Dietary Proanthocyanidins Modulate Melatonin levels in Plasma and the expression pattern of Clock Genes in the hypothalamus of Rats.

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Abbreviations: all-trans retinoic acid (ARA), brain and muscle ARNT-like protein-1(BMAL1), circadian locomotor output cycles kaput (CLOCK), clock controlled gens (CCGs), cryptochrome (CRY), grape seed proanthocyanidin extract (GSPE), 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCOAR) monounsaturated fatty acids (MUFA), nicotinamide phosphoribosyl transferase (NAMPT), period (PER), polyunsaturated fatty acids (PUFA), proanthocyanidins (PAs), nuclear Overhauser effect spectroscopy (NOESY), principal component analysis (PCA), nuclear receptor subfamily 1 group D (Nr1d1, also known as REV-ERBα), retinoic acid-related orphan receptor alpha (RORα), suprachiasmatic nucleus (SCN), tetramethylsilane (TMS), zeitgeber time (ZT).

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Abstract

Scope: Circadian rhythms allow organisms to anticipate and adapt to environmental changes; food components can adjust internal rhythms. Proanthocyanidins (PAs), improve cardiovascular risk factors that exhibit circadian oscillations. Therefore, the aim of the current study was to determine whether PAs can modulate body rhythms.

Methods and results: Male Wistar rats were orally gavaged with 250 mg grape seed proanthocyanidin extract (GSPE)/kg body weight at zeitgeber time (ZT) 0, light on. Phenotypic biorhythm was evaluated by measuring the concentration of plasma melatonin and metabolites, using MNR-metabolomics, at several ZT. Remarkably, GSPE treatment maintained nocturnal melatonin levels at ZT3 and altered the oscillations of some metabolites in plasma. Quantification of expression of clock-core (*Clock, Bmal1, Per2, Rorα, Rev-erbα*) and clock-controlled (*Nampt*) genes in the hypothalamus by RT-PCR showed that this phenotypic alteration was concomitant with the modulation of the expression pattern of *Bmal1* and *Nampt*. However, GSPE did not modulate the nocturnal expression of clock genes when administered at ZT12 (light off).

Conclusions: PAs could have chronobiological properties, although their activity depends largely on the time of administration.

Introduction

Circadian rhythms are the approximately 24-hour endogenous oscillations of most biological processes that are entrained to the environment by external cues called zeitgebers, thus allowing organisms to anticipate environmental changes and to adapt the time of day and food availability [1]. The most important zeitgeber is light. The hypothalamic suprachiasmatic nucleus (SCN) integrates direct photic input from the retina. No other region in the body is able to accomplish that function; thus, SCN is considered the central and master clock [2]. Nonetheless, many other peripheral and cerebral oscillators (peripheral clocks) that emit rhythms in a self-autonomous manner but are synchronized by outputs from the central clock and by external cues such as fasting-feeding time or temperature cycling are present throughout the body [3]. The SCN contains approximately 10,000 neurons in two anatomic subdivisions: a ventral "core" region and a dorsal "shell" region, which must be well coupled to integrate incoming information from light and other peripheral clocks [4]. In fact, complete SCN lesions abolish circadian rhythmicity [5,6], but implantation of fetal SCN tissue can partially restore them [7], thus demonstrating an essential role of SCN in maintaining circadian rhythms.

The SCN synchronizes peripheral clocks and body rhythms by hormonal signals and neural connections [3]. In this regard, melatonin receives special attention as it is a robust hormonal signal that indicates the time of environmental darkness and is even secreted during the dark phase of the circadian cycle in nocturnal animals, when these animals are also increasing their activity [8]. In fact, the sympathetic innervations of the pineal gland, where melatonin is primarily synthesized, that connect the rhythmic activity of the SCN with the rhythmic release of melatonin is the first output pathway that provides a circadian message to the organism through general circulation at night that is driven by the SCN master clock, therefore, making the SCN an anatomical target for chronotherapeutic studies [9].

At the molecular level, the master clock consists of an autoregulatory transcription–translation feedback loop cycling with a periodicity of approximately 24 h, driving the positive branch of this loop by the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein-1 (BMAL1), which activate the transcription of the Period (*Per*) and cryptochrome (*Cry*) genes after their own heterodimerization. After reaching a critical concentration, their protein products, PER and CRY, translocate to the nucleus and inhibit the activity of the CLOCK:BMAL1 heterodimer, thus leading to a decrease in *Per* and *Cry* expression. Additionally, the active CLOCK:BMAL1 heterodimer also promotes the transcription of the retinoic acid-related orphan receptor alpha (Rorα) and the nuclear receptor subfamily 1, group D (Nr1d1, also known as Rev-erbα), its activator and repressor, respectively, generating another regulatory loop. Finally, the CLOCK:BMAL1 heterodimer enhances the transcription of metabolic genes or clock controlled genes (CCGs), such as nicotinamide phosphoribosyl transferase (*Nampt*), which has been implicated in many metabolic and biochemical processes. Here lies the tight relationship among light, SCN and metabolism or physiology [1,10].

Proanthocyanidins (PAs) are a class of polyphenols present in vegetables, fruits, cacao, nuts and some beverages such as red wine or tea; therefore, its presence in the human diet is considerably high [11]. Several studies that used various *in vitro* and animal models demonstrate that PAs have a vast range of health effects, such as improving insulin resistance [12] and decreasing inflammation [13], hypertension [14], oxidative stress [15] and lipid abnormalities[16], thus reducing metabolic syndrome [17] and cardiovascular diseases [18]. Interestingly, all these processes exhibit circadian rhythms [19] and humans with disrupted circadian rhythms have increased risk of developing symptoms of metabolic syndrome [20,21].

Although light is considered the master zeitgeber, the feeding-fasting cycle [22] as well as dietary components, such as dietary fat [23] and resveratrol [24], also work as external cues that synchronize biological rhythms. Therefore, the aim of the current study was to determine whether PAs can modulate internal body rhythms and the central clock. Body rhythm has been evaluated by analyzing plasma melatonin and metabolite concentrations at several zeitgeber times (ZT). The capacity of PAs to modulate the SCN has been determined by measuring the expression pattern of clock core and clock-controlled genes in the hypothalamus while administering PAs diurnally or at night or even in concert with circadian disruption using jet-lagged rats.

Materials and methods

Grape seed proanthocyanidin extract composition

Grape seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The composition of GSPE is as follows [25]: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechingallate (89 μmol/g), epigallocatechingallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g) and hexameric procyanidins (0.38 μmol/g).

Animals

All procedures involving the care and use of animals were reviewed and approved by The Animal Ethics Committee from the Universitat Rovira i Virgili (Permit number 4249 by Generalitat de Catalunya).

Ninety eight-week-old male Wistar rats (Crl: WI (Han)) were purchased from Charles River (Barcelona, Spain) and fed a standard chow diet (STD, Panlab 04, Barcelona, Spain) and tap water ad libitum. Rats were divided in three groups, according to the zeitgeber time (ZT) when GSPE was administered.

Administration of GSPE at ZT0: Forty-two rats were singly caged in animal quarters at 22ºC with a 12-h light/dark cycle (light from 9:00 to 21:00 P.M.). After three weeks of adaptation, rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water in a single dose at ZT0 (9:00 am, light on). Rats were sacrificed by decapitation at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24 (n=3 for control and n=3 for GSPE treated groups).

Administration of GSPE at ZT12: Twenty-four rats were singly caged in animal quarters at 22ºC with a 12-h light/dark cycle (light from 21:00 pm to 9:00 am). After three weeks of adaptation, the rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT12 (9:0 am, light off). Rats were sacrificed by decapitation at ZT12, ZT13, ZT15, ZT18 (n=3 for control and n=3 for GSPE treated groups).

Administration of GSPE to Jet-lagged rats: Twenty-four rats were singly caged in animal quarters at 22ºC with a 12-h light/dark cycle (light from 15:00 pm to 03:00 am). After three weeks of adaptation, rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT6 (9:00 am, middle of light day) and immediately moved to a dusk room (ZT12), thus rats received a jet lag of 6 hours. Rats were sacrificed by decapitation at ZT12, ZT13, ZT15, ZT18 (n=3 for control and n=3 for GSPE treated groups).

For the three experiments, blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant and plasma was obtained by centrifugation. Plasma was frozen at -80ºC until melatonin and metabolomic analysis. The hypothalamus was excised and frozen immediately in liquid nitrogen and stored at -80ºC until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA from hypothalamus was extracted using TRIzol reagent and an RNeasy Lipid Tissue Mini Kit (Qiagen, 74804, Barcelona, Spain) according to both manufacturer protocols. RNA was quantified using spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at λ=260 nm and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Applied Biosystems (4368814, Madrid, Spain)

mRNA quantification by real-time qRT-PCR

A total of 10 ng cDNA was subjected to quantitative RT-PCR amplification using SYBR Green PCR Master Mix from Bio-Rad (172-5200, Barcelona, Spain). The forward and reverse primers of the analyzed genes are shown in Table 1. Reactions were run on a quantitative real-time PCR system (CFX96 touch of Bio-Rad, Barcelona, Spain); the thermal profile settings were 50ºC for 2 min and 95ºC for 2 min and then 40 cycles at 95ºC for 15 s and 60ºC for 2 min. Finally, statistical data were converted and normalized to the linear form using the 2^{-}CT ($\Delta\Delta C_T$) calculation [26]. The relative expression of the clock genes was normalized to cyclophilin mRNA levels.

Melatonin measurement

Melatonin plasma levels were assayed using an ELISA method following the manufacturer's instructions (RE54021 IBL international, Hamburg, Germany).

MRN analysis and sample preparation

MRN analysis and sample preparation was performed according to the method described by Vinaixa et al[27] for untargeted metabolomics. From 400 µL of the plasma sample, 200 µL were placed in a tube with 1800 µL of a methanol:water mixture (8:1) for an aqueous extraction (methanol from Panreac Química S.A. Barcelona, Spain). The other 200 µL were placed in a tube with 3 mL of a chloroform:methanol mixture (2:1) for lipid extraction (chloroform from Panreac Química S.A. Barcelona, Spain). The mixtures were vortexed vigorously and centrifuged for 10 min at 4500 rpm (4ºC). For the aqueous extraction, the pellet was washed twice with additional methanol:water (8:1), vortexed and centrifuged, combining the liquid phases. Finally, the upper aqueous phases were partially dried in a nitrogen stream to remove methanol and quickly frozen. The lipid extraction was completely dried in a nitrogen stream. For NMR measurements, the hydrophilic extracts were reconstituted in 600 µl of D_2O containing 0.67mM trimethylsilylpropionic acid (TSP). The lipophilic extracts were subsequently extracted in 700 µl of $CDCl_3/CD_3OD$ (2:1) containing 1.18mM tetramethylsilane (TMS). Samples were then vortexed, homogenized for 20 min, and centrifuged for 15 min at 6000×g at 4°C. Finally, redissolved samples were placed into 5 mm NMR tubes. ^{1}H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm CPTCI triple resonance (^{1}H, ^{13}C, ^{31}P) gradient cryoprobe. One-dimensional ^{1}H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with irradiation power of 75 Hz was applied during recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample and varied from 6.57 to 6.99 ms. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each ^{1}H spectrum. The exponential line broadening applied before Fourier transformation was of 0.3 Hz. The frequency domain spectra were phased and baseline-corrected using TopSpin software (version 2.1, Bruker).

The acquired ^{1}H NMR spectra were phased, baseline-corrected, and referenced to the chemical shift of residual A signal at B ppm. Pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, and Chenomx databases were used as references for metabolite identification. In addition, we assigned metabolites by ^{1}H–^{1}H homonuclear correlation (COSY and TOCSY) and ^{1}H–^{13}C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After baseline correction, specific ^{1}H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package.

Data (pre-) processing, data analysis, and statistical calculations were performed in R.

Data and statistical analysis

Results are presented as the mean with the associated standard error (SE) of three animals. The data were analyzed using two-way ANOVA, comparing simultaneously the effect of GSPE at all ZT studied. In the case of significance, one-way ANOVA was applied, comparing GSPE-treated and control groups at each ZT, in order to determine the specific ZT when GSPE induce significant effects. ANOVA was performed using SPSS statistical software (version17.0 for Windows; SPSS, Inc.). P values<0.05 were considered statistically significant.

Multivariate analysis was performed using R software for Windows [28]. The full set of NMR metabolomics data were subjected to principal component analysis (PCA) using the *pca* function of the mixOmics package [29] with three principal components using the centering and scaling options. The rest of the options were left at default. Visual 3D representations were constructed with the Rcmdr R package [30].

Results

Acute administration of GSPE at Zeitgeber Time 0 modulates melatonin and metabolite levels in plasma.

The capacity of PAs to modulate circadian rhythms was evaluated by measuring the circadian oscillation of several metabolites and melatonin in the plasma of the rats maintained on a 12-h light/12-hdark cycle and treated with an acute dose of 250 mg GSPE /kg body weight at zeitgeber time (ZT) 0, when the light was turned on.

Melatonin levels in plasma were measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24 (Fig.1). The results show that GSPE treatment significantly increased plasma melatonin levels at ZT3, when control animals had the lowest level. The effect of GSPE modulating plasma melatonin was still significant after 24 hours of GSPE administration.

Plasma metabolomics, carried out using RMN, was performed at ZT0, ZT6, ZT12 and ZT24. Forty-nine metabolites were identified, including amino acids, glucose, lactate, 3-hydroxybutyrate, glycerol, triglycerides, cholesterol and some phospholipids and fatty acids (Tables 2, 3 and 4). GSPE treatment significantly affected the circadian oscillations of tyrosine, serine, glycerolphosphocholine, oleic acid and monounsaturated fatty acids (MUFA). Moreover, the circadian oscillation of glucose, pyruvate, citrate, valine, leucine, lysine, tyrosine, glutamine, isoleucine, histidine, serine, cholesterol, esterified cholesterol, phosphatidylcholine, linoleic acid, MUFA, polyunsaturated fatty acids (PUFA) and *all-trans* retinoic acid (ARA) showed a significant interaction between time and GSPE.

We subjected the full set of NMR data from samples at ZT0, ZT6, ZT12 and ZT24 to PCA analysis (Fig. 2). Because the value at each ZT for GSPE- or vehicle-treated animals is derived from material pooled from three animals and clustering techniques are not recommended in such small groups, we did not use this multivariate analysis for classification. Instead, we assessed the separation between the projection of the data for each animal (scores) as a measure of whole metabolic variability through the 24-hour period. Remarkably, the scores for GSPE-treated animals were clearly condensed when compared with the control animals (Fig. 2A). The differences were not due to increased variability within a given ZT but to increased variability between ZTs, as observed in fig. 2B when the scores for ZT6 and ZT12 animals, as representative groups, are plotted for both GSPE and control conditions.

Taken together, these results indicate that GSPE treatment profoundly alters melatonin and metabolite oscillation in plasma and that GSPE modulate internal body circadian rhythms.

Acute administration of GSPE at ZT0 altered *Bmal1* and *Nampt* expression pattern in the hypothalamus.

Because the central clock is the master regulator of body circadian rhythms, we further studied whether this modulation of phenotypic rhythm by GSPE was associated with modification of clock gene expression in the hypothalamus. Therefore, we evaluated the expression pattern of *Clock* and *Bmal1* (clock core genes), *Per2* (component of the negative loop of the circadian clock), *Rorα* and *Rev-erbα* (nuclear receptors, the expression of which is regulated by CLOCK:BMAL1 and that act as activator or repressor of Bmal1 gene expression, respectively), *Nampt* (metabolic gene that its expression is regulated directly by CLOCK:BMAL1) and *HmgCoAR* (metabolic gene that has circadian rhythm expression but not directly controlled by CLOCK:BMAL1) in the hypothalamus.

Of the two clock core genes, only *Bmal1* (Fig. 3A) responded significantly to GSPE whereas *Clock* (Fig. 3B) remained similar to that of the control animals. The pattern of *Bmal1* expression was mainly altered after 3 and 6 hours of GSPE administration.

The mRNA pattern of the *Bmal1* targeted genes, *Rorα, Rev-erbα, Per2* and *Nampt* (Fig.3 C-F, respectively) was modulated in different way by GSPE. In this sense, *Rorα* and *Rev-erbα* mRNA levels remained similar to those of control animals at all the times studied. On the contrary, the expression pattern of *Nampt* was significantly altered by GSPE. Despite GSPE administration did not alter significantly *Per2* expression pattern, a significant interaction between GSPE and ZT was observed for this gene.

The mRNA pattern of *HmgCoAR* (Figure 3G), a gene not directly controlled by *Bmal1*, was not affected by the administration of GSPE.

Altogether, these results indicate that GSPE could adjust the central clock by modulating *Bmal1* and *Nampt* pattern expression.

Acute administration of GSPE at ZT12 affected the expression of only Rev-erbα

Next, we studied whether PAs could modulate the central clock independently of the time during the circadian cycle they are administered. Thus, GSPE was administered at ZT12 (light turned off) and the expression of clock and clock-controlled genes was determined at four time points, ZT12, ZT13, ZT15 and ZT18 in the hypothalamus.

GSPE, administered at ZT12, induced slight effects on the mRNA levels of the clock core genes *Bmal1* (Fig. 4A) and *Clock* (Fig. 4B) as well as the mRNA levels of the *Bmal1*-controlled clock genes *Rorα* (Fig. 4C) and *Per2* (Fig. 4E). However, GSPE significantly affected the mRNA levels of *Rev-erbα* (Fig.4D), and the expression of *Nampt* and *Rev-erbα* showed a significant interaction between time and GSPE. GSPE treatment at ZT12 did not affect the expression of *HmgCoAR* (Fig. 4G)

Plasma metabolites were analyzed at ZT12 and ZT15 (Table 5). No significant differences were observed in any metabolite after GSPE administration at ZT12, which is in accordance with the few effects of GSPE on the hypothalamic expression pattern of clock genes when it was administered at ZT12.

Overall, these results indicate that GSPE modulated the circadian rhythms more strongly when it was administered at ZT0 (starting of day) than at ZT12 (starting of night).

Acute administration of GSPE modulated the pattern expression of clock genes in the hypothalamus of jet-lagged rats

Finally, the capacity of GSPE to modulate the central clock was evaluated in a simulation of circadian disruption using rats subjected to a 6 hours jetlag. GSPE was administered to rats at ZT6 (middle of light period), and the rats were then moved to ZT12 (light off). The capacity of GSPE to modulate core and related clock genes, was evaluated at ZT12, ZT13, ZT15 and ZT18.

GSPE, administered at the beginning of jetlag, did not modulate *clock* (Fig. 5B), *Rev-erbα* (Fig. 5D) or *HmgCoAR* (Fig. 5G), whereas the expression pattern of *Bmal1* (Fig. 5A), *Rorα* (Fig. 5C), *Per2* (Fig. 5E) and *Nampt* (Fig. 5F) were altered significantly when compared with the jet-lag control group. Remarkably, *Bmal*, and *Nampt* were again the most sensitive genes to GSPE, similar to when GSPE was administered at ZT0. Both when GSPE was administered at ZT0 and in the jet-lag situation, the mRNA levels of *Bmal1* and *Nampt* decreased significantly after GSPE treatment.

Discussion

Light and meal timing entrain circadian rhythms, but specific components in foods could also be important signals. For instance, resveratrol adjusts the circadian rhythms of locomotor activity and body temperature in animals [31,32] and alters clock gene expression in cultured fibroblast [24] and rat organs [33]. Therefore, the aim of this work was to determine the capacity of an acute dose of GSPE to modulate biological rhythms and the expression pattern of clock genes in the hypothalamus.

Melatonin is a robust indicator of the internal body time [9]. Additionally, plasma metabolites exhibit circadian oscillations, and blood metabolomics has been proposed as a method to analyze internal body time [34]. Remarkably, GSPE administered at ZT0 strongly increased plasma melatonin levels in the middle of the light period, maintaining similar levels as at dusk, and altered the circadian oscillation of several plasma metabolites, such some amino acids and lipids. Moreover, GSPE treatment masked changes in metabolite concentration that were very evident in the control animals at the ZT studied. Therefore, acute GSPE treatment at ZT0 actually affected biological rhythms in the rats.

To study the relationship between PAs and the molecular clock, we focused on the hypothalamus, where the SCN integrates direct input from light and information from other oscillators present throughout the body, thus acting as a master synchronizer [3]. The SCN contains approximately 10,000 neurons in two anatomic subdivisions, which must be well coupled as demonstrated by the fact that the period of an intact SCN is more precise than the period of independently oscillating SCN neurons [4]. Therefore, assaying the whole hypothalamus allows the study of the intact SCN. However, all hypothalamic nuclei also express clock genes and their phases are not synchronized with those of SCN [35,36]. Thus, the expression values of clock core and clock-controlled genes at each ZT presented in this work could not fit completely with those described for SCN.

Three different experimental approaches were used to determine whether PAs can modulate the expression pattern of clock genes in the hypothalamus: GSPE treatment at ZT0, at the beginning of the light phase; at ZT12, at the beginning of the dusk phase; and at ZT6 with a jetlag of 6 hours. The data clearly show that GSPE adjusted the pattern expression of *Bmal1* and *NAMPT* in the hypothalamus when was administered at ZT0 or in jet-lagged rats, whereas the administration at ZT12 caused minimal effects on clock-core and clock-controlled genes. Thus, GSPE is primarily active when administered during the day period, indicating that the effectiveness of PAs to modulate hypothalamic clock gene expression depends largely on the time of administration. In fact, SCN cells have been established to be extensively coupled during the day, when the cells exhibit synchronous neural activity, but to be minimally coupled during the night, when the cells are electrically silent [37]. So, this discrepant functionality of SCN cells between day and night could determine the effectiveness of PAs. Moreover, exposure to light has been observed to cause shifts in the phase of the SCN clock primarily during the subjective night in nocturnal rodents, whereas non-photic cues trigger these shifts mainly during the subjective day [3]. Therefore, GSPE could act as a non-photic cue, triggering the central clock system during the light period. Nonetheless, no studies have determined whether PAs reach the hypothalamus, although some studies in Wistar rats have elucidated the distribution of flavanols and their metabolites to different tissues, e.g., the brain [38,39] and ruled out the capacity of flavanols to cross the blood-brain barrier. Even, an experiment with a rat model of Parkinson's disease with chronic oral tangeretin administration (10 mg/kg/day for 28 days) confirmed a significant level of this citrus flavonoid in the hypothalamus, even at higher concentrations than in liver and plasma [40]. Alternatively, PAs could adjust the central clock by acting at intestinal levels through the brain-gut axis, which send gut cues to the brain by neuronal and hormonal mechanisms [41]. Thus, more studies are needed to define the molecular mechanism by which PAs could adjust the central clock.

Among the observed effects of GSPE treatment at ZT0 on the pattern expression of clock core and clock-controlled genes in the hypothalamus, special attention should be paid to *Bmal1*, which was significantly modulated by GSPE. Because *clock* was not affected by GSPE, which is consistent with its constitutive expression in the SCN [42], the modification of *Bmal1* pattern expression could explain the modification of the clock-controlled gene *Nampt*, according to the defined role of *Bmal1* as master regulator of the molecular clock system [43]. Interestingly, *Bmal1* was also targeted by GSPE in jet-lagged rats.

SCN synchronizes circadian rhythm of the whole body by hormonal signals and neural connections [3]. In particular, nocturnal secretion of melatonin by the pineal gland is directly controlled by the SCN [44]. Remarkably, GSPE administered at ZT0 triggered high plasma melatonin levels at middle light day. GSPE extract does not contain melatonin; thus the alteration of the melatonin concentration could be due to the adjustments induced by GSPE in the SCN. However, the SCN expresses

melatonin receptors [45].Thus, the inverse mechanism, including an initial effect of GSPE on melatonin secretion followed by melatonin action on SCN, cannot be ruled out.

Because GSPE kept dawn levels of melatonin during the light period and modulated the expression of clock genes in the hypothalamus when it was administered at ZT0 and not at ZT12, it can be suggested that PAs act as a non-photic signal of dusk. These findings agree with the xenohormesis hypothesis, which proposes that heterotrophs are able to sense chemical cues synthesized by plants, such as polyphenols, in response to stress[46]. In fact, circadian rhythms allow anticipation of environmental changes and adaptation to the time of day and food availability. Thus, PAs could provide cues to animals about environmental conditions by modulating biological rhythms.

In conclusion, GSPE treatment could modulate biological rhythms by maintaining high levels of melatonin during the light period. Moreover, GSPE altered the expression pattern of clock genes in the hypothalamus when it was administered during the light period but not at dusk, indicating that the ability of PAs to modulate the hypothalamic clock gene expression depends largely on the time of administration. Specifically, *Bmal1* emerges as a target of GSPE. Therefore, PAs could have chronobiologic properties. However, as rats are nocturnal animals, assessing the ZT when PA-rich foods can entrain circadian rhythms in humans will be necessary.

Author Contribution

A.R.L., A.A.A., M.J.S., L.A. and C.B. designed the experiments. A.R.L., L.B.E. and E.S. performed the experimental procedures and the molecular analysis. J.M.D.B. performed the PCA analysis, and A.R.L. performed the statistical and cosinor analyses. All the authors participated in the interpretation and discussion of data. A.R.L., J.M.D.B., L.A. and C.B. wrote the manuscript. All the authors contributed to and approved the final manuscript.

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Conflict of Interest

The authors have declared no conflicts of interest.

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Fig. 1

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 0 modulate melatonin plasma levels in rats.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (9:00 am, light on), and plasma melatonin was measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24. The graph shows the mean±s.e. for each data point (n=3). ZT points are not equally distributed in the X-axis. T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables, by two factors ANOVA.* significant difference between control and GSPE-treated groups at the same ZT by one-way ANOVA

Fig. 1

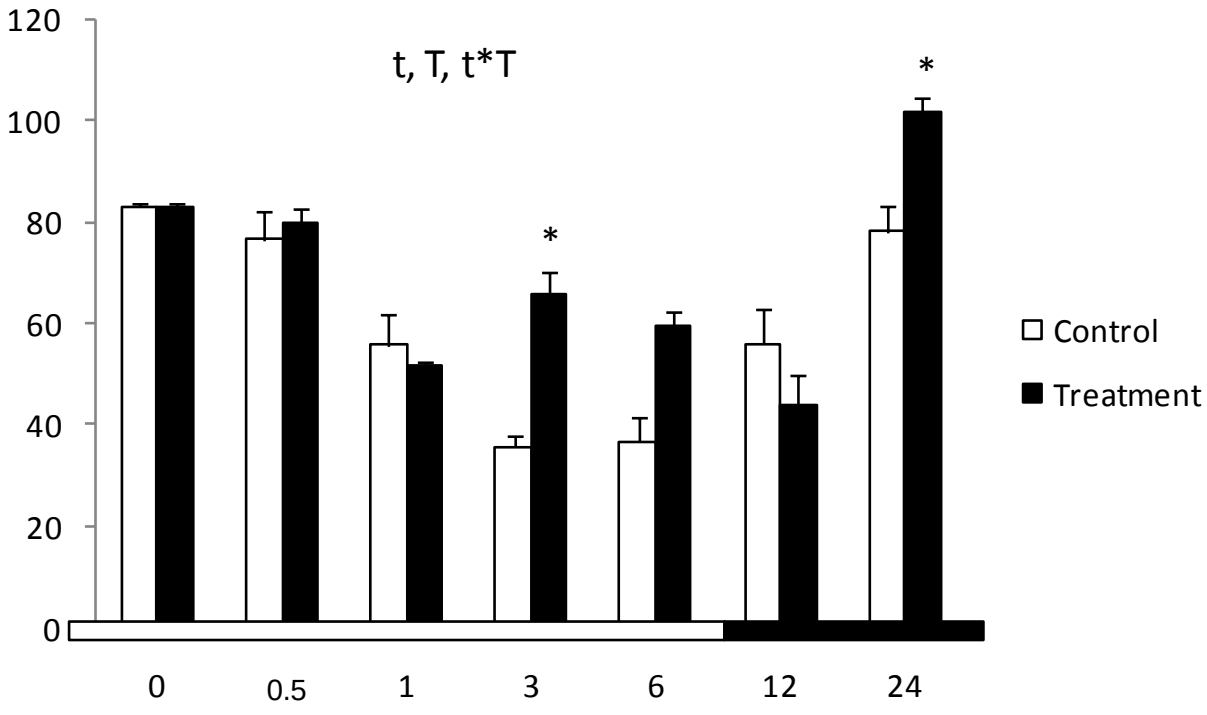

Fig. 2

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 0 modulate metabolite circadian oscillation in rats.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (9:00 am, light on) and plasma metabolites were measured by NMR metabolomics at ZT0, ZT6, ZT12 and ZT24. (A) The values of abundance of all metabolites were subjected to a principal component analysis. Scores for each animal are represented in the three dimensions defined by PC1, PC2 and PC3 (explaining a 72%, 8% and 6% of the variance respectively) as blue dots for the control group and green dots for the GSPE-treated group. Ellipsoids containing the 50% of the scores are represented for each group. (B) Heat map depicting temporal patterns of changing metabolites. The fold-change respect the ZT0 of each metabolite (rows) is represented as explained in the color key insert for each ZT (columns). Metabolites patterns are clustered by euclidean distance as depicted in the dendogram.

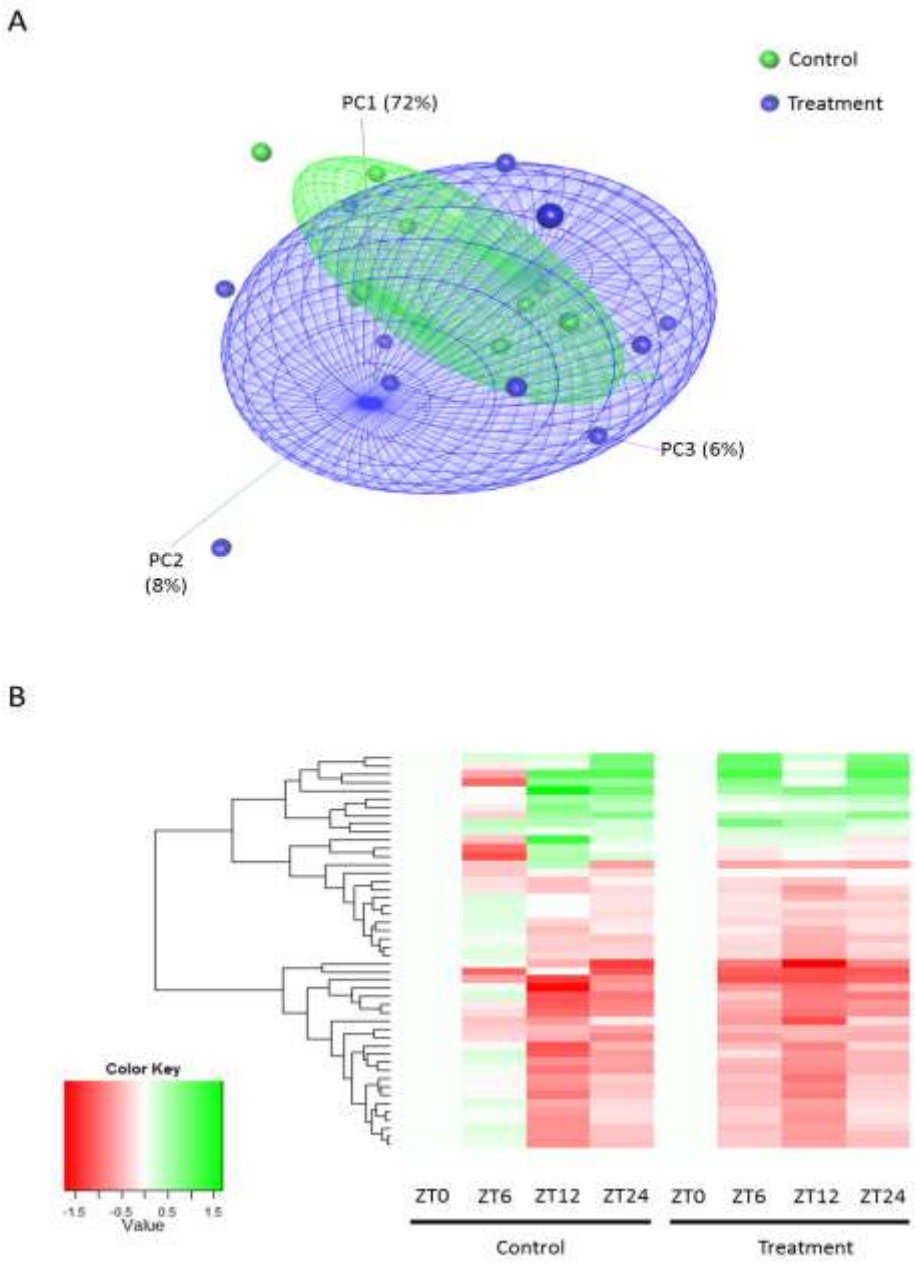

Fig. 3

Effects of an oral dose of grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 0 on the expression pattern of clock-core and clock-controlled genes in the hypothalamus.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (light on), and mRNA levels were measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24 in the hypothalamus. A: *Bmal1*, B: *Clock*, C: *Rorα*, D*: Rev-erbα*, E: *Per2*, F: *Nampt* and G: *HmgCoAR*. Each graph shows the mean±s.e. for ZT point (n=3). ZT points are not equally distributed in the X-axis. T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables, by two factors ANOVA.* significant difference between control and GSPE-treated groups at the same ZT by one-way ANOVA

Fig. 3

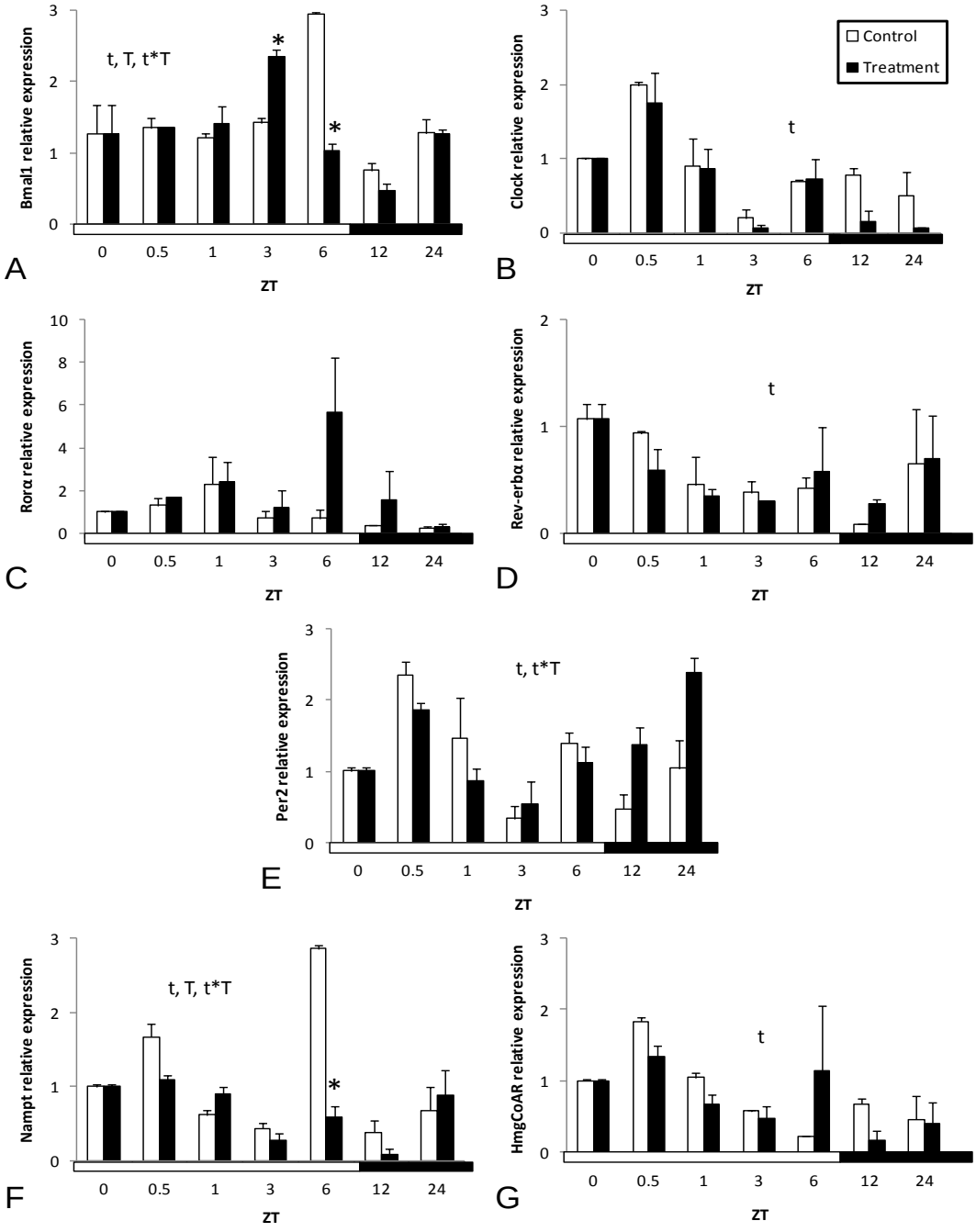

Fig. 4

Effects of an oral dose of grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 12 on the expression pattern of clock core and clock-controlled genes in the hypothalamus.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT12 (light off), and mRNA levels were measured at ZT12, ZT13, ZT15 and ZT18 in the hypothalamus. A: *Bmal1*, B: *Clock*, C: *Rorα*, D*: Rev-erbα*, E: *Per2*, F: *Nampt* and G: *HmgCoAR*. Each graph shows the mean±s.e. for ZT point (n=3). ZT points are not equally distributed in the X-axis. T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables, by two factors ANOVA. * significant difference between control and GSPE-treated groups at the same ZT by one-way ANOVA

Fig 4.

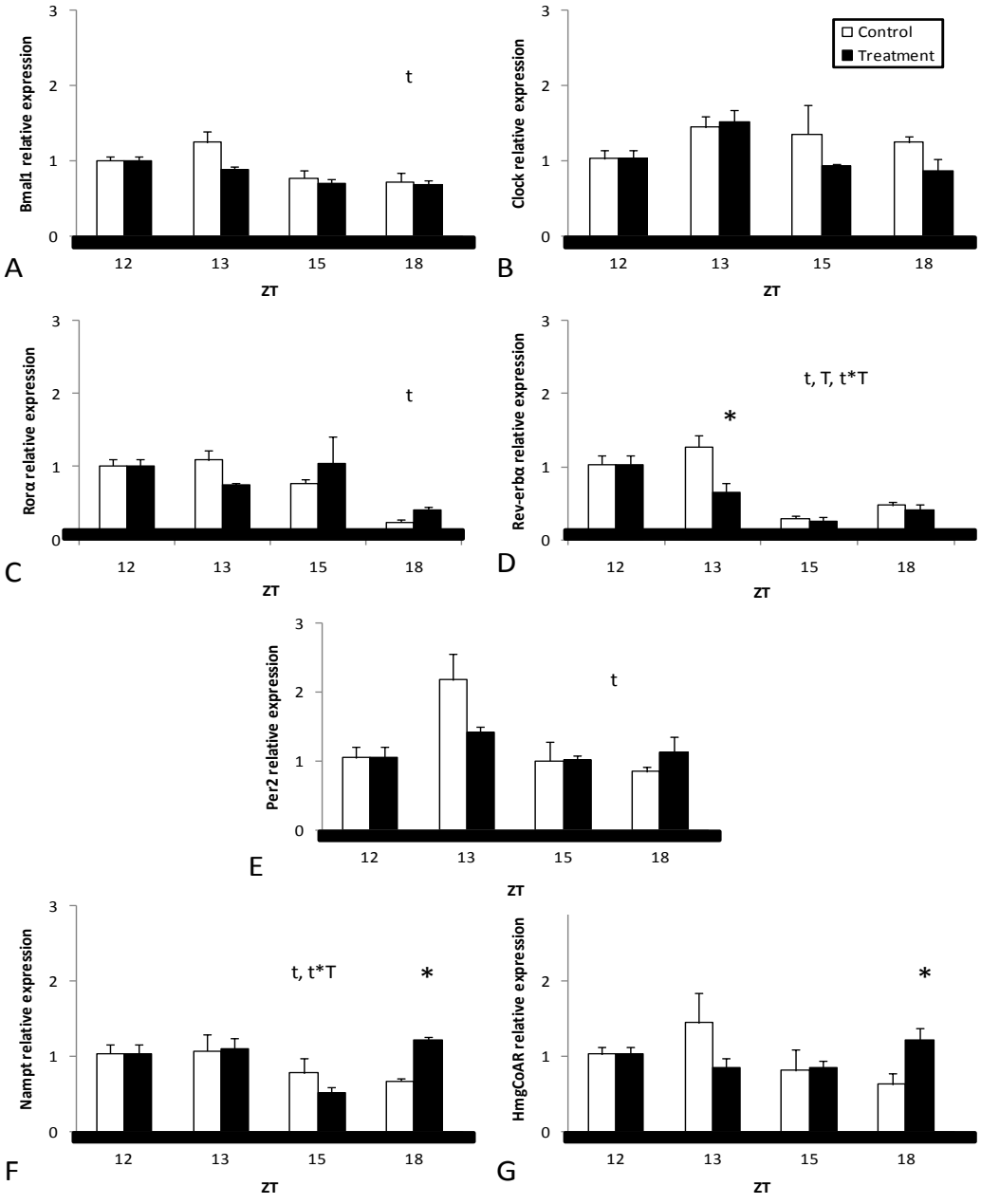

Fig. 5

Effects of an oral dose of grape seed proanthocyanidin extract (GSPE) administered to jet-lagged rats on the expression pattern of clock core and clock-controlled genes in the hypothalamus.
Rats were orally gavaged with tap water (control group) or 250 mg GSPE /kg body weight dissolved in tap water at ZT6 (middle of light period) and moved to ZT12 (light turn off). mRNA levels were measured at ZT12, ZT13, ZT15 and ZT18 in the hypothalamus. A: *Bmal1*, B: *Clock*, C: *Rorα*, D*: Rev-erbα*, E: *Per2*, F: *Nampt* and G: *HmgCoAR*. Each graph shows the mean±s.e. for ZT point (n=3). ZT points are not equally distributed in the X-axis. T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables, by two factors ANOVA. * significant difference between control and GSPE-treated groups at the same ZT by one-way ANOVA

Fig. 5

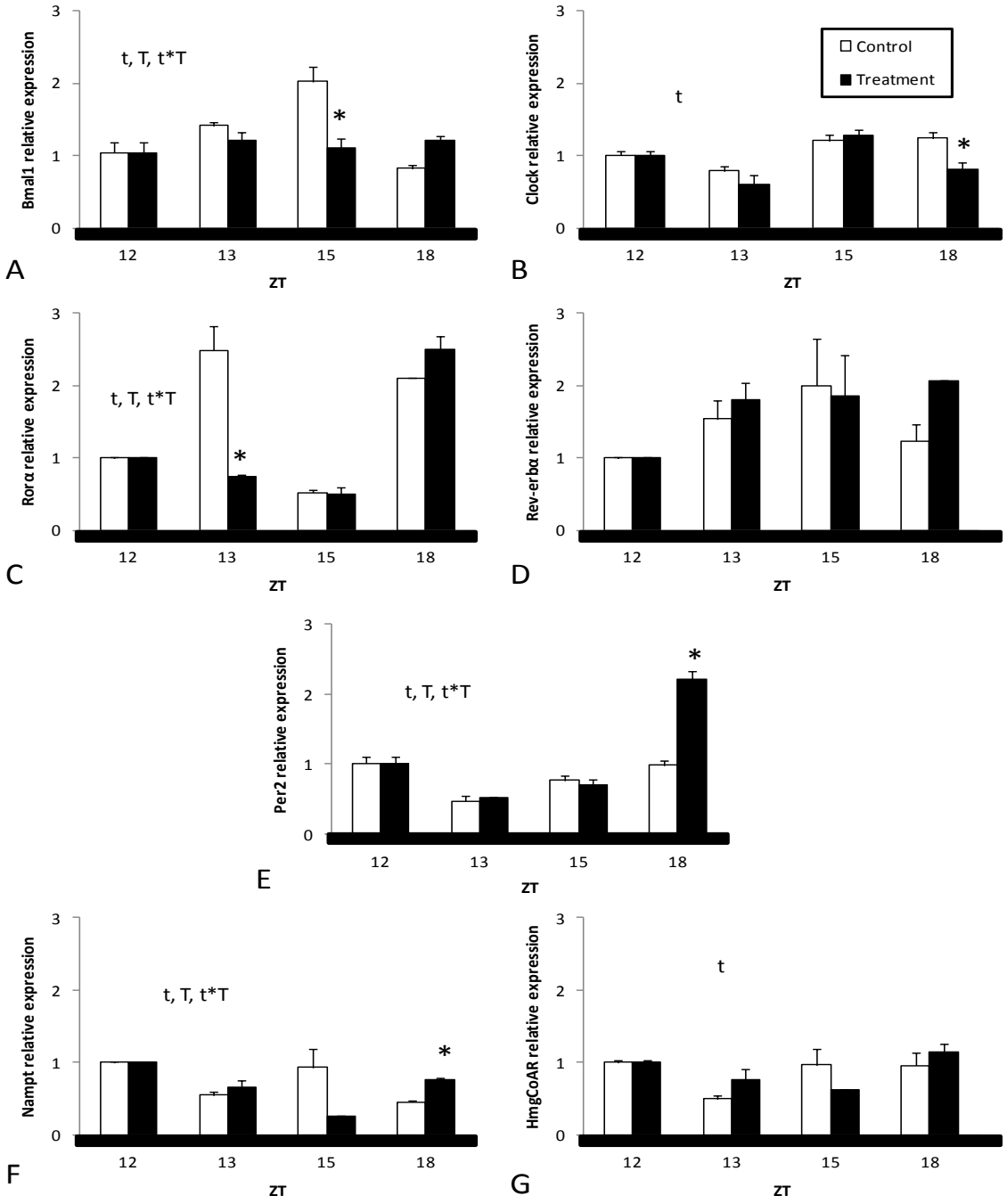

Table 1. Primer sequences

	Forward	***Reverse***
Bmal1	5′-GTAGATCAGAGGGCGACGGCTA-3′	5′-CTTGTCTGTAAAACTTGCCTGTGAC-3′
Clock	5′-TGGGGTCTATGCTTCCTGGT-3′	5′-GTAGGTTTCCAGTCCTGTCG-3′
Per2	5′-CGGACCTGGCTTCAGTTCAT-3′	5′-AGGATCCAAGAACGGCACAG-3′
Rorα	5′-GAAGGCTGCAAGGGCTTTTTCAGGA-3′	5′-CCAAACTTGACAGCATCTCGA-3′
Rev-erbα	5′-CTGCTCGGTGCCTAGAATCC-3′	5′-GTCTTCACCAGCTGGAAAGCG-3′
Nampt	5′-CTCTTCACAAGAGACTGCCG-3′	5′-TTCATGGTCTTTCCCCCACG-3′
HmgCoAR	5′- GAAACCCTCATGGAGACGCA-3′	5′- ACCTCTGCTGAGTCACAAGC-3′
Cyclophilin	5′-CTTCGAGCTGTTTGCAGACAA-3′	5′-AAGTCACCACCCTGGCACATG-3′

Bmal1 (Arntl) : aryl hydrocarbon receptor nuclear translocator-like; *Clock*: circadian locomotor output cycles kaput; *Per2*: period circadian clock 2; *Rorα* (Rora): RAR-related orphan receptor A; *Rev-erbα* (Nr1d1): nuclear receptor subfamily 1, group D, member 1; *Nampt:* nicotinamide phosphoribosyl transferase; *HmgCoAR*: 3-hydroxy-3-methyl-glutaryl-CoA reductase; *Cyclophylin* (Ppia): Cyclophilin A.

Table 2. Circadian oscillation of plasma glucose and related metabolites concentration of rats administered at Zeitgeber Time (ZT) 0 with an oral dose of a grape seed proanthocyanidin extract (250mg /kg body weight)

Metabolite (mM)		ZT0	ZT6	ZT12	ZT24	Annova	P-value
Glucose	C	6.61±0.09	6.07±0.03	6.87±0.15	5.29±0.15	T	0.146
	GSPE	6.63±0.08	6.22±0.10	6.06±0.18	6.75±0.03	T*t	0.001*
Lactate	C	5.71±0.64	6.41±0.28	3.60±0.64	4.23±0.07	T	0.811
	GSPE	5.70±0.44	4.91±0.75	3.42±0.44	6.58±1.35	T*t	0.208
Pyruvate	C	0.20±0.02	0.25±0.00	0.10±0.10	0.12±0.00	T	0.632
	GSPE	0.22±0.01	0.15±0.03	0.13±0.01	0.17±0.02	T*t	0.005*
Citrate	C	0.45±0.06	0.56±0.00	0.22±0.01	0.26±0.01	T	0.713
	GSPE	0.43±0.05	0.38±0.06	0.30±0.03	0.30±0.00	T*t	0.044*
Formate	C	0.08±0.00	0.07±0.01	0.04±0.00	0.05±0.00	T	0.484
	GSPE	0.07±0.01	0.07±0.02	0.05±0.00	0.06±0.01	T*t	0.881
Methylsuccinate	C	0.24±0.02	0.33±0.00	0.14±0.01	0.13±0.00	T	0.661
	GSPE	0.23±0.03	0.33±0.04	0.18±0.00	0.13±0.01	T*t	0.055
Dihydroxyacetone	C	0.75±0.05	0.94±0.06	0.75±0.04	0.57±0.02	T	0.291
	GSPE	0.74±0.03	0.64±0.00	0.63±0.03	0.70±0.02	T*t	0.117
Mannose	C	0.13±0.03	0.11±0.01	0.08±0.01	0.08±0.01	T	0.768
	GSPE	0.11±0.02	0.08±0.01	0.09±0.01	0.08±0.01	T*t	0.934

Rats were gavaged at ZT0. Each value is the mean ± standard error of three animals. C, control animals; GSPE, group treated with a grape seed proanthocynidin extract. T, effect of proanthocyanidins; T*t, interaction between proanthocyanidins and Zeitgeber Time.

Table 3. Circadian oscillation of plasma amino acids and related metabolites concentration of rats administered at Zeitgeber Time (ZT) 0 with an oral dose of a grape seed proanthocyanidin extract (250mg /kg body weight)

Metabolite (mM)		ZT0	ZT6	ZT12	ZT24	Annova	P-value
Alanine	C	1.13±0.11	1.38±0.06	0.72±0.02	0.38±0.05	T	0.526
	GSPE	1.12±0.17	1.02±0.07	0.79±0.02	0.97±0.11	T*t	0.143
Serine	C	1.14±0.03	1.46±0.01	1.10±0.11	0.79±0.10	T	0.028*
	GSPE	1.13±0.01	0.93±0.16	0.07±0.07	1.00±0.02	T*t	0.09*
Threonina	C	1.01±0.05	1.13±0.20	1.20±0.00	0.66±0.10	T	0.154
	GSPE	1.02±0.03	0.84±0.16	0.55±0.03	0.80±0.08	T*t	0.175
Dimethyl-glycine	C	0.09±0.00	0.11±0.00	0.03±0.01	0.06±0.01	T	0.330
	GSPE	0.08±0.01	0.06±0.01	0.05±0.00	0.06±0.00	T*t	0.019*
Tyrosine	C	0.18±0.01	0.20±0.00	0.11±0.00	0.14±0.01	T	0.024*
	GSPE	0.17±0.01	0.13±0.01	0.09±0.00	0.14±0.01	T*t	0.06*
Glutamine	C	1.73±0.13	2.12±0.09	1.25±0.05	1.30±0.09	T	0.222
	GSPE	1.72±0.15	1.42±0.14	1.31±0.04	1.48±0.13	T*t	0.026*
Glutamate	C	1.17±0.09	1.38±0.01	0.72±0.08	1.00±0.15	T	0.077
	GSPE	1.15±0.10	0.93±0.09	0.72±0.06	0.90±0.05	T*t	0.172
Leucine	C	0.48±0.02	0.55±0.00	0.28±0.03	0.34±0.01	T	0.285
	GSPE	0.47±0.03	0.38±0.05	0.29±0.02	0.41±0.04	T*t	0.016*
Isoleucine	C	0.58±0.05	0.72±0.02	0.41±0.01	0.40±0.01	T	0.312
	GSPE	0.57±0.04	0.47±0.07	0.41±0.01	0.52±0.05	T*t	0.015*
Valine	C	0.59±0.02	0.63±0.01	0.31±0.03	0.38±0.01	T	0.135
	GSPE	0.58±0.01	0.43±0.05	0.32±0.02	0.44±0.02	T*t	0.007 *
Lysine	C	0.74±0.06	0.93±0.01	0.37±0.00	0.44±0.03	T	0.357
	GSPE	0.73±0.08	0.57±0.14	0.52±0.02	0.47±0.01	T*t	0.016*
Histidine	C	0.17±0.01	0.22±0.00	0.10±0.00	0.08±0.00	T	0.629
	GSPE	0.16±0.02	0.12±0.03	0.12±0.00	0.12±0.00	T*t	0.006*
Phenylalanine	C	0.10±0.00	0.13±0.00	0.08±0.01	0.08±0.01	T	0.06
	GSPE	0.11±0.01	0.08±0.01	0.07±0.00	0.08±0.00	T*t	0.095
Tryptophan	C	0.19±0.00	0.16±0.01	0.16±0.00	0.18±0.01	T	0.740
	GSPE	0.18±0.02	0.16±0.01	0.14±0.01	0.18±0.00	T*t	0.915
Methionine	C	0.47±0.02	0.47±0.20	0.21±0.02	0.20±0.00	T	0.868
	GSPE	0.46±0.04	0.36±0.08	0.25±0.05	0.25±0.02	T*t	0.237
Taurine	C	1.73±0.21	1.51±0.10	1.20±0.01	1.43±0.17	T	0.294
	GSPE	1.72±0.17	1.41±0.24	1.08±0.10	0.75±0.14	T*t	0.833
Methylhistidines + xanthine	C	0.06±0.02	0.04±0.01	0.04±0.02	0.02±0.01	T	0.687
	GSPE	0.05±0.03	0.03±0.01	0.02±0.00	0.03±0.00	T*t	0.828
Urea	C	0.45±0.02	0.31±0.00	0.52±0.03	0.50±0.00	T	0.818
	GSPE	0.44±0.04	0.38±0.06	0.45±0.00	0.53±0.02	T*t	0.575
Creatine	C	1.30±0.57	0.92±0.02	0.43±0.00	0.71±0.01	T	0.766
	GSPE	1.29±0.63	0.57±0.05	0.55±0.00	0.62±0.05	T*t	0.939
Cadaverine	C	0.02±0.06	0.01±0.00	0.03±0.00	0.03±0.04	T	0.275

	GSPE	0.01±0.04	0.03±0.17	0.03±0.01	0.02±0.01	T*t	0.008*
Tymidine	C	0.02±0.00	0.02±0.00	0.02±0.00	0.33±0.00	T	0.897
	GSPE	0.03±0.01	0.02±0.00	0.03±0.00	0.03±0.00	T*t	0.793
Cytosine	C	0.97±0.00	1.21±0.00	0.48±0.00	0.60±0.00	T	0.822
	GSPE	0.96±0.02	0.74±0.01	0.67±0.00	0.64±0.00	T*t	0.236
Allantoin	C	0.15±0.01	0.14±0.01	0.18±0.01	0.13±0.01	T	0.825
	GSPE	0.14±0.02	0.14±0.01	0.13±0.02	0.13±0.01	T*t	0.989

Rats were gavaged at ZT0. Each value is the mean ± standard error of three animals. C, control animals; GSPE, group treated with a grape seed proanthocynidin extract. T, effect of proanthocyanidins; T*t, interaction between proanthocyanidins and Zeitgeber Time.

Table 4. Circadian oscillation of plasma lipid metabolites concentration of rats administered at Zeitgeber Time (ZT) 0 with an oral dose of a grape seed proanthocyanidin extract (250mg /kg body weight)

Metabolite (mM)		CT0	CT6	CT12	CT24	Annova	P-value
Triglycerides	C	0.25±0.00	0.30±0.11	0.24±0.01	0.35±0.14	T	0.826
	GSPE	0.25±0.00	0.39±0.11	0.17±0.00	0.27±0.02	T*t	0.846
Linoleicacid	C	0.12±0.01	0.04±0.02	0.19±0.02	0.22±0.01	T	0.293
	GSPE	0.11±0.02	0.16±0.01	0.13±0.01	0.21±0.02	T*t	0.004*
Oleicacid	C	0.22±0.02	0.14±0.02	0.20±0.02	0.21±0.01	T	0.033*
	GSPE	0.23±0.01	0.22±0.01	0.22±0.01	0.22±0.00	T*t	0.153
MUFA	C	0.22±0.00	0.04±0.02	0.14±0.01	0.30±0.06	T	0.030*
	GSPE	0.23±0.00	0.33±0.07	0.10±0.03	0.36±0.01	T*t	0.026*
PUFA	C	0.21±0.03	0.28±0.06	0.63±0.02	0.43±0.01	T	0.167
	GSPE	0.21±0.02	0.39±0.03	0.45±0.06	0.34±0.01	T*t	0.028*
omega-3 fattyacids	C	0.04±0.20	0.04±0.01	0.06±0.01	0.06±0.00	T	0.730
	GSPE	0.03±0.02	0.05±0.00	0.05±0.01	0.06±0.01	T*t	0.924
ARA+EPA	C	0.04±0.23	0.03±0.00	0.11±0.01	0.09±0.01	T	0.155
	GSPE	0.04±0.25	0.07±0.01	0.07±0.00	0.06±0.00	T*t	0.010*
Freeglycerol	C	1.53±0.05	1.59±0.02	1.10±0.14	1.05±0.15	T	0.140
	GSPE	1.52±0.04	1.18±0.08	0.96±0.07	1.05±0.10	T*t	0.411
3-hydroxy-butyrate	C	0.73±0.01	0.63±0.03	0.44±0.04	0.30±0.00	T	0.817
	GSPE	0.72±0.01	0.46±0.08	0.52±0.02	0.48±0.06	T*t	0.669
Acetates	C	0.79±0.12	0.76±0.02	0.32±0.04	0.40±0.04	T	0.838
	GSPE	0.78±0.15	0.52±0.10	0.44±0.01	0.42±0.02	T*t	0.789
Total cholesterol	C	0.24±0.09	0.19±0.01	0.52±0.03	0.52±0.03	T	0.549
	GSPE	0.23±0.10	0.46±0.06	0.33±0.00	0.43±0.00	T*t	0.007*
Esterifiedcholesterol	C	0.15±0.01	0.13±0.02	0.38±0.02	0.34±0.00	T	0.332
	GSPE	0.14±0.02	0.34±0.02	0.24±0.00	0.34±0.02	T*t	0.037*
Cholate	C	0.20±0.02	0.27±0.00	0.12±0.01	0.12±0.01	T	0.219
	GSPE	0.20±0.03	0.15±0.03	0.13±0.01	0.16±0.01	T*t	0.013*
Phosphocholines	C	0.29±0.12	0.12±0.00	0.64±0.06	0.77±0.04	T	0.963
	GSPE	0.28±0.15	0.66±0.07	0.21±0.07	0.64±0.02	T*t	0.002*
Glycerol-phosphocholine	C	0.81±0.09	1.14±0.04	0.86±0.00	0.70±0.15	T	0.025*
	GSPE	0.80±0.08	0.71±0.12	0.55±0.07	0.69±0.02	T*t	0.155
Choline	C	0.69±0.01	0.09±0.00	0.04±0.00	0.06±0.01	T	0.948

	GSPE	0.68±0.02	0.06±0.00	0.05±0.00	0.07±0.00	T*t	0.309
Plasmalogen	C	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	T	0.831
	GSPE	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	T*t	0.504
Sphingomyelin	C	0.03±0.00	0.04±0.00	0.04±0.00	0.04±0.00	T	0.437
	GSPE	0.02±0.00	0.06±0.00	0.04±0.00	0.04±0.00	T*t	0.325

Rats were gavaged at ZT0. Each value is the mean ± standard error of three animals. C, control animals; GSPE, group treated with a grape seed proanthocynidin extract. T, effect of proanthocyanidins; T*t, interaction between proanthocyanidins and Zeitgeber Time. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, all-trans retinoic acid; EPA, Eicosapentaenoic acid.

Table 5. Plasma metabolite levels of rats administered at Zeitgeber Time 12 with an oral dose of a grape seed proanthocyanidin extract (250mg /kg body weight) after 3 hours of gavage.

Metabolite (mM)	Control	GSPE	ANOVA p-value
Glucose	6.65±0.04	6.71±0.49	0.92
Lactate	5.16±0.29	6.35±0.52	0.62
Pyruvate	0.16±0.00	0.16±0.00	1.00
Citrate	0.36±0.02	0.34±0.01	0.50
Methylsuccinate	0.15±0.01	0.15±0.01	1.00
Dihydroxy acetone	0.62±0.03	0.64±0.01	0.60
Mannose	0.07±0.00	0.05±0.01	0.45
Alanine	1.00±0.04	0.95±0.02	0.60
Serine	0.98±0.02	0.93±0.04	0.34
Threonina	0.78±0.02	0.75±0.03	0.58
Dimethyl-glycine	0.06±0.00	0.05±0.00	0.20
Tyrosine	0.12±0.00	0.11±0.01	0.65
Glutamine	1.49±0.06	1.50±0.03	0.66
Glutamate	0.91±0.04	0.80±0.03	0.17
Leucine	0.37±0.03	0.34±0.00	0.42
Isoleucine	0.43±0.03	0.38±0.02	0.23
Valine	0.43±0.03	0.38±0.03	0.30
Lysine	0.47±0.03	0.40±0.02	0.50
Histidine	0.01±0.01	0.01±0.01	0.71
Phenylalanine	0.09±0.00	0.07±0.00	0.26
Tryptophan	0.15±0.01	0.16±0.01	0.54
Methionine	0.25±0.01	0.23±0.02	0.51
Taurine	1.41±0.03	1.22±0.03	0.38
Methylhistidines + xanthine	0.03±0.00	0.04±0.01	0.63
Urea	0.34±0.03	0.31±0.01	0.46
Creatine	0.05±0.05	0.05±0.05	0.25
Cadaverine	0.65±0.04	0.60±0.02	0.41
Triglycerides	0.52±0.04	0.52±0.01	0.98
Linoleic acid	0.23±0.00	0.25±0.00	0.77
Oleic acid	0.20±0.02	0.21±0.01	0.81
MUFA	0.35±0.01	0.35±0.03	0.78
PUFA	0.51±0.02	0.55±0.04	0.73
omega-3 fatty acids	0.07±0.00	0.07±0.00	0.81

ARA+EPA	0.11±0.00	0.12±0.01	0.74
Free glycerol	0.99±0.04	0.94±0.04	0.41
3-hydroxy-butyrate	0.39±0.01	0.39±0.02	0.90
Acetates	0.41±0.00	0.43±0.04	0.74
Total cholesterol	0.60±0.02	0.67±0.10	0.80
Esterified cholesterol	0.45±0.01	0.43±0.07	0.95
Cholate	0.13±0.01	0.12±0.01	0.62
Phosphocholines	0.91±0.02	0.90±0.07	0.97
Glycerol-phosphocholine	0.55±0.01	0.55±0.03	0.95
Choline	0.06±0.00	0.05±0.00	0.47
Plasmalogen	0.02±0.00	0.02±0.00	0.90
Sphingomyelin	0.05±0.01	0.06±0.00	0.90
Tymidine	0.02±0.00	0.04±0.00	0.18
Cytosine	0.02±0.00	0.04±0.01	0.21
Formate	0.05±0.00	0.04±0.00	0.45
Allantoin	0.12±0.00	0.12±0.00	0.94

Rats were gavaged at ZT0. Each value is the mean ± standard error of three animals. GSPE, group treated with a grape seed proanthocynidin extract; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, all-trans retinoic acid; EPA, Eicosapentaenoic acid.