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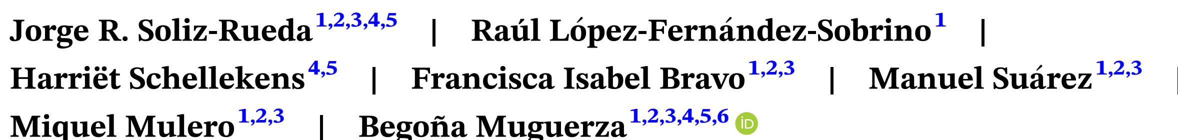
Received: 24 September 2024 | Accepted: 10 January 2025

DOI: 10.1002/biof.70000

RESEARCH ARTICLE

Biofactors WILEY

Clock system disruption in male Fischer 344 rats fed cafeteria diet and administered sweet treats at different times: The *zeitgeber* role of grape seed flavanols



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Funding information

Ministerio de Ciencia, Innovación y Universidades, Grant/Award Numbers: AGL2016-77105-R, PID2021-128813OB-I00, BES-2017-080919, PRX21/00592, 2021URV-RQ-01; Biostime Institute for Nutrition and Care - Geneva; Food for Health Ireland through the EI Technology Centre award, Grant/Award Number: TC20180025

Abstract

Current lifestyles include calorie-dense diets and late-night food intake, which can lead to circadian misalignment. Our group recently demonstrated that sweet treats before bedtime alter the clock system in healthy rats, increasing metabolic risk factors. Therefore, we aimed to assess the impact of the sweet treat consumption time on the clock system in rats fed a cafeteria diet (CAF). Moreover, since flavanols have demonstrated beneficial effects in metabolic disorders and clock gene modulation, we also investigated whether these phenolic compounds can restore the circadian disruption caused by these altered dietary patterns. For this, 64 Fisher rats were fed CAF for 9 weeks. In the last 4 weeks, animals were daily administered a low dose of sugar (160 mg/kg) as a sweet treat at 8 a.m. (ZT0) or 8 p.m. (ZT12). Two other groups received 25 mg/kg of grape seed flavanols in addition to sweet treats. Finally, the animals were sacrificed at different time points (9 a.m., 3 p.m., 9 p.m., and 3 a.m.). The results showed that metabolic and circadian disturbances by CAF may be influenced by the time of sugar administration, slightly reinforcing the alterations in diurnal rhythmicity of serum biochemical parameters, hormones, and hypothalamic genes with bedtime snacking. Flavanols improved metabolic health and restored the oscillation of biochemical parameters, hormones, and clock and appetite-signaling genes, showing greater effects at ZT12. These results highlight the importance of meal timing in influencing physiological and metabolic outcomes, even under calorie-dense diets. Moreover, they also suggest the *zeitgeber* role of flavanols, modulating the clock system and contributing to an improved metabolic profile under different feeding pattern conditions.

KEYWORDS

Chrononutrition, circadian rhythms, cosinor, metabolism, snack timing, *zeitgebers*

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1 | INTRODUCTION

Living organisms exhibit autonomous oscillations that play a crucial role in physiological and metabolic adaptations to the time of day.[1] Indeed, diurnal oscillations have been described in processes such as the sleep–wake cycle, body temperature, blood pressure, and gastrointestinal tract function, and their alterations have been associated with metabolic disorders.[1,2] The central clock, in the hypothalamic suprachiasmatic nucleus (SCN), and peripheral clocks in various tissues are regulated by clock genes via self-regulatory transcriptional-translation feedback loops.[3] Thus, the CLOCK/BMAL1 (circadian locomotor kaput protein production cycles/brain and muscle ARNT 1) heterodimer regulates the circadian machinery by promoting transcription of period (PER1 and PER2) and cryptochrome (CRY1 and CRY2) proteins. These proteins lead to a negative feedback loop with an approximately 24-h cycle.[1,4] In addition, the heterodimer CLOCK/BMAL1 activates nuclear receptors, including RORα, RORβ, REV-ERBα, and REV-ERBβ, which stabilize the central clock by modulating *Bmal1* and *Clock* transcription.[1,4] The heterodimer also stimulates the expression of metabolic genes, such as nicotinamide phosphoribosyltransferase (*Nampt*) and the aforementioned *Rev-erbα*, involved in energy metabolism and appetite pathways in the hypothalamic arcuate nucleus (Arc).[5–9] This region contains two neuronal populations, orexigenic NPY-AGRP (neuropeptide Y-agouti-related protein) neurons, which reduce energy expenditure (EE) and increase food intake in response to ghrelin, and anorexigenic POMC-CART (pro-opiomelanocortin-cocaine-amphetamine-regulated transcript) neurons, which enhance EE and promote satiety in response to leptin.[5,10]

Despite the precise regulation of the clock system by the central clock, external cues, also called *zeitgebers*, can modulate or alter the synchronization of this system.[3,11] The central clock is reset by light, the main *zeitgeber*, which is detected by the retina via the retinohypothalamic tract (RHT). This triggers signals via the autonomic nervous system or circulating humoral factors, including melatonin and cortisol (corticosterone in rodents), to keep peripheral tissue clocks synchronized.[1] However, in the twentieth century, remarkable lifestyle changes have occurred in modern occidental society. These modifications include alterations of the natural light/dark phases, such as shift work and social jetlag, and variations in dietary patterns (ultra-processed and high-calorie-dense foods, meal timing). All these alterations cause a misalignment between external cues and the internal clock and have been associated with the development of metabolic diseases.[1,12–15] These metabolic disorders, together with other comorbidities such as depression, sleep disturbances, cognitive dysfunction, and steatohepatitis are part of the recently defined "Circadian Syndrome."[2,16] In this regard, diet composition and timing stand out as crucial *zeitgebers* or modulators of our clock system.

In fact, the effects of a calorie-dense diet on the misalignment of biological rhythms have also been extensively described in animal studies.[17,18] For instance, changes in the expression of clock genes, nuclear receptors regulating clock transcription factors, and clock-controlled genes involved in fuel utilization have been reported in the hypothalamus and hepatic and adipose tissues of mice fed a high-fat diet.[19] In addition, loss of diurnal rhythmicity of hormones involved in the control of hypothalamic–pituitary–adrenal (HPA), hypothalamic–pituitary-gonadal (HPG), and hypothalamic–pituitary-thyroid (HPT) axes, such as cortisol/corticosterone, thyroid-stimulating hormone (TSH), and testosterone, has been observed in both rodents and humans fed high-fat diets.[19,20] Recently, altered diurnal oscillations of hepatic metabolites have also been reported in rats fed a cafeteria (CAF) diet.[21] This energy-dense and unhealthy diet, a model of a human obesogenic diet to study metabolic syndrome (MetS), is also able to disrupt the expression of clock genes in peripheral tissues such as *Bmal1* and *Per1/2* in the gut, *Bmal1* and *Rev-erbα* in the liver, and *Clock* and *Rev-erbα* in the adipose tissue.[22,23]

Nevertheless, not only is food composition crucial for maintaining the synchronization of the clock system, but the timing of food intake is now gaining importance.[13,24] Indeed, the role of meal timing (i.e., day- or night-time-restricted feeding) in the modulation of the clock system was demonstrated in mice.[25] Recent evidence in both humans and rodents also suggests that snacking can induce time-of-day-dependent effects on the organism.[26–29] For instance, Begemann et al. reported that snack consumption in the early hours of the resting phase increased energy resorption and body weight (BW) gain and produced changes in the peripheral clocks, locomotor activity, and body temperature in male mice.[26,27] Similarly, our group recently observed that a low dose of sugar (160 mg/kg), equivalent to only 2.5 g (half a teaspoon) in humans,[30] administered as a sweet treat to healthy rats before sleep promoted disruption of the central clock, an increase in BW gain, and changes in energy metabolism.[28] However, it should be noted that these experiments were conducted in healthy animals with a controlled diet, which may not fully reproduce the complexities of a more varied or calorically dense obesogenic diet. Interestingly, a recent clinical trial in healthy women demonstrated that the timing of chocolate consumption over 2 weeks produced different effects on

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circadian parameters, hunger perception, and gut microbiota composition.[29] These results suggest that, even in a human and varied dietary context, snack time could still exert significant effects on circadian regulation.

Interestingly, it has been recently observed that bioactive compounds, such as flavanols, can influence the clock system with a *zeitgeber* role.[23] This raises the question of whether interventions targeting circadian misalignment, such as bioactive compounds, could mitigate the detrimental effects of altered dietary patterns. Flavanols, a sub-class of flavonoids that are abundant in several foods, such as wine, tea, cocoa, and grape seeds, have been associated with multiple health benefits, including protective effects against obesity and MetS, conditions closely linked to circadian disruption, as mentioned above.[31] Different molecular mechanisms have been shown to be involved in the efficacy of flavanols, including epigenetic modifications, which have recently emerged as important mediators of their properties.[32] In this context, our group has reported that a grape seed (poly)phenol-rich extract (GSPE) can modulate central and peripheral clock genes in both healthy and CAF diet-induced obese rats.[33,34] In addition, these flavanols have shown modulatory effects on hepatic and central clocks in rats under jet-lag conditions.[35–37] Thus, the interaction of these phenolic compounds with the clock system has been recently suggested as another mechanism involved in their beneficial effects, particularly in contexts of dietary-induced circadian disruption.[23,38–40]

Therefore, the aim of the present study was to investigate whether long-term consumption of a low dose of sugar at two different times can influence, in a time-dependent manner, the deleterious effects of a human obesogenic diet on the central clock and metabolism in rats. Additionally, this study aimed to determine whether the administration of grape seed flavanols can ameliorate the circadian disruption caused by these variations in dietary patterns. To this end, CAF-fed rats were administered a low dose of sugar, without or with GSPE, at 8 a.m. or 8 p.m. (ZT0 and ZT12, respectively). Animals were sacrificed at 4 different points on the day (ZT1, ZT7, ZT13, and ZT19) to analyze diurnal oscillations of the hypothalamic clock genes, major biochemical parameters, and serum hormones over a 24-h period.

2 | EXPERIMENTAL PROCEDURES

2.1 | Grape seed (poly)phenol-rich extract

The extract used in this study was obtained from white grape seed and it is mainly composed of catechin, epicatechin, gallic acid, epicatechin gallate, and dimers, trimers, and tetramers of proanthocyanidins.[36] The main compounds present in this extract are shown in Table S1. This extract was provided by Les Dérives Résiniques et Terpéniques (Dax, France).

2.2 | Animal procedures

Sixty-four 13-week-old male Fischer 344 rats from Charles River Laboratories (Barcelona, Spain) were housed in pairs under standard (STD) laboratory conditions at 22°C and 12 h light/dark cycle (with ZT0 marking the time of lights on as a reference for counting the hours of the day), with ad libitum access to food (STD chow diet) and drinking water. After a one-week acclimatization period, rats were weighed and randomly divided into four groups ($n = 16$), and all groups were fed CAF diet for 9 weeks. During the last 4 weeks of this period, two animal groups were administered with low-fat sweetened condensed milk (60.8% sugars, 8.9% proteins, and 0.2% fats) diluted 1/5 in water (v/v) as a sweet treat (160 mg of sugar/kg of rat) at 8:00 a.m. (CAFZT0) or 8:00 p.m. (CAFZT12). The other two groups, in addition to the sweet treats, also received GSPE (25 mg/kg) at these 2 different times (CAFZT0-GSPE and CAFZT12-GSPE, respectively). GSPE was dissolved in diluted low-fat sweetened condensed milk, improving its taste, which allowed the animal to voluntarily consume the dose by syringe. To obtain four different time points in a 24-h period to analyze the diurnal oscillations, each group was randomly divided into four groups ($n = 4$) and sacrificed at ZT1, ZT7, ZT13, and ZT19 (9:00 a.m., 3:00 p.m., 9:00 p.m., and 3:00 a.m.) (Figure S1). The initial BW (week 1) of the different groups was 372.81 g (±3.62) for CAFZT0, 373.13 g (±2.28) for CAFZT12, 374.81 g (±4.57) for CAFZT0-GSPE, and 378.25 g (±4.63) for CAFZT12-GSPE. The CAF diet was prepared freshly every day and contained bacon, cookies with *paté*, cookies with cheese, carrots, *ensaïmada* (pastry), STD chow (72% carbohydrates, 8% fats, and 19% proteins; Safe-A04c, Scientific Animal Food and Engineering, Barcelona, Spain), and sweetened milk (22% sucrose *w/v*), and its caloric distribution was 55% carbohydrates, 33.6% fats, and 11.4% proteins (Table S2).[41] Moreover, to avoid the effect of our intervention at different times to give the dose, all rats were in the same room, and we ensured that all rats approached the end of the box at two times of administration. BW and food intake were recorded weekly throughout the experimental period. At the end of the experiment, the animals were deprived of access to food for 3 h and sacrificed by decapitation. Blood was collected in non-heparinized tubes, incubated for 1 h at

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room temperature, and immediately centrifuged at 1200g for 15 min at 4°C to collect serum. To extract the hypothalamus, we followed the same procedure described previously[28] and it was rapidly frozen in liquid nitrogen and stored at −80°C until further analyses.

Animal experiments were approved by the Animal Ethics Committee of Universitat Rovira i Virgili (reference number 9495) and carried out in accordance with Directive 86/609/CEE of the Council of the European Union and the procedures established by the Departament d'Agricultura, Ramaderia i Pesca of Generalitat de Catalunya (Barcelona, Spain).

2.3 | Indirect calorimetry

Indirect calorimetry was performed 1 week before sacrifice in all groups after receiving the low dose of sugar without and with GSPE. This procedure was carried out using an Oxylet Pro System (Panlab, Barcelona, Spain) and with the Metabolism 2.1.02 software program (Panlab, Barcelona, Spain) as previously described,[35] with minor modifications. Briefly, due to the differences in the metabolic activity of the rodents during the day and night, the results of the light and dark phases were separated. In addition, to avoid differences due to starting the procedure at different times, the data analyzed for each phase were narrowed down by eliminating the first 6 h after dosing. Thus, each phase was analyzed for 6 h, from ZT5 to ZT11 for the light phase and from ZT17 to ZT23 for the dark phase.

2.4 | Serum analysis

Glucose, total cholesterol (TC), and triglycerides (TAG) (QCA, Amposta, Tarragona, Spain) and total non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany) were analyzed by colorimetric enzymatic assays according to the manufacturer's instructions.

Serum hormone levels were measured by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ). Briefly, after sample preparation as previously described,[28] the samples were loaded into an SPE cartridge previously conditioned. The compounds were eluted with 500 μL of methanol, 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, analytical column was Zorbax Eclipse C18 (150 × 2.1 mm) from Agilent Technologies.

2.5 | 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 S3 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as a percentage of the rats sacrificed at ZT1 of the group CAF ZT0 using the $2^{-\Delta\Delta Ct}$ method with *Ppia* gene as an endogen control, as reported by Schmittgen and Livak.[42]

2.6 | Statistical analysis

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

To analyze the diurnal rhythmicity of the different parameters, we used cosinor-based rhythmometry method. This method makes it possible to calculate the diurnal oscillation rhythmicity parameters over a 24-h period, such as mean adjusted to the diurnal rhythm, amplitude (difference between the maximum value and the mean value of a wave), or acrophase (time at which the peak of a rhythm occurs). We considered the presence of diurnal oscillations when the model of the expressions of each gene fitted the cosine curves ($p < 0.05$). As previously described,[28] a script developed by our group was

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used for this analysis. Sample size was calculated by multiple regression test using G Power software (Düsseldorf, Germany) according to previous results obtained for melatonin level,[35] setting an alpha significance level of 0.05 to achieve 80% power. Moreover, for a more accurate estimation of the oscillatory parameters, we selected four equidistant time points that satisfy the Nyquist theorem, which stipulates a sampling frequency must be at least twice the highest frequency that is intended to be studied.[43] Previous studies also confirm that this sample size is adequate to estimate and model the diurnal oscillation of different parameters at 4 different time points during 24 h.[44,45]

3 | RESULTS

3.1 | Sugar administration before sleep did not modify body weight gain in cafeteria-fed rats, while grape seed flavanols administered at ZT12 reduced it

Animals fed a CAF diet progressively gained BW during the experimental period (Figure 1A,B). CAFZT0 and CAFZT12 rats gained 56.62% and 56.78%, respectively, of their initial BW. No differences were observed in the area under the curve (AUC) analysis of BW gain (Figure 1C), food intake (Table S4), or EE (Figure 1D) in animals administered sugar as a sweet treat before sleep (at ZT0) compared to animals that received sugar at the beginning of their active phase (at ZT12). GSPE administration caused a decrease in BW gain when it was administered at ZT12 ($p < 0.001$ and $p = 0.049$ for interaction between time and treatment effect, respectively) (Figure 1A,B). In concordance with these results, AUC analysis showed a greater decrease in BW gain in rats administered the extract at ZT12 ($p = 0.003$) than in those administered at ZT0 ($p = 0.052$) (Figure 1C). Interestingly, although no differences in food or energy intake were observed (Table S4), EE was stimulated by GSPE administration at ZT12 (Figure 1D). This group exhibited a 7.51% higher EE over the whole day compared to rats that received only sugar simultaneously ($p = 0.001$). Moreover, this increase tended to be greater than that observed in rats administered GSPE at ZT0 ($p = 0.062$). When EE was analyzed separately for the light (Figure 1E) and dark (Figure 1F) phases, GSPE administration at ZT0 showed

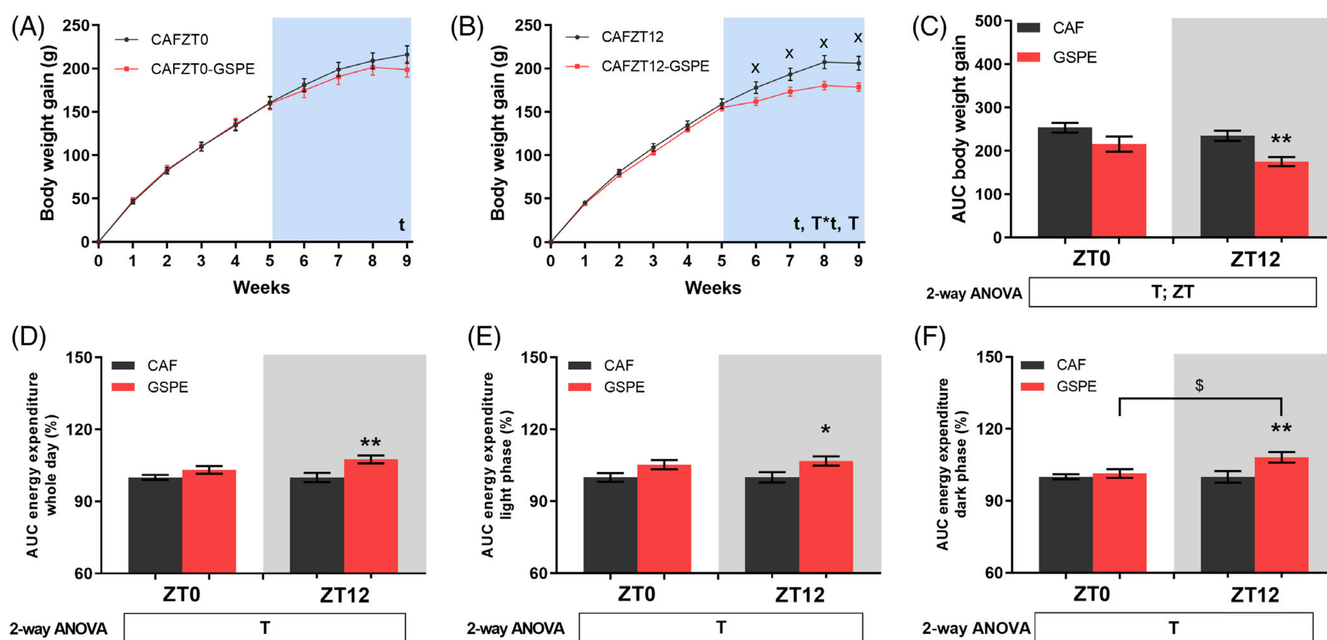

FIGURE 1 Body weight gain and energy expenditure. Body weight gain throughout the 9 weeks of the experiment in animals fed a cafeteria diet and administered a low dose of sugar without and with grape seed flavanols at ZT0 (A) and ZT12 (B) during the last 4 weeks. Area under the curve (AUC) of body weight gain during the supplementation period (C). AUC of energy expenditure for the whole day (D), light phase (E), and dark phase (F). Values are expressed as the mean ± SEM ($n = 14$–16). t, time effect; T, treatment effect; t × T, interaction between time and treatment effect; ZT, treatment time effect. x indicates significant differences ($p < 0.05$) between CAF and CAF-GSPE at each time point, using repeated measures ANOVA followed by Student's *t*-test. * and $ indicate significant differences by treatment effect and treatment time effect, respectively, as determined by two-way ANOVA followed by LSD post-hoc test ($p < 0.05$). ZT0 and ZT12: supplementation time at 8 a.m. and 8 p.m., respectively; CAF and GSPE: rats fed cafeteria diet and administered a sweet treat without and with 25 mg/kg grape seed (poly)phenol-rich extract, respectively.

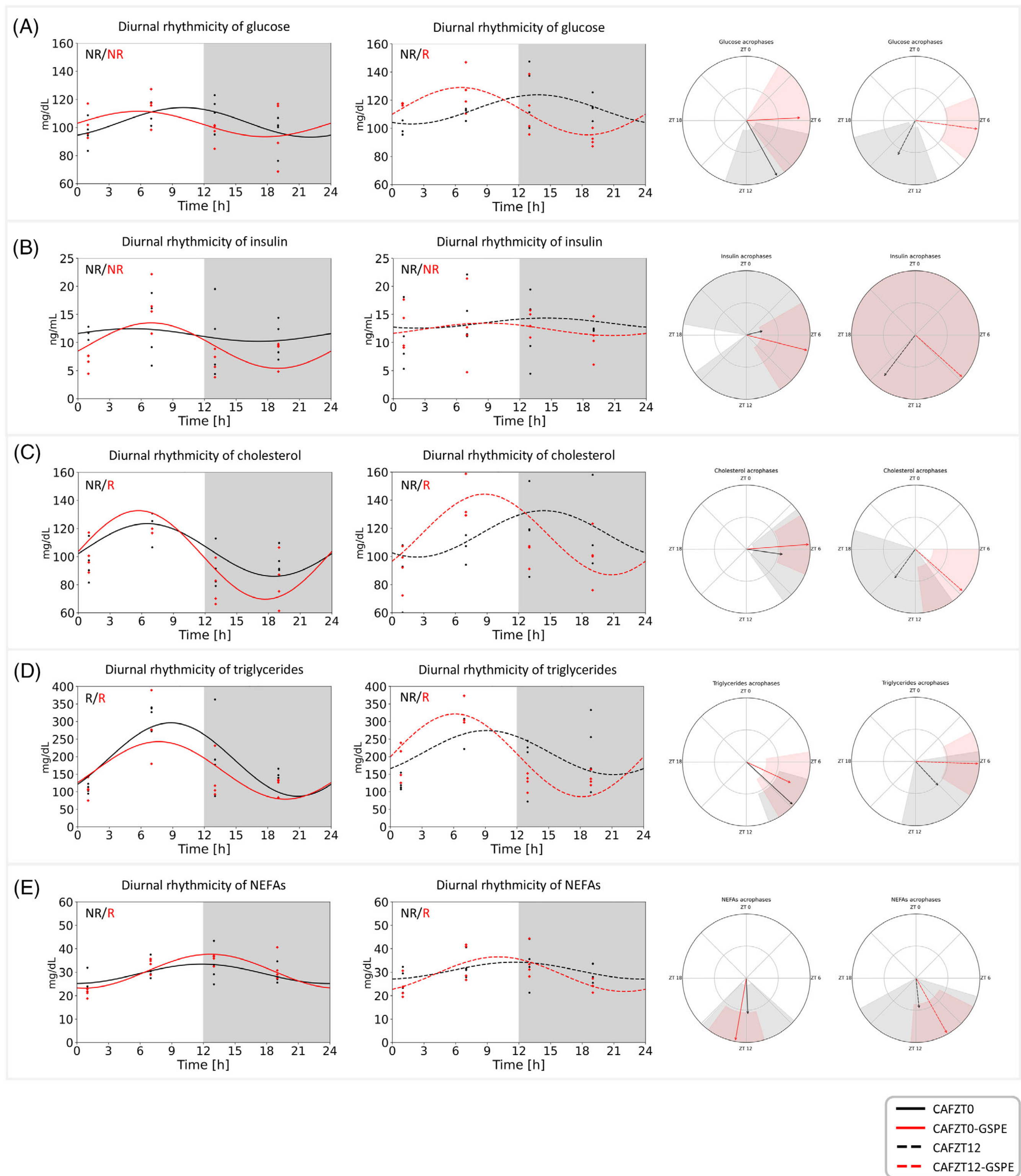

FIGURE 2 Serum biochemical parameters. The left side of the figure shows diurnal rhythmicity estimated using the cosinor method for groups administered at ZT0 and ZT12. The right side shows acrophases (indicated by the arrows) and their amplitudes (indicated by the arrow size) for groups administered at ZT0 and ZT12. Serum glucose (mg/dL) (A), insulin (ng/mL) (B), total cholesterol (mg/dL) (C), triglycerides (mg/dL) (D), and total NEFAs (mg/dL) (E). ZT: zeitgeber time; CAFZT0 and CAFZT12: rats fed a cafeteria diet and administered with a sweet treat at ZT0 (8 a.m.) or ZT12 (8 p.m.), respectively; CAFZT0-GSPE and CAFZT12-GSPE: rats administered a sweet treat with 25 mg/kg grape seed (poly)phenol-rich extract at ZT0 or ZT12, respectively; R: significantly rhythmic; NR: no rhythmic.

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a trend toward increased EE during the light phase ($p = 0.076$), with no significant differences observed in the dark phase compared to the CAFZT0 group. In contrast, GSPE administration at ZT12 resulted in a significant increase in EE during both the light ($p = 0.015$) and dark ($p = 0.005$) phases compared to the CAFZT12 group.

3.2 | The timing of sugar administration influenced the rhythmicity of some biochemical parameters in cafeteria-fed rats, while grape seed flavanols restored their altered diurnal oscillation

Most serum biochemical parameters showed no diurnal oscillations in rats with altered dietary patterns (CAFZT0 and CAFZT12) using the cosinor method (Figure 2 and Table S5). Regarding serum glucose, a tendency to display oscillation was observed in both groups that received sugar ($p = 0.052$ and $p = 0.094$ for CAFZT0 and CAFZT12, respectively). However, the oscillation models showed that while the acrophase of the CAFZT0-group oscillation was during the light phase (at ZT10), the acrophase of the CAFZT12-group oscillation was during the darkness phase (at ZT14), showing a tendency to be different ($p = 0.076$) by cosinor method (Figure 2A and Table S5). Additionally, sugar administration at ZT12 resulted in an increase in glucose levels at the ZT19 time point ($p = 0.043$) compared to animals receiving sugar at ZT0 by the Mann–Whitney test (Figure S2A). As a result of GSPE administration at ZT12, a clear diurnal rhythmicity was observed in glucose levels ($p = 0.008$). Moreover, the oscillation pattern of this parameter was nearly opposite between the CAFZT12-GSPE group and the CAFZT12 group (Table S5 and Figure 2A), showing significant differences in the acrophases of their oscillations for serum glucose levels ($p < 0.001$) (Table S6). Additionally, GSPE treatment at ZT12 led to increased glucose levels at ZT1 ($p = 0.029$) and decreased glucose levels at ZT19 ($p = 0.021$) compared to the CAFZT12 group by the Mann–Whitney test (Figure S2A). No diurnal oscillation was detected in glucose levels for the CAFZT0-GSPE group (Figure 2A and Table S5). For serum insulin (Figure 2B), no diurnal oscillation was detected for the CAFZT0 and CAFZT12 groups, nor for the group that received GSPE treatment at ZT12 (Table S5). However, the group receiving GSPE treatment at ZT0 showed a tendency to display diurnal rhythmicity ($p = 0.055$), with an acrophase around ZT7 (Table S5). No differences were found using the Mann–Whitney test among the different groups at each time point (Figure S2B).

Other biochemical parameters, such as total cholesterol, triglycerides, and total NEFAs, were also studied (Figure 2C–E). Regarding serum cholesterol (Figure 2C) and triglyceride levels (Figure 2D), only when CAF-fed rats received sugar at ZT0 did they display or show a tendency to exhibit diurnal oscillation ($p = 0.080$ and $p = 0.004$, respectively, for cholesterol and triglycerides), while the CAFZT12 group exhibits no diurnal oscillation for triglycerides or serum cholesterol by the cosinor method (Table S5). However, no diurnal rhythmicity was detected for any CAF-fed group administered with sugar in serum NEFAs (Figure 2E and Table S5). In addition, the oscillation models of both groups were opposite for serum cholesterol levels, with the acrophase of the CAFZT0-group oscillation around ZT7, whereas the acrophase of the CAFZT12-group oscillation would be around ZT14 ($p = 0.009$) (Table S6). When the different time points were analyzed, only a tendency to increased serum cholesterol levels in the CAFZT12 group compared to the CAFZT0 group at ZT13 ($p = 0.083$) was found by Mann–Whitney test (Figure S2C). No differences were detected at any time point for serum triglycerides or NEFAs by the Mann–Whitney test (Figure S2D–E). Interestingly, the GSPE-treated groups at ZT0 and ZT12 showed diurnal oscillations in serum cholesterol levels ($p = 0.005$ and $p = 0.029$, respectively), triglycerides ($p = 0.018$ and $p = 0.003$, respectively), and NEFAs ($p = 0.001$ and $p = 0.018$, respectively) (Table S5). Additionally, some differences were detected in these parameters by the Mann–Whitney test when the animals were administered the extract. Serum triglyceride levels tended to increase at ZT1 when GSPE was administered at ZT12 ($p = 0.089$) (Figure S2D). GSPE treatment at ZT12 resulted in decreased serum NEFA levels at ZT19 ($p = 0.047$) compared to the CAFZT12 group (Figure S2E). No differences in serum cholesterol were detected because of GSPE administration at any time point using the Mann–Whitney test (Figure S2C).

3.3 | Differential effects of sugar administration time on the rhythmicity of corticosterone and its receptors, while grape seed flavanols restored the diurnal oscillation of serum testosterone at any time and serum melatonin only at ZT12 in cafeteria-fed animals

Diurnal oscillations in serum corticosterone levels were detected in all experimental groups, with acrophases detected at the onset of the dark phase (Figure 3A and Table S5). These acrophases were different depending on the time of sugar administration between CAFZT0 and CAFZT12 groups ($p = 0.042$), being the acrophase of the CAFZT0-group oscillation around ZT17, while the

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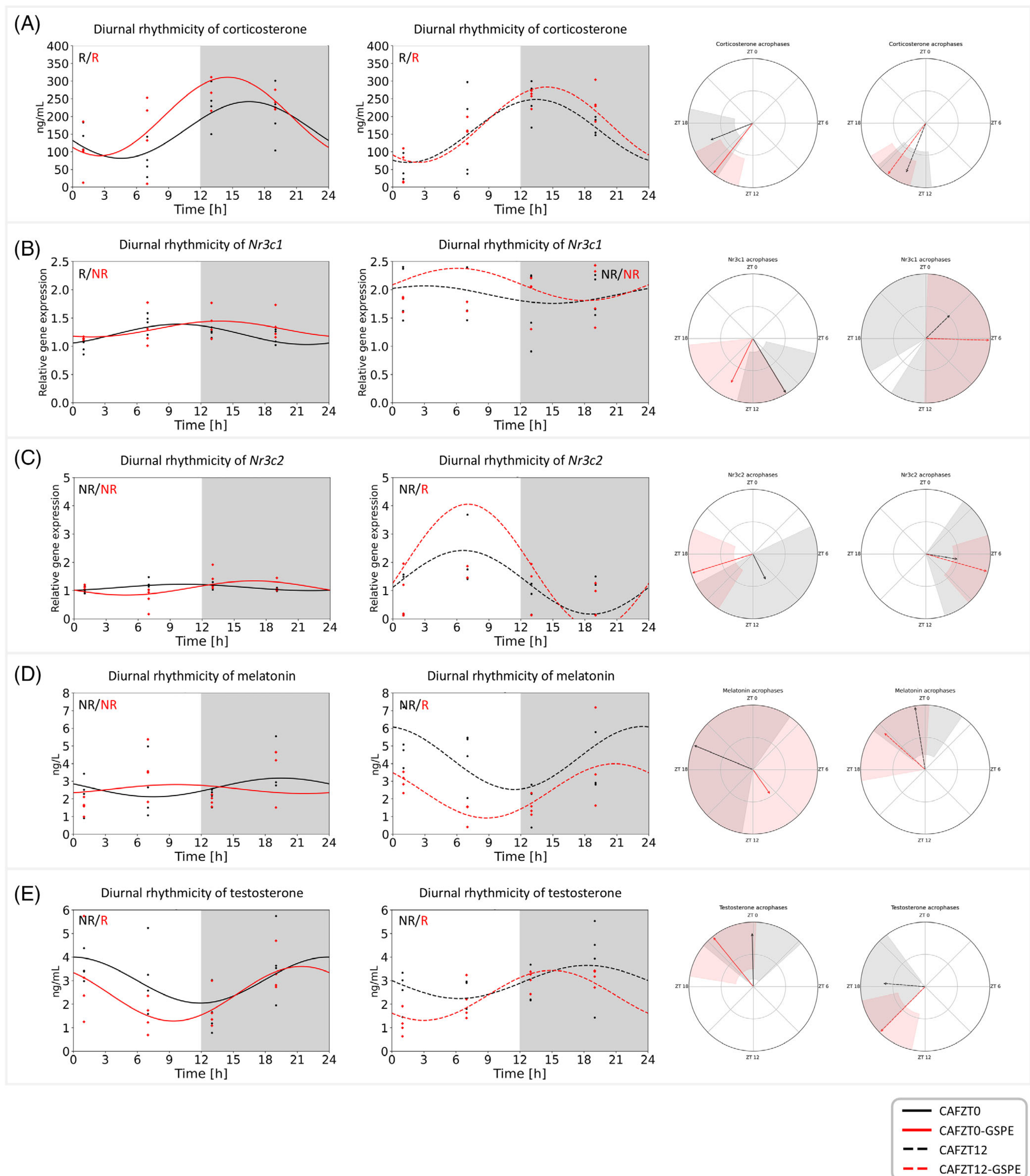

FIGURE 3 Serum hormones and glucocorticoid receptor genes. The left side of the figure shows diurnal rhythmicity estimated using the cosinor method for groups administered at ZT0 and ZT12. The right side shows acrophases (indicated for the arrows) and their amplitudes (indicated by the arrow size) for groups administered at ZT0 and ZT12. Serum corticosterone (ng/mL) (A), hypothalamic *Nr3c1* (B), and *Nr3c2* (C) gene expressions (relative gene expressions), serum melatonin (ng/L) (D), and testosterone (ng/mL) (E). ZT: zeitgeber time; CAFZT0 and CAFZT12: rats fed a cafeteria diet and administered with a sweet treat at ZT0 (8 a.m.) or ZT12 (8 p.m.), respectively; CAFZT0-GSPE and CAFZT12-GSPE: rats administered a sweet treat with 25 mg/kg grape seed (poly)phenol-rich extract at ZT0 or ZT12, respectively; R: significantly rhythmic; NR: no rhythmic.

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acrophase of the CAFZT12-group oscillation was around ZT14 (Table S6). Additionally, a tendency to increase serum corticosterone levels at ZT1 ($p = 0.083$) when the animals received sugar at ZT0 compared to the CAFZT12 group was observed by the Mann–Whitney test (Figure S3A). Although no differences in acrophase and amplitude were observed between animals treated with GSPE and those that only received sugar (Table S6), a higher fit of the oscillation model at both administration times was observed with GSPE treatment ($p = 0.005$ and $p < 0.001$, respectively, for CAFZT0-GSPE and CAFZT12-GSPE) (Table S5). No differences were detected at any time point for corticosterone levels by the Mann–Whitney test (Figure S3A). Regarding genes encoding for glucocorticoid receptors, the oscillations of gene expression of *Nr3c1* (Figure 3B) and *Nr3c2* (Figure 3C) genes were modified by sugar administration at different times of the day (Table S7). Only when sugar was administered at ZT0, the diurnal oscillation of *Nr3c1* gene expression was detected ($p = 0.021$) by the cosinor method. This absence of rhythmicity for the ZT12 group could be due to the observed increase in gene expression at ZT1 ($p = 0.029$) and ZT19 ($p = 0.021$) and a tendency to increase at ZT7 ($p = 0.083$). Sugar was administered to animals at ZT12 compared to the CAFZT0 groups by the Mann–Whitney test (Figure S3B). No diurnal rhythmicity was detected in the GSPE-treated groups at ZT0 and ZT12 by the cosinor method (Table S7). In the case of the *Nr3c2* gene, both CAF-fed groups that received sugar showed tendency to display diurnal oscillation ($p = 0.072$ and $p = 0.058$, respectively, for CAFZT0 and CAFZT12). However, these oscillations are considerably different between the two groups. Thus, differences between the amplitudes of the CAFZT0 and CAFZT12 groups ($p = 0.017$) were observed by the cosinor method (Table S8). Additionally, when the animals received sugar at ZT12, an increased *Nr3c2* gene expression was detected at ZT7 ($p = 0.043$) by the Mann–Whitney test (Figure S3C). GSPE treatment promoted the diurnal oscillation of the expression of this gene only when the extract was administered at ZT12 ($p = 0.049$) (Table S7). No differences were detected at any time point for *Nr3c1* and *Nr3c2* gene expressions by the Mann–Whitney test in GSPE-treated animals compared to CAFZT0 and CAFZT12, respectively, for each dose time (Figure S3B,C).

Interestingly, no diurnal oscillation was detected for serum melatonin levels for CAF-fed groups that received sugar at both ZT0 and ZT12 (Figure 3D and Table S5) by the cosinor method. Only the GSPE-treated group at ZT12 showed a clear diurnal oscillation for serum melatonin levels ($p = 0.023$) with an acrophase around ZT21 (Table S5), whereas no rhythmicity was detected for the GSPE-treated group at ZT0. Furthermore, for the groups receiving sugar, no diurnal rhythmicity was detected for serum testosterone (Figure 3E). Nevertheless, both GSPE-treated groups at both ZT0 ($p = 0.042$) and ZT12 ($p = 0.001$) showed a clear oscillation with acrophase in the dark phase (Table S5) by cosinor analysis. However, no differences were detected at any time point for these two hormones by the Mann–Whitney test (data not shown). The two main thyroid hormones were also studied. However, no oscillations were detected for either group in serum T3, T4, and T3-to-T4 ratio (Figure S4 and Table S6), nor differences between groups at each time point by the Mann–Whitney test (data not shown).

3.4 | Diurnal rhythm patterns of hypothalamic appetite signaling genes were altered differently according to sugar administration time in cafeteria-fed animals, while grape seed flavanol treatment at ZT12 restored the diurnal rhythmicity of leptin and modulated appetite signaling gene expression

Diurnal rhythmicity of serum, leptin was not detected for the CAFZT0 and CAFZT12 groups (Figure 4A and Table S5) by the cosinor method, and the administration of GSPE at ZT0 did not restore the rhythmicity of this parameter. In contrast, diurnal oscillation was detected for the group treated with GSPE at ZT12 ($p = 0.018$), showing acrophase in the light phase (Figure 4A and Table S5). No differences were detected at any time point for serum leptin by the Mann–Whitney test (data not shown). Hypothalamic *Cart* gene expression tended to display diurnal rhythmicity in all 4 experimental groups by cosinor method (Figure 4B and Table S7). Diurnal oscillations in the expression of this gene differed between the CAF-fed groups and their corresponding GSPE-treated groups at both ZT0 ($p = 0.043$) and ZT12 ($p = 0.029$) (Table S8). Moreover, the acrophases of both groups receiving sugar and GSPE at ZT0 were detected in the light phase, whereas the acrophases of CAFZT12 and CAFZT12-GSPE were in the dark phase, being significant between the CAFZT0 and CAFZT12 groups ($p = 0.002$) (Table S8). Additionally, an increased gene expression ($p = 0.043$) for *Cart* gene was observed at ZT19 time point by Mann–Whitney test (Figure S5A). Hypothalamic *Pomc* gene expression showed diurnal oscillation for both groups receiving sugar ($p = 0.008$) and GSPE ($p = 0.016$) at ZT0, with acrophases in the dark phase (around ZT16) (Figure 4C and Table S7). No diurnal oscillations in the expression of this gene were observed for the CAFZT12 or CAFZT12-GSPE groups. A tendency to enhance gene

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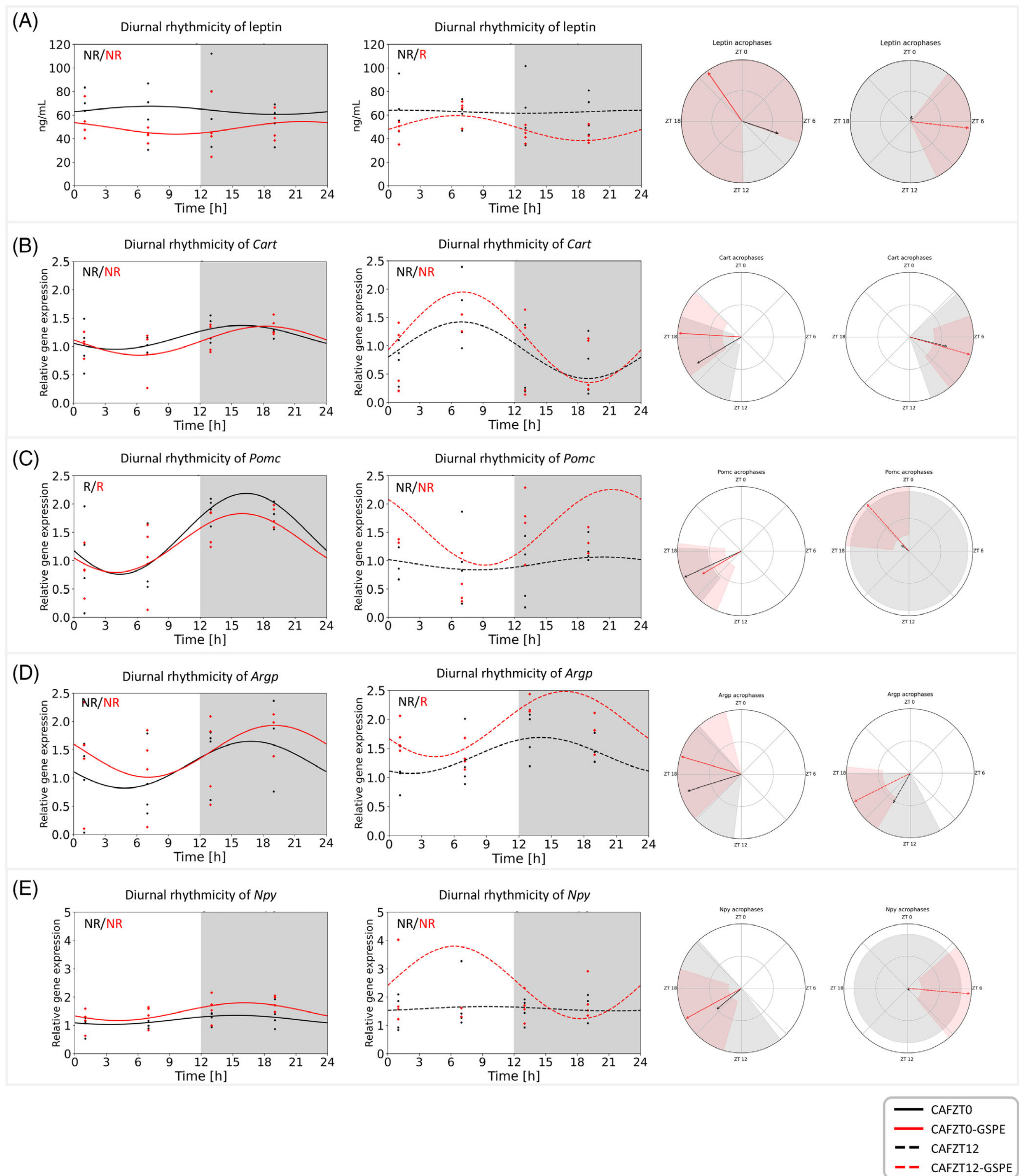

FIGURE 4 Serum leptin and hypothalamic appetite signaling genes. The left side of the figure shows diurnal rhythmicity estimated using the cosinor method for groups administered at ZT0 and ZT12. The right side shows acrophases (indicated for the arrows) and their amplitudes (indicated by the arrow size) for groups administered at ZT0 and ZT12. Serum leptin (ng/mL) (A), hypothalamic *Cart* (B), *Pomc* (C), *Argp* (D), and *Npy* (E) gene expressions (relative gene expressions). ZT: zeitgeber time; CAFZT0 and CAFZT12: rats fed a cafeteria diet and administered with a sweet treat at ZT0 (8 a.m.) or ZT12 (8 p.m.), respectively; CAFZT0-GSPE and CAFZT12-GSPE: rats administered a sweet treat with 25 mg/kg grape seed (poly)phenol-rich extract at ZT0 or ZT12, respectively; R: significantly rhythmic; NR: no rhythmic.

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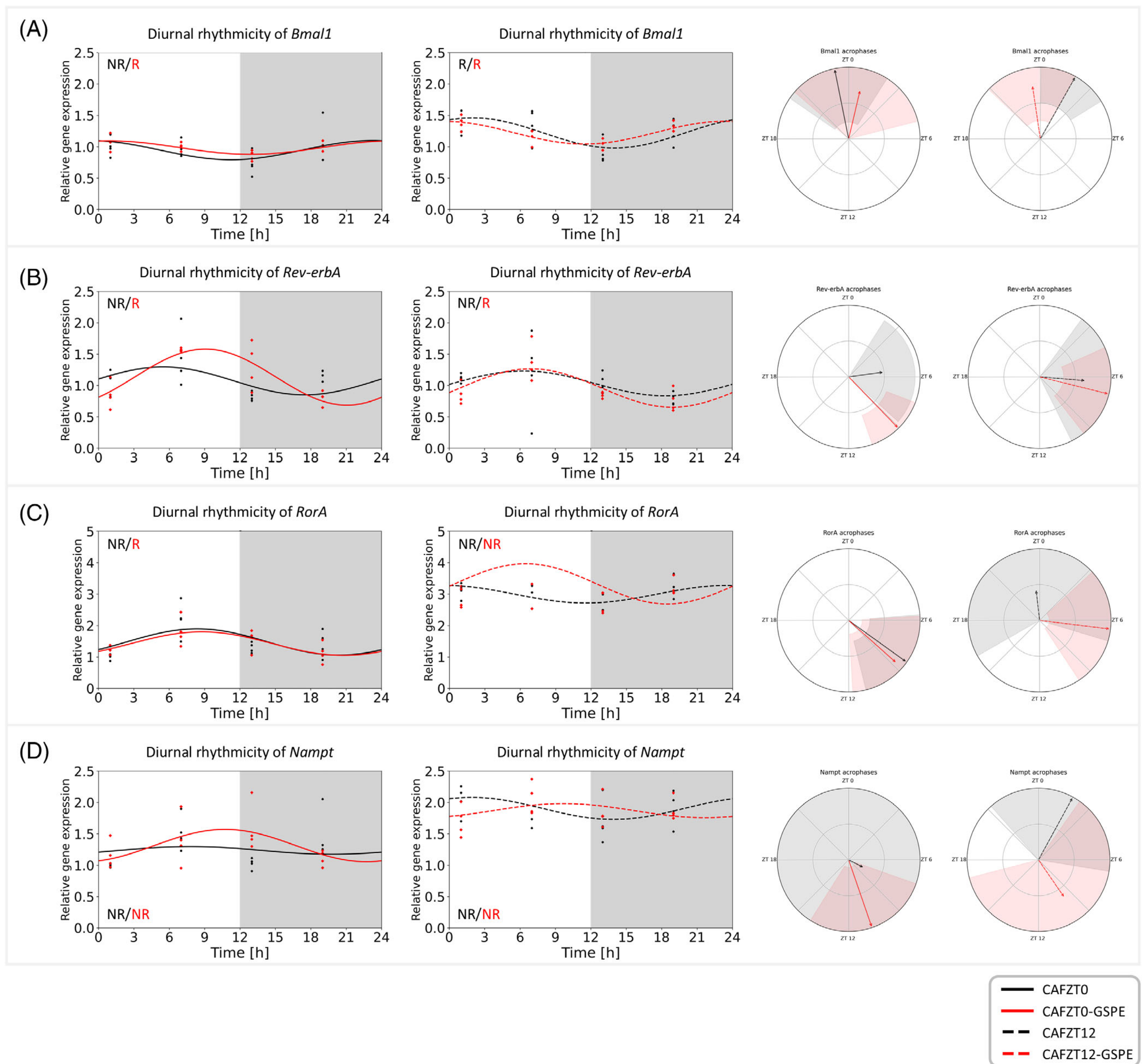

FIGURE 5 Hypothalamic clock genes. The left side of the figure shows diurnal rhythmicity estimated using the cosinor method for groups administered at ZT0 and ZT12. The right side shows acrophases (indicated for the arrows) and their amplitudes (indicated by the arrow size) for groups administered at ZT0 and ZT12. Hypothalamic *Bmal1* (A), *Rev-erbα* (B), *Rorα* (C), and *Nampt* (D) gene expressions (relative gene expressions). ZT: zeitgeber time; CAFZT0 and CAFZT12: rats fed a cafeteria diet and administered with a sweet treat at ZT0 (8 a.m.) or ZT12 (8 p.m.), respectively; CAFZT0-GSPE and CAFZT12-GSPE: rats administered a sweet treat with 25 mg/kg grape seed (poly) phenol-rich extract at ZT0 or ZT12, respectively; R: significantly rhythmic; NR: no rhythmic.

expression was detected for this gene at ZT13 ($p = 0.083$) and an increase at ZT19 ($p = 0.021$) when the animals received sugar at ZT0 compared to ZT12 (Figure S5B) by the Mann–Whitney test. Additionally, although no rhythmicity was detected in the animals that received the GSPE dose at ZT12, a tendency to increase *Pomc* expression with GSPE treatment at ZT1 ($p = 0.083$) and ZT13 ($p = 0.083$) compared to the CAFZT12 group was observed by the Mann–Whitney test (Figure S5B). Regarding *Argp* gene (Figure 4D), only the animals treated with GSPE at ZT12 displayed diurnal oscillation for the expression of this gene with an acrophase around ZT16 (Table S7). Furthermore, GSPE treatment at ZT12 resulted in a tendency to increase *Argp* expression at both ZT13 ($p = 0.083$) and ZT19 ($p = 0.085$) by the Mann–Whitney test (Figure S5C). In the case of hypothalamic

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Npy gene expression (Figure 4E), no diurnal rhythmicity was detected using the cosinor method in any group. No differences were detected at any time point for *Npy* gene expression by the Mann–Whitney test (Figure S5D).

3.5 | Sugar administration time modified the rhythmicity of the hypothalamic expression of *Bmal1* in cafeteria-fed animals, while grape seed flavanols modulated the expression of clock genes in the hypothalamus

To determine the state of the central clock in an obesogenic context, the diurnal rhythmicity of the expression of hypothalamic clock genes was analyzed using the cosinor method (Figure 5 and Table S7). Interestingly, the administration of a low dose of sugar at ZT0 altered the diurnal oscillation of *Bmal1* gene expression in the hypothalamus, losing the diurnal oscillation observed in the CAFZT12 group ($p = 0.032$) (Figure 5A and Table S7). The acrophases were also different between both groups receiving sugar at different times ($p < 0.001$), being around ZT23 and ZT2 for the CAFZT0 and CAFZT12 groups, respectively (Table S8). Additionally, the expression of this gene at ZT1 ($p = 0.043$) and ZT7 ($p = 0.043$) was decreased when the animals received sugar at ZT0 compared to the ZT12 group by the Mann–Whitney test (Figure S6A). Interestingly, GSPE administration at ZT0 resulted in the restoration of diurnal oscillation in the expression of this gene ($p = 0.014$), showing an acrophase around ZT1 (Table S7). Animals administered GSPE at ZT12 also displayed diurnal oscillations in *Bmal1* expression ($p = 0.001$). Nevertheless, a significant difference ($p < 0.001$) between the acrophases of the animals administered GSPE and sugar at ZT12 was observed by the cosinor method (Table S8). While the acrophase of the CAFZT12 group was around ZT2, that of the GSPE-treated animals was around ZT23 (Table S7). No differences were detected at any time point between sugar-administered and GSPE-treated groups for *Bmal1* expression by the Mann–Whitney test (Figure S6A). No diurnal oscillation of *Rev-erbα* gene expression was detected for both CAF-fed groups that received sugar at both times, ZT0 and ZT12 (Figure 5B). Only an increased gene expression at ZT13 was detected when animals received sugar at ZT12 compared to the CAFZT0 group ($p = 0.043$) by the Mann–Whitney test (Figure S6B). GSPE treatment at ZT0 and ZT12 resulted in rhythmicity recovery ($p < 0.001$ and $p = 0.006$, respectively) (Table S7). In both cases, the acrophase was detected in the light phase around ZT9 and ZT7 for CAFZT0-GSPE and CAFZT12-GSPE groups, respectively (Table S7). Moreover, GSPE treatment at ZT0 resulted in increased *Rev-erbα* expression at ZT13 compared to the CAFZT0 group ($p = 0.043$) and, for this time point, a tendency to decrease gene expression when the animals were treated with GSPE at ZT12 ($p = 0.053$) compared to CAFZT12 by the Mann–Whitney test (Figure S6B). Additionally, increased gene expression at ZT1 in the CAFZT12-GSPE group compared to CAFZT12 was detected ($p = 0.045$) by the Mann–Whitney test (Figure S6B). In the case of *Rorα* gene expression (Figure 5C), no diurnal oscillation was detected in the CAFZT0 group, whereas CAFZT12 animals showed a tendency to oscillate ($p = 0.088$) (Table S7). Moreover, an increase of gene expression of this gene was detected at all time points when the animals received sugar at ZT12 compared to the CAFZT0 groups by the Mann–Whitney test (Figure S6C). Interestingly, only the GSPE-treated group at ZT0 showed diurnal oscillation in *Rorα* gene expression ($p = 0.020$) with acrophase around ZT9 (Tables S7 and S8), while GSPE administration at ZT12 did not result in the recovery of diurnal oscillation. Additionally, an increase in expression with GSPE treatment at ZT0 was observed at time point ZT1 compared to the CAFZT0 group ($p = 0.043$) by the Mann–Whitney test (Figure S6C). Finally, for the *Nampt* gene expression, no diurnal rhythmicity was detected for any group, only GSPE treatment at ZT0 tended ($p = 0.092$) to show oscillation for *Nampt* expression (Figure 5D and Table S7). Sugar administration at ZT12 resulted in an increase of gene expression at ZT1 ($p = 0.021$) and ZT13 ($p = 0.021$) time points compared to animals that received sugar at ZT0 by the Mann–Whitney test (Figure S6D). Furthermore, GSPE treatment at ZT0 resulted in increased *Nampt* expression at ZT13 compared to the CAFZT0 group ($p = 0.021$) by the Mann–Whitney test (Figure S6D).

4 | DISCUSSION

The important impact on the body clock system of food compositions has been evidenced.[17–19] Moreover, the time of the day at which food is consumed is also considered as crucial for the maintenance of circadian system.[13,24] Indeed, unbalanced diets such as late-night food intake have been related to alterations of the diurnal rhythms and changes in behavior, metabolism, and some physiological processes, increasing the risk of developing MetS.[1,26–29] In this context, flavanols have demonstrated extensive beneficial effects on metabolic disorders related to MetS.[31] Our group has recently demonstrated GSPE effects on central and peripheral clocks.[23,33,34] Indeed, the interaction of phenolic compounds with the clock

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system has been suggested by our group as another potential mechanism involved in phenolic beneficial effects in obesity.[23] Therefore, the aim of the present study was to evaluate, for the first time to our knowledge, the effects of a long-term consumption of a sweet treat at two different times in an obesogenic context and whether the administration of grape seed flavanols can ameliorate the circadian disruption caused by these variations in dietary patterns. To achieve these purposes, four groups were fed a CAF diet for 9 weeks and administered a sweet treat with or without GSPE (25 mg/kg of BW) at ZT0 or ZT12 for the last 4 weeks, being sacrificed at four different times to study their clock system.

To understand the impact of sweet treat consumption at the beginning of the active phase (CAFZT12) or at the beginning of the resting phase (CAFZT0) in rats fed a human calorie-dense diet, the groups that received sweet treats at different times were compared. Some studies have demonstrated the impact on BW gain when a snack or sweet treat is administered before bedtime for a long term in both humans[29] and animals.[27,28] In fact, in our previous study an increased BW gain was observed when healthy rats received sweet treats at ZT0 for 4 weeks compared to rats receiving sweet treats at ZT12.[28] However, in the present study, no differences were found in BW gain when sugar was administered at ZT0 or ZT12 in an obesogenic context. This discrepancy in these results may be due to the fact that healthy animals are on a controlled diet and the effects of this dose of sugar (160 mg/kg) are more marked than in obese rats fed a CAF diet, whose diet is higher in sugar and fat. In addition, the CAF diet includes milk with 22% sucrose which is similar to a sweet treat and could be consumed ad libitum by animals. Although no significant changes were found in BW gain, sugar administration at different times slightly affected the oscillation of serum glucose levels. Specifically, the acrophase of serum glucose oscillations shifted depending on when sugar was administered. Both groups, CAFZT0 and CAFZT12, showed an acrophase around ZT12 (beginning of the dark phase), in contrast to healthy rats, where the acrophase occurred during the light phase.[28] However, in both groups the oscillations were not significant, which contrasts with our previous findings where only rats receiving sugar at ZT12 displayed clear diurnal rhythmicity, while those receiving sugar at ZT0 lost this rhythmicity.[28] Interestingly, these time-dependent changes in the acrophase of glucose level oscillations coincide with changes in the oscillatory patterns of *Bmal1* gene expression. Indeed, the deletion of this gene has been reported to decrease insulin secretion and disrupt glucose levels, suggesting that *Bmal1* plays a crucial role in glucose metabolism.[46] Our results showed that, in both cases, the oscillation patterns revealed that the highest *Bmal1* gene expression coincided with the greatest drop in glucose levels, following patterns dependent on the timing of sugar administration. Additionally, a clear oscillation was observed only in the CAFZT12 group, while no oscillation was detected in animals receiving sugar at ZT0. However, although rhythmicity was evident in the CAFZT12 group, the *Bmal1* oscillation did not align with the pattern observed in healthy rats, where the peak occurred around ZT22.[28] In addition, changes in the diurnal oscillation of *Cart* and *Pomc* gene expression were observed depending on the time of sweet treat administration. These changes could be due to the observed modifications in the oscillation of *Bmal1* gene expression, which, through other clock genes such as *Nampt* and *Rev-erbα*, are able to modulate the Arc in rats.[8,9,47] In this context, our results showed that *Cart* and *Pomc* gene expressions were lower during the light phase in rats supplemented with sugar at ZT0, while *Cart* gene expressions were lower during the dark phase, and *Pomc* gene expression did not display diurnal oscillation in rats supplemented with sugar at ZT12. These results are in concordance with those obtained in the previous study in healthy rats, where the direction of the oscillation in *Cart* gene expression changed according to the time of sweet treat administration, showing opposite oscillations between the two groups.[28] In addition, the oscillation models of the *Pomc* gene showed similar sugar administration time-dependent patterns in healthy rats.[28]

An important hormone in the circadian control of metabolism is cortisol in humans or corticosterone in rodents. This hormone plays a key role in the HPA axis and exhibits marked diurnal rhythmicity,[48,49] thus its circadian disruption could lead to diseases of glucocorticoid sensitivity or resistance disorders.[50] Furthermore, the rhythmic maintenance of corticosterone release appears to be regulated through the action of the clock system on the expression of the *Nr3c1* gene, which encodes for a glucocorticoid receptor.[48,51–53] Specifically, a cross-regulation between *Nr3c1* and *Rev-erbα* that affects the expression of both receptors was reported.[54] Our results showed a shift in corticosterone oscillations depending on the time of sugar administration. Similar findings were observed in healthy rats, where the acrophase of the ZT0 sugar-administered group shifted compared to the ZT12 group.[28] This displacement could be caused by changes in the oscillations of the glucocorticoid receptor genes, *Nr3c1* and *Nr3c2*, both of which displayed patterns dependent on the time of sugar intake. These results highlight the influence of sweet treat consumption at different times of the day, promoting changes in the central clock and energy homeostasis. However, these changes were not as pronounced as in healthy rats.[28] This could be explained by the high fat

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and carbohydrate content of the CAF diet that leads to its capacity to induce a clinical picture of obesity and other comorbidities characteristic of the MetS and, especially, by its ability to induce circadian disruption.[21,41]

In view of these results, the GSPE effects as a circadian modulator were evaluated in this obesogenic context caused by these variations in dietary patterns. GSPE administration reduced BW gain compared to obese rats that only received sugar at ZT12 (CAFZT12), but this reduction was not observed with supplementation at ZT0. Regarding this, other studies have found effects on BW gain after morning administration (ZT0) of GSPE to CAF-fed rats, but the doses of GSPE were higher than the 25 mg/kg used in this study,[41] or the extract was administered in combination with other bioactive compounds.[55] Additionally, GSPE administration stimulated EE over the whole day in rats receiving sugar at ZT12, whereas this effect was limited to the light phase in those that received sugar at ZT0. Although other studies reported an increase in EE after GSPE administration when treatment was given in the morning (ZT0), the doses were also higher than those used in this study.[56,57] The greater decrease in BW gain in animals administered at ZT12 could be due to the stimulation of EE by GSPE. Furthermore, the difference between the GSPE-administered groups in terms of decreased BW gain and stimulating EE could be related to the recently reported changes in GSPE bioavailability depending on the time of day it is consumed.[58]

CAF diet-induced obesity has been shown to alter lipid and glucose profiles,[59] as well as disrupt the rhythmicity of glucose metabolism and hepatic diurnal oscillations.[21,33,60,61] In this study, most of the biochemical parameters studied did not display diurnal oscillations in serum. For animals with late-night snack intake (ZT0), GSPE treatment tended to restore rhythmicity in serum insulin but not glucose levels. In contrast, in the group that received sugar at ZT12, rhythmicity was observed for serum glucose but not insulin. Regarding triglycerides, cholesterol, and NEFAs, GSPE treatment improved their rhythmicity under both feeding conditions. In this regard, GSPE has demonstrated its ability to improve the lipid or glucose profile in an obesogenic context.[62–65] In addition, GSPE showed circadian modulating capacity of central clock and peripheral clock components in the liver, such as *Bmal1*, *Rev-erbα*, and *Rorα* genes, that are closely related to an improvement of lipid and glucose metabolism.[33,36,66–69] Specifically in the hypothalamus, GSPE treatment modulated diurnal oscillation of *Bmal1* and *Nampt* when administered at ZT0 and *Rev-erbα* when administered at ZT12, as previously observed in healthy rats.[34]

Interestingly, the restoration of *Bmal1* rhythmicity by GSPE treatment appeared to play a crucial role in modulating the oscillatory patterns of other clock genes and biochemical parameters, as it is the master regulator of the molecular clock system and has a fundamental role in glucose metabolism.[46,47] For instance, in sugar-supplemented animals at ZT0, GSPE restored the oscillatory patterns of *Bmal1*, *Rev-erbα*, and *Rorα*, which could explain the recovery of rhythmicity in triglycerides, cholesterol, and NEFAs. In fact, the absence or disruption of *Rev-erbα* promotes an increase in serum triglycerides and BW in mice.[8] Moreover, *Rev-erbα* expression has been related to the maintenance of the central clock and the regulation of lipid metabolism in an obesogenic context.[70] In contrast, GSPE did not restore *Bmal1* oscillations as it was not lost in CAFZT12 animals, but it did shift its acrophase to the dark phase, aligning with oscillation patterns observed in healthy rats.[28] Interestingly, this change was accompanied by the restoration of serum glucose rhythmicity, which is in alignment with the aforementioned interaction between *Bmal1* and the regulation of glucose metabolism. Similar to ZT0 animals, lipid parameters were restored, together with *Rev-erbα* expression. In addition, hypothalamic gene expression of *Nampt* is also related to energy metabolism and the control of food intake through appetite signaling genes; thus, it has been reported that NAMPT administration in the Arc of rats promoted increased food intake and decreased *Cart* expression.[9] The regulation of *Argp* or *Pomc* genes and genes related to mitochondrial function have also been related to *Nampt* expression.[5,6,9] In this sense, appetite signaling genes overall did not show rhythmicity for sugar-administered groups, which would be in agreement with the results obtained for clock genes, which lose rhythmicity. Interestingly, together with the recovery of the diurnal oscillation of some clock genes, rhythmicity was observed in the expression of genes such as *Cart* or *Pomc* for GSPE-treated animals with late-night snack intake, while *Cart* and *Argp* for GSPE-treated animals that received sugar at ZT12. Finally, serum leptin only displayed diurnal oscillation with GSPE treatment in sugar-supplemented animals at ZT12. This hormone, secreted by fat cells, exhibits important functions in the regulation of food intake and energy metabolism, and leptin resistance may promote the development of obesity and MetS. In humans, leptin shows a marked diurnal oscillation 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 eating behavior.[71,72] In our study, although the restoration of leptin rhythmicity in GSPE-treated animals is displaced by 3 h with respect to healthy animals,[28] this coincided with the recovery of the *Rev-erbα* gene oscillation. These results align with previous studies that have shown that the absence of *Rev-erbα* alters feeding behavior and promotes leptin resistance.[8]

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Melatonin, a key circadian marker, increases during the dark phase, being a crucial signal for the organism to recognize the light/dark cycle and synchronize peripheral clocks.[73] In this study, melatonin diurnal oscillation was significantly affected in all CAF-fed groups that did not receive GSPE. Interestingly, GSPE treatment in sugar-supplemented animals at ZT12 restored melatonin rhythmicity, an effect not observed in animals with late-night snack intake (ZT0). Previous studies in healthy rats reported that GSPE administration at ZT0 increased melatonin levels, extending its circulation beyond dawn, whereas this effect was not observed when administered at ZT12.[34] These discrepancies in the results could be due to the fact that healthy rats did not have diet-induced alterations of the clock system. In contrast, in this study the diurnal oscillation of corticosterone was not affected in either group. As mentioned above, this hormone plays a fundamental role in maintaining the rhythmicity of energy metabolism.[48,49] Although glucocorticoid receptors did not show clear rhythmicity in these obese rats, they were not sufficient to break the rhythmicity of corticosterone levels. Interestingly, the observed slight shift between the two groups in both feeding pattern conditions was restarted by the administration of GSPE, locating the acrophase of both treated groups around ZT15. Testosterone and thyroid hormones act as modulators of the HPG and HPT axes, respectively. These axes are important in processes such as lipid and carbohydrate metabolism and are closely related to biological rhythms.[74,75] In this study, the rhythmicity of these hormones was not detected in sugar-administered groups, which could lead to metabolic disorders. This effect was particularly relevant for thyroid hormones, as their diet-dependent diurnal oscillation is crucial for regulating feeding patterns.[76,77] Although GSPE administration restored the rhythmicity of testosterone levels at both dosing times, thyroid hormones did not recover the rhythmicity.

5 | CONCLUSIONS

On one hand, long-term consumption of sugar as a sweet treat in rats fed a human obesogenic diet caused slight but notable changes in the diurnal oscillation of different parameters, depending on the time of sugar administration. Specifically, this low dose of sugar altered in a time-of-day-dependent manner the oscillation of hypothalamic *Bmal1* and glucocorticoid receptor gene expression, as well as serum corticosterone levels. Changes in the rhythmicity of anorexigenic genes such as *Cart* and *Pomc*, which are involved in energy homeostasis, were also observed. These findings, together with alterations in glucose, triglycerides, and cholesterol levels, suggest that the timing of sweet treat consumption differentially affects CAF-fed animals. Although these changes are slight, they could lead to long-term changes in eating behavior and metabolism due to alterations in central clock signaling and regulation of food intake. On the other hand, GSPE treatment restored the diurnal oscillation of the central clock and several cardiometabolic parameters altered by these dietary patterns. GSPE effectively stimulated EE and reduced BW gain, especially in sugar-supplemented animals at ZT12. In addition, GSPE treatment restored rhythmicity in hypothalamic clock genes, appetite signaling genes, and key serum parameters such as glucose, insulin, leptin, cholesterol, and triglycerides. Notably, melatonin levels, a critical marker of the light/dark cycle, also recovered oscillation only in animals supplemented with sugar and treated with GSPE at ZT12. These results highlight the potential of the GSPE as a *zeitgeber*, modulating the central clock and metabolic rhythms under obesogenic conditions. Importantly, there is evidence that circadian disruption by the CAF diet affects males and females differently[21] and our study is limited to male rats. Thus, studying the effect of these dietary patterns in females would be of interest. In conclusion, these findings showed the importance of circadian rhythms in the regulation of metabolism and energy homoeostasis, and how the time of consumption of a small, sweet snack can slightly influence the clock system even in rats fed a human obesogenic diet. Furthermore, our results also reinforce the hypothesis suggesting the role of GSPE as a *zeitgeber*, showing modulatory effects on the central clock and restoring the diurnal oscillation of different parameters in these altered feeding patterns.

AUTHOR CONTRIBUTIONS

Jorge R. Soliz-Rueda: Conceptualization; data curation; formal analysis; investigation; methodology; writing – original draft. **Raúl López-Fernández-Sobrino**: Data curation; formal analysis; investigation; methodology. **Harriët Schellekens**: Conceptualization; funding acquisition; supervision; writing – reviewing and editing. **Francisca Isabel Bravo**: Conceptualization; funding acquisition. **Manuel Suárez**: Conceptualization; funding acquisition. **Miquel Mulero**: Conceptualization; funding acquisition. **Begoña Muguerza**: Conceptualization; funding acquisition; supervision; investigation; writing – reviewing and editing.

ACKNOWLEDGMENTS

The authors would like to thank Niurka Llópiz and Rosa Pastor for their help and technical assistance. They also thank Pol Herrero and Antonio del Pino of the Center for Omics Sciences (COS) Joint Unit of the Univesitat Rovira

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i Virgili-Eurecat for their contribution to hormone determination. This project was funded by the Spanish Ministry of Science and Innovation MCIN/AEI/10.13039/501100011033/FEDER "Una manera de hacer Europa" (Grant number: AGL2016-77105-R and PID2021-128813OB-I00). J. R. S.-R. is the recipient of a grant for the hiring of predoctoral research staff (Grant number: BES-2017-080919, FPI) from the Spanish Ministry of Science and Innovation MCIN/AEI/10.13039/501100011033 and FSE "El FSE invierte en tu futuro." B. M. thanks the Spanish Ministry of Universities for the support received in the framework of the mobility programs "Salvador de Madariaga" (Grant number: PRX21/00592) and "Grants for the Requalification of the Spanish University System" (Grant number: 2021URV-RQ-01). H. S. received funding from the Geneva BIOSTIME Institute Nutrition & Care (BINC) Foundation and Food for Health Ireland through the EI Technology Centre award (TC20180025).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Soliz-Rueda JR, López-Fernández-Sobrino R, Schellekens H, Bravo FI, Suárez M, Mulero M, et al. Clock system disruption in male Fischer 344 rats fed cafeteria diet and administered sweet treats at different times: The *zeitgeber* role of grape seed flavanols. BioFactors. 2025;51(1):e70000. https://doi.org/10.1002/biof.70000

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