvement of *Nampt*, pathways such as lipolysis or glycolysis and Krebs cycle are altered by changes in feeding patterns that modify the clock system.[42,52,53] In our study, the fact that ZT0-supplemented rats showed only 6 metabolites with diurnal rhythmicity while rats supplemented at ZT12 showed 17 metabolites with rhythmicity indicated a change in overall metabolic profile of the rats depending on the timing of administration. Furthermore, it has been reported that T3/T4 ratio has diurnal rhythm that is determined by feeding patterns, which is lost when there is a change in these patterns.[54,55] According to this, in addition to the metabolic alterations found in ZT0 group, a loss of oscillation of the T3/T4 ratio was observed in these animals, while it is maintained in the ZT12 group. Additionally, glucocorticoid receptors are important regulators of metabolic rhythmicity in the central nervous system. These regulate glucocorticoid effects, such as cortisol in humans or corticosterone in rodents, via the hypothalamic–pituitary–adrenal (HPA) axis.[56,57] In addition, the circadian maintenance of corticosterone release appears to be regulated through the action of the clock system on the expression of glucocorticoid receptors such as Nr3c1.[56,58–60] More specifically, it is known that there is a regulation between *Nr3c1* and *Rev-erbα* that affects the expression of both receptors.[61] Therefore, disruption of the normal rhythmicity of their expression could lead to diseases of glucocorticoid sensitivity or resistance.[62,63] The results of this study showed differences in the circadian pattern of serum corticosterone concentration, which may have resulted from the changes on *Nr3c1* and *Nr3c2* receptors and differently shaped clock genes produced by sugar supplementation at different times of the day.

4 | MATERIALS AND METHODS

4.1 | Animal procedures

The animals used were 13-week-old male Fischer 344 rats from Charles River Laboratories (Barcelona, Spain) housed in pairs under standard laboratory conditions at 22°C and 12 h light–dark cycle. Rats were weighed and randomly divided into two groups ($n = 16$ per group) and fed ad libitum with STD diet (2.90 kcal·g^{-1}; A04, Panlab, Barcelona, Spain) for 2 weeks. After this acclimation time, a low dose (diluted 1/5) of low-fat sweetened condensed milk (SCM) (60.8% glucose, 8.9% protein, and 0.2% fat) was administered as a sweet treat by syringe to the animals at 8:00 a.m. and 8:00 p.m. (ZT0 and ZT12, respectively), for 4 weeks. This amount of SCM consisted of 160 mg of sugar per kg of rat, which is equivalent to 2.5 g in humans (half teaspoon). To avoid any potential effect of our intervention being given at different times of the day, all 32 rats were held in the same room. In addition, we made sure that all rats approached the end of the box at each of the two supplementation times. Finally, all non-invasive procedures were carried out in this room. BW and food intake were recorded weekly throughout the experimental procedure. To obtain four different time points over a period of 24 h, the animals were sacrificed at four different points on the day ($n = 4$), including 1, 7, 13, and 19 h after the last dose (ZT1, ZT7, ZT13, and ZT19) (Figure S1). At the end of the experimental period, the animals were deprived of food for 3 h before each of these time points and sacrificed by decapitation in a different room prepared for this purpose. Blood was collected after decapitation in non-heparinized tubes, incubated for 1 h at room temperature, and immediately centrifuged at 1200*g* for 15 min at 4°C to collect the serum. To extract the hypothalamus, the whole brain was first removed and placed face down. In this position, the hypothalamus is located between the frontal optic chiasm and the posterior mammillary body. After determining the position of the hypothalamus, it was excised along the edge to obtain the entire hypothalamus,[33] which was rapidly frozen in liquid nitrogen and stored at −80°C until further analyses.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and the Generalitat de Catalunya approved all the procedures (reference number 9495, 18/09/19) and were carried out in accordance with Directive 86/609EEC of the Council of the European Union and the procedure established by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. All materials submitted is in accordance with good publishing practice in physiology.[34]

4.2 | Serum biochemical parameters and hormone levels

Enzymatic colorimetric assays were used for the analysis of glucose, total cholesterol, and triglycerides (QCA, Amposta, Tarragona, Spain) and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany) according to the manufacturer's instructions. ELISA kits were performed to determine insulin and leptin serum concentrations (EMD Millipore Corporation, 290 Concord Road, Billerica, MA 01821, USA).

The serum hormone concentrations were measured by liquid chromatography coupled with triple quadrupole mass spectrometry (LC-QqQ). Serum samples were thawed at 4°C. A volume of 50 µL of serum was mixed with 250 µL of methanol containing the internal standard (2 ng/mL). Then, the mixture was vortexed and centrifuged for 5 min at 4°C and 25 200*g*. The supernatant was transferred to a new tube and mixed with 700 µL of 0.1% formic acid in water. The sample was loaded into an SPE cartridge previously conditioned with methanol and 0.1% formic acid in water. The cartridge was washed with 0.1% formic acid in water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45°C and reconstituted with 50 µL of water:methanol (60:40, v/v) and transferred to a glass vial for analysis. The hormones detected were melatonin, corticosterone, triiodothyronine (T3), thyroxine (T4), and testosterone.

4.3 | Metabolomics analysis

Metabolomic analysis in the 32 rat serum samples was performed using gas chromatography coupled with quadrupole time-of-flight mass spectrometry (GC-qTOF). The extraction was performed by adding 400 µL of methanol:water (8:2) containing internal standard mixture to serum samples (approx. 100 µL). Then, the samples were mixed, incubated at 4°C for 10 min, centrifuged at 25 200*g* 4°C for 10 min, and the supernatant was evaporated to dryness before compound derivatization (methoximation and silylation). The derivatized compounds were analyzed by GC-qTOF (model 7200 of Agilent, USA). The chromatographic separation was based on Fiehn method[35] using a J&W Scientific HP5-MS (30 m × 0.25 mm i.d., 0.25 µm film capillary column, and helium as carrier gas using an oven program from 60 to 325°C). Ionization was done by electronic impact (EI), with electron energy of 70 eV, and operating in full scan mode. Identification of metabolites was performed using commercial standards and by matching their EI mass spectrum and retention time to

metabolomic Fiehn library (from Agilent) which contains more than 1400 metabolites. After putative identification of metabolites, these were semi-quantified in terms of internal standard response ratio.

4.4 | Gene expression analysis

The total RNA was extracted from the hypothalamus using E.Z.N.A.® Micro RNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. The RNA yield was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems. Barcelona, Spain) for analyzing the expression of the samples. A Labnet MultiGene Gradient PCR Thermal Cycler (Sigma-Aldrich. Madrid, Spain) was used for reverse transcription. The reaction was performed according to the instructions of the manufacturer. The cDNA was subjected to a quantitative reverse transcriptase polymerase chain reaction amplification using iTaq Universal SYBR Green Supermix (Bio-Rad, Madrid, Spain) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Madrid Spain). The primers used for the different genes are described in Table S7 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated by normalizing to MS-ZT0 at ZT1 group using the $2^{-\Delta\Delta Ct}$ method with *Ppia* gene as an endogenous control, as reported by Schmittgen and Livak.[36]

4.5 | Statistical analysis

BW gain and cumulative food intake data were represented as means ± standard error of mean (SEM) for each group and Shapiro–Wilk and Levene test were used to verify the normality and homogeneity of variance of these data, respectively. Differences between groups were assessed by repeated-measures ANOVA followed by LSD post hoc test. The rest of parameters and gene expressions were considered non-parametric data. Kruskal–Wallis test or Mann–Whitney test were used to analyze this data, as indicated in the respective figure legend. These statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

To analyze the diurnal oscillations, we used cosinor-based rhythmometry method. For this, a script was developed by J.R. S-R. using PyCharm software (v.2018.2.4, JetBrains s.r.o., Prague, Czech Republic) with Python version 3.7.4, and diurnal rhythmicity estimated was plotted using CosinorPy package (v.1.1).[37] We considered the presence of diurnal rhythmicity when the model of each gene expression fits the cosine curves ($p < 0.05$). In addition, this method allowed for obtaining rhythmicity parameters such as the MESOR (diurnal rhythm-adjusted mean), amplitude (the difference between the peak and the mean value of a wave), or acrophase (the time at which the peak of a rhythm occurs) of the diurnal oscillations.

5 | CONCLUSION

In conclusion, daily low-dose sugar consumption as a sweet treat in healthy rats showed differential effects depending on the timing. Noticeable metabolic and circadian effects were observed when this sweet treat was administered at the beginning of the light phase, a time when rats rest. This low-dose sugar supplementation at the beginning of the resting phase caused changes in the circadian patterns of expression of central clock genes such as *Bmal1* or *Rev-erbα* and significant alterations in genes such as *Nampt* and *Cart* or glucocorticoid receptor genes, suggesting a change in feeding patterns through altered signaling of food intake and in which the hedonic center could play a crucial role. Thus, despite finding no change in total food intake over the 24 h of the day, the results suggest that a higher intake may be induced during the resting phase after receiving the low dose of sugar promoting important changes in metabolism such as loss of diurnal rhythmicity of hormones, metabolites, and an increase in BW gain in the rats administered sugar before bedtime.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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