INTERACTION BETWEEN CIRCADIAN RHYTHM AND GRAPE SEED PROANTHOCYANIDINS IN HEALTHY AND OBESE RATS: POTENTIAL ROLE AS A CENTRAL AND PERIPHERICAL CLOCK SYNCHRONIZER

Jorge Ricardo Soliz Rueda

Interaction between circadian rhythm and grape seed proanthocyanidins in healthy and obese rats: potential role as a central and peripherical clock synchronizer

JORGE RICARDO SOLIZ RUEDA

DOCTORAL THESIS

TARRAGONA 2022

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FAIG CONSTAR que aquest treball, titulat "Interacció entre el ritme circadià i les proantocianidines de llavor de raïm en rates sanes i obeses. Potencial com a sincronitzador del rellotge central i perifèric", que presenta *Jorge Ricardo Soliz* Rueda per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia de la universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctoral.

HAGO CONSTAR que el presente trabajo, titulado "Interacción entre el ritmo circadiano y las proantocianidinas de semillas de uva en ratas sanas y obesas. Potencial como sincronizador del reloj central y periférico", que presenta *Jorge Ricardo Soliz Rueda* para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de la universidad Rovira i Virgili y cumple con los requisitos para la obtención de la mención Internacional de Doctorado.

I STATE that the present study, entitled "Interaction between circadian rhythm and grape seed proanthocyanidins in healthy and obese rats: potential role as a central and peripherical clock synchronizer", presented by *Jorge Ricardo Soliz Rueda* for the award of the degree of Doctor, has been carried out under my supervision at the Department de Bioquímica i Biotecnologia from the University Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 7 abril 2022

El/s director/s de la tesi doctoral
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Esta tesis está enmarca en el proyecto PLAN NACIONAL: RETOS DE LA SOCIEDAD (2016-77105-R) cuyo objetivo es desarrollar una alimentación integrada para mantener el peso corporal y prevenir el riesgo de patologías relacionadas con la obesidad como la hipertensión. Jorge Ricardo Soliz Rueda es beneficiario de una ayuda a la contratación de personal investigador predoctoral para la formación de doctores (FPI) (BES-2017-080919) para la realización de esta tesis doctoral concedida por el Ministerio de Ciencia e Innovación del Gobierno de España y FSE "El FSE invierte en tu futuro".

This thesis is framed within the PLAN NACIONAL: RETOS DE LA SOCIEDAD (2016-77105-R) project, that aims to develop an integrated food to maintain body weight and to prevent the risk of obesity related pathologies such as hypertension. Jorge Ricardo Soliz Rueda is the recipient of a grant for the hiring of predoctoral research staff for training of PhDs (FPI) (BES-2017-080919) to carry out this doctoral thesis awarded by the Spanish Ministry of Science and Innovation and FSE “El FSE invierte en tu futuro”.

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A mi familia,

a mis amigos,

a los míos

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"*La distancia puede causar nostalgia,*

pero nunca olvido"

En memoria de Jorge Rueda Castellón y

Rita Castro de Soliz, mis abuelos.

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LIST OF PUBLICATIONS

A) Published papers included in this thesis:

Ávila-Román J, **Soliz-Rueda JR**, Bravo FI, Aragonès G, Suárez M, Arola-Arnal A, Mulero M, Salvadó MJ, Arola L, Torres-Fuentes C, Muguerza B. Phenolic compounds, and biological rhythms: Who takes the lead? *Trends in Food Science & Technology*. July 2021, Volume 113, Pages 77-85. Impact Factor (2020): 12.563. SI Journal Citation Reports © Ranking 3/143 (Decile 1) in Food Science & Technology.

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B) Papers in preparation included in this thesis:

Soliz-Rueda JR, López-Fernández-Sobrino R, Schellekens H, Torres-Fuentes C, Arola L, Bravo FI, Muguerza B. Administration time of a low dose of sugar causes changes in the central clock and alters the metabolism in healthy rats.

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E) Other papers in preparation:

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LIST OF CONFERENCE PAPERS

A) Poster communications:

Soliz-Rueda JR, Arreaza-Gil V, Torres-Fuentes C, Arola-Arnal A and Muguerza B. Grape seed proanthocyanidins attenuate the activity changes trigged by the disruption of photoperiod rhythms in cafeteria diet fed rats. *XI Seminario sobre Alimentación y Estilos de Vida Saludables* – Barcelona, Cataluña, **España 2019**.

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Soliz-Rueda JR, Cortés-Espinar AJ, Ávila-Román J, Mulero M and Muguerza B. Grape seed proanthocyanidin extract (GSPE) improves cardiovascular risk factors and restores the circadian rhythm of the central clock in diet-induced obese rats. *Doctoral Students Meeting from la Xarxa d'Innovació Alimentària (XIA) and the Food Quality and Design Group from Wageningen University* – Barcelona, Catalonia, **Spain 2022**.

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B) Oral communications:

Soliz-Rueda JR and Muguerza B. Grape seed proanthocyanidins extract attenuates the effects after an abrupt disruption of the photoperiod in obese rats. *I Jornada interuniversitaria del Doctorado en Nutrigenómica y Nutrición Personalizada* – Palma de Mallorca, Illes Balears, **España 2020**.

Soliz-Rueda JR, López-Fernández-Sobrino R, Bravo FI and Muguerza B. Interacción de los ritmos biológicos con la efectividad de los compuestos fenólicos de pepita de uva. *I Jornadas Nutracéutica. Compuestos bioactivos y nutracéuticos* – Tarragona, Cataluña, **España 2022**.

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Cuesta-Marti C, **Soliz-Rueda JR**, Schverer M, Torres-Fuentes C, Mazzocchi M, O'Keeffe GW, Stanton C, Dinan TG, Cryan JF, Clarke G and Schellekens H. *B. Longum*-PSY001 supplementation modulates hypothalamic and hippocampal expression of genes related to food intake and stress in two independent mouse strains. Keystone Symposia 2022 in Neuronal Control of Appetite and Gut-Brain Axis. Banff, **Canada 2022**.

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SUMMARY

Biological rhythms play an important role in the physiological and metabolic adaptation of the organism to the time of day, circadian rhythm, or year, seasonal rhythm. External cues or modulators called *zeitgebers*, such as light or food, play a crucial role in the reprogramming or altering these oscillations. Circadian rhythm disruption due to modern lifestyles such as changes in light/dark cycles or dietary patterns has been related to the development of metabolic disorders. In this context, phenolic compounds such as proanthocyanidins (PACs) have demonstrate to exert beneficial effects on metabolic disorders. Moreover, polyphenols have shown their capacity to modulate some components of the clock system. Indeed, the interaction of phenolic compounds with biological rhythms has been recently pointed out by our group as a potential mechanism involved in their beneficial effects. Then, the aim of this thesis was to elucidate whether PACs act through biological rhythms and to investigate whether these phenolic compounds can restore biological rhythms in different perturbation situations, such as changes in light-dark cycles or in dietary patterns. For this, circadian rhythms of healthy and cafeteria (CAF) diet obese Fischer 344 rats, the effects of different situations of perturbation of these rhythms and the properties of PACs to restore these disruptions were studied. CAF diet caused disruption of central clock genes and alteration of metabolism circadian rhythms. PACs demonstrated to act as *zeitgebers* mitigating the circadian disruption, notably the restoration of the circadian rhythm of melatonin. In addition, in a jet lag situation caused by a sudden light/dark shift, PACs mitigated the circadian disruption in a photoperiod-dependent manner. Thus, PACs have an important role as modulators of central and peripheral circadian rhythm, which may mediate the beneficial effects of these phenolic compounds on metabolic syndrome.

RESUMEN

Los ritmos biológicos desempeñan un papel importante en la adaptación fisiológica y metabólica del organismo a momento del día, ritmo circadiano, o del año, ritmo estacional. Las señales o moduladores externos llamados *zeitgebers*, como la luz o la comida, desempeñan un papel crucial en la reprogramación o alteración de estas oscilaciones. La alteración del ritmo circadiano debida a los estilos de vida modernos, como los cambios en los ciclos de luz/oscuridad o los patrones dietéticos, se ha relacionado con el desarrollo de trastornos metabólicos. En este contexto, los compuestos fenólicos como las proantocianidinas (PACs) han demostrado ejercer efectos beneficiosos sobre los trastornos metabólicos. Además, los polifenoles han demostrado su capacidad para modular algunos componentes del sistema del reloj. De hecho, la interacción de los compuestos fenólicos con los ritmos biológicos ha sido señalada recientemente por nuestro grupo como un mecanismo potencial implicado en sus efectos beneficiosos. Entonces, el objetivo de esta tesis fue dilucidar si los PACs actúan a través de los ritmos biológicos e investigar si estos compuestos fenólicos pueden restaurar los ritmos biológicos en diferentes situaciones de perturbación, como los cambios en los ciclos de luz-oscuridad o en los patrones dietéticos. Para ello, se estudiaron los ritmos circadianos de ratas Fischer 344 sanas y con dieta de cafetería (CAF), los efectos de diferentes situaciones de perturbación de estos ritmos y las propiedades de los PACs para restaurar estas perturbaciones. La dieta CAF causó la disrupción de los genes del reloj central y la alteración de los ritmos circadianos del metabolismo. Los PACs demostraron actuar como *zeitgebers* mitigando la disrupción circadiana, destacando la restauración del ritmo circadiano de la melatonina. Además, en una situación de jet lag causada por un cambio repentino de luz/oscuridad, los PACs mitigaron la alteración circadiana de forma dependiente del fotoperiodo. Por lo tanto, los PACs tienen un papel importante como moduladores del ritmo circadiano central y periférico, que puede mediar los efectos beneficiosos de estos compuestos fenólicos en el síndrome metabólico.

RESUM

Els ritmes biològics tenen un paper important en l'adaptació fisiològica i metabòlica de l'organisme un moment del dia, ritme circadià, o de l'any, ritme estacional. Els senyals o moduladors externs anomenats *zeitgebers*, com la llum o el menjar, tenen un paper crucial en la reprogramació o alteració d'aquestes oscil·lacions. L'alteració del ritme circadià deguda als estils de vida moderns, com ara els canvis en els cicles de llum/foscor o els patrons dietètics, s'ha relacionat amb el desenvolupament de trastorns metabòlics. En aquest context, els compostos fenòlics com les proantocianidines (PACs) han demostrat exercir efectes beneficiosos sobre els trastorns metabòlics. A més, els polifenols han demostrat la seva capacitat per modular alguns components del sistema del rellotge. De fet, la interacció dels compostos fenòlics amb els ritmes biològics ha estat assenyalada recentment pel nostre grup com un mecanisme potencial implicat en els efectes beneficiosos. Aleshores, l'objectiu d'aquesta tesi va ser dilucidar si els PACs actuen a través dels ritmes biològics i investigar si aquests compostos fenòlics poden restaurar els ritmes biològics en diferents situacions de pertorbació, com els canvis en els cicles de llum/foscor o en els patrons dietètics. Per això, es van estudiar els ritmes circadians de rates Fischer 344 sanes i amb dieta de cafeteria (CAF), els efectes de diferents situacions de pertorbació d'aquests ritmes i les propietats dels PACs per restaurar aquestes pertorbacions. La dieta CAF va causar la disrupció dels gens del rellotge central i l'alteració dels ritmes circadians del metabolisme. Els PACs van demostrar actuar com a *zeitgebers* mitigant la disrupció circadiana, destacant la restauració del ritme circadià de la melatonina. A més, en una situació de jet lag causada per un canvi sobtat de llum/foscor, els PACs van mitigar l'alteració circadiana de forma depenent del fotoperíode. Per tant, els PACs tenen un paper important com a moduladors del ritme circadià central i perifèric, que pot intervindre els efectes beneficiosos d'aquests compostos fenòlics en la síndrome metabòlica.

LIST OF ABBREVIATIONS

ACC	acetyl coenzyme A carboxylase
AgRP	agouti-related protein
ALAS1	aminolevulinate synthase 1
AMPK	adenosine monophosphate kinase
BMAL1	brain and muscle Arnt-like protein-1
BMAL2	brain and muscle Arnt-like protein-2
BW	body weight
CAF	cafeteria diet
cAMP	cyclic adenosine monophosphate
CHGA	chromogranin A
CK1δ	Casein kinases δ
CK1ε	Casein kinases ε
CLOCK	circadian locomotor output cycles kaput
CRY	Cryptochrome
DEC1	Deleted in esophageal cancer 1
Dio2	type II iodothyronine deiodinase
Dio3	type III iodothyronine deiodinase
ERR	estrogen-related receptor
ERα	estrogenic receptor-α
EYA3	the eyes-absent 3
FOXO1	Forkhead box O1
GSPE	Grape Seed Proanthocyanidin Extract
HMGCR	enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase
HNF4α	hepatocyte nuclear factor 4 alpha
L12	photoperiod with 12 hours of light
L18	photoperiod with 18 hours of light
L6	photoperiod with 6 hours of light
LXR	hepatic X receptor
ME	median eminence

MeS	Metabolic syndrome
NAD	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NPY	Neuropeptide Y
NURR1	nuclear receptor related 1 protein
PACs	proanthocyanidins
PD	pars distalis
PER	Period
PGC1α	coactivator peroxisome proliferators–activated receptor gamma coactivator 1-alpha
POMC-CART	pro-opiomelanocortin—cocaine-amphetamine-regulated transcript
PPARα	Peroxisome proliferator–activated receptor alpha
PT	pars tuberalis
Rev-Erbα	orphan nuclear hormone receptor reverse-erb alpha
RHT	retinohypothalamic tract
Ror	retinoic acid orphan receptor
SCN	hypothalamic suprachiasmatic nucleus
SIRT1	sirtuin 1
SIX1	Six (sine oculis)-binding homeodomain factor 1
SREBP1c	sterol regulatory element-binding protein 1c
STD	standard chow diet
T2	inactive diiodothyronine
T3	triiodothyronine
T4	thyroxine
TEF	thyrotroph embryonic factor
TH	thyroid hormone
TPH	hypothalamic-pituitary-thyroid axis
TRH	TSH-releasing hormone
TRα	thyroid hormone receptor-α

TSHR	TSHβ receptor
TSHβ	thyrotropin beta subunit

INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
INTERACTION BETWEEN CIRCADIAN RHYTHM AND GRAPE SEED PROANTHOCYANIDINS IN HEALTHY AND OBESE RATS: POTENTIAL ROLE AS A CENTRAL AND PERIPHERICAL CLOCK SYNCHRONIZER
Jorge Ricardo Soliz Rueda

1.1. Background

The interaction between an organism and its environment allows him to adapt to circumstances in order to be more efficient and survive in different situations. In this context, the sun is a very important cue since it provides energy, in the form of light and heat, that enables life and is an external signal that governs the cycles of the Earth. Thus, the translation around the sun, which triggers the different seasons of the year, and the rotation of the Earth on its own axis, which generates daily light-dark cycles, are the main factors to which the organism must adapt and whose periodicity marks the biological rhythms.

These Biological rhythms play an important role in the physiological and metabolic adaptation of the organism to the environmental variations. This adaptation to the time of day (circadian rhythm) or of the year (seasonal rhythm) allows the optimization of metabolism and energy expenditure [1,2]. In this regard, processes such as cardiovascular activity, endocrine system, blood pressure, body temperature, sleep-wake cycle, kidney activity, gastrointestinal tract and liver metabolism are regulated to a greater or lesser extent by the circadian rhythm, whose period is usually around 24 hours [3–6]. In addition, the number of daylight hours during these 24 hours also regulates these processes by changing the light-dark cycle and allows the organism to have a reference of the time of the year in which it finds itself.

The control center of circadian rhythm is located in the hypothalamic suprachiasmatic nucleus (SCN), which synchronizes the organism with the external environment via the retina through retinohypothalamic tract (RHT) [2,7]. In turn, from the SCN a variety of signalling pathways are triggered in order to communicate with the peripheral tissues to orchestrate and synchronize the metabolism to the temporal signals of the environment [7]. In this context, external signals or modulators called Zeitgebers, such as light, the most important one, or food, play a crucial role in altering or reprograming the circadian rhythm [2,8,9]. Thus, the circadian rhythm disturbance due to modern

lifestyles (shift work, artificial light, fast food, eating time, etc.) are related to the development of metabolic disorders that in the long term could lead to type 2 diabetes, cardiovascular diseases, overweight and obesity [10–14]. These metabolic disorders typical of the metabolic syndrome (MeS), together with other comorbidities such as depression, sleep disturbances, cognitive dysfunction and steatohepatitis could form part of "Circadian Syndrome" [15].

Phenolic compounds are a group of molecules produced by plants as response to stress [16] which have been demonstrated to exhibit healthy properties. Proanthocyanidins (PACs) are a class of polyphenols constituted by polymers of flavonoids and its gallate derivatives. PACs are abundant in many foods, such as wine, tea, cocoa and fruits and its consumption has been related to multiple beneficial effects [17]. Grape Seed Proanthocyanidin Extract (GSPE) is an extract obtained from white grape seed, whose phenolic profile is mainly composed of catechin, epicatechin, gallic acid, epicatechin gallate and dimers, trimers, and tetramers of proanthocyanidins [18]. This extract has been extensively studied by our group and has demonstrated remarkable effects on metabolism, showing beneficial activities on different key aspects of MeS [17,19–24]. Several molecular mechanisms have been demonstrated to be involved in the effectivity of PACs, including epigenetic modifications which have recently emerged as important mediators of their properties [25]. In addition, different studies have demonstrated that these phenolic compounds can modulate both central and peripheral biological rhythms in healthy animals under jet-lag conditions, but also in CAF fed obese rats [26–29]. In this regard, the interaction of these phenolic compounds with the clock system has been recently pointed out by our group as other potential mechanism involved in their beneficial effects [30].

1.2. Biological rhythm machinery

The circadian rhythm is well studied, and its regulatory mechanisms are well known. As previously mentioned, the circadian rhythm has its regulatory center

in the hypothalamic SCN, which is composed of about 10,000 to 15,000 neurons. This central clock, pacemaker of the organism and its peripheral clocks, has the capacity to work autonomously, without any external input, but it can also be reprogrammed by different Zeitgebers. Light, the most important Zeitgeber of the central clock, is perceived by RHT [2]. Thus, once this signal has been captured, central clock provides signals, via the autonomic nervous

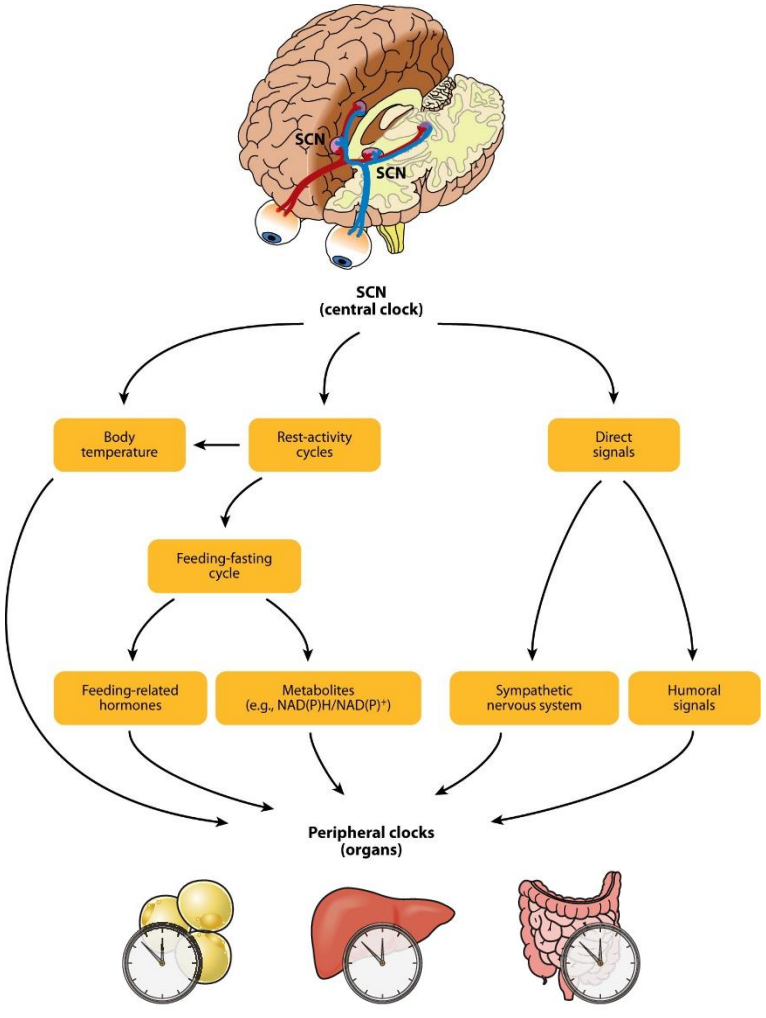

***Figure 1. Peripheral clock entrainment pathways**. Central clock can synchronize peripheral clocks via multiple pathways. The rest-activity cycle drives the feeding-fasting cycle which are strong Zeitgebers for some organs. In addition, body temperature cycles appear to play an important role in resetting peripheral clocks. Moreover, central clock also uses humoral and neural signals that are more direct. All these pathways are controlled to a greater or lesser extent by the SCN with the aim of adjusting peripheral clocks. Figure adapted from Dibner C, et al. 2010.*

system or circulating humoral factors such as melatonin and cortisol [7], to various peripheral oscillators to maintain rhythmicity and ensure a temporally coordinated physiology (Figure 1). These peripheral clocks are present in almost all other mammalian tissues, such as liver, muscle, pancreas, and adipose tissue, where they maintain circadian rhythms and regulate tissue-specific gene expression and functionality [7]. Although peripheral clocks are synchronized by the central clock, they are modulated by behavioral signals such as physical activity and, most notably, fasting-feeding cycles [4]. Therefore, although circadian clock regulates multiple metabolic pathways, metabolites availability and feeding behavior can also influence rhythms, being the central clock and peripheral clocks two systems that are reciprocally regulated. Thus, food entrains the liver clock, whereas light acts through the brain clock to control feeding time.

In mammals, the circadian machinery is an intracellular mechanism sharing the same molecular components in SCN neurons and peripheral cells (Figure 2). The central feature of this molecular rhythmicity is the transcription–translation autoregulatory feedback loop which cycles with a periodicity of 24 h [31]. The transcriptional activators of this feedback system are the circadian locomotor output cycles kaput (CLOCK) and the brain and muscle Arnt-like protein-1 (BMAL1). CLOCK and BMAL1 form a heterodimer that can activate the transcription upon binding to E-box (5'-CACGTG -3') and E-box-like sequences found in the promoters of circadian-responsive genes. Its target factors Period (PER) and Cryptochrome (CRY) are rhythmically accumulated in the cytoplasm, oligomerize after reaching an appropriate concentration and translocate to the nucleus where interact with CLOCK and BMAL1 inhibiting their own transcription. All the aforementioned clock genes exhibit a 24h transcriptional oscillation in SCN cells and peripheral tissues, except for Clock that has been shown not to oscillate in the SCN [2,32–34].

CLOCK-BMAL1 dimer also activates the circadian expression of the orphan nuclear hormone receptor reverse-erb alpha (REV-ERBα) and retinoic acid

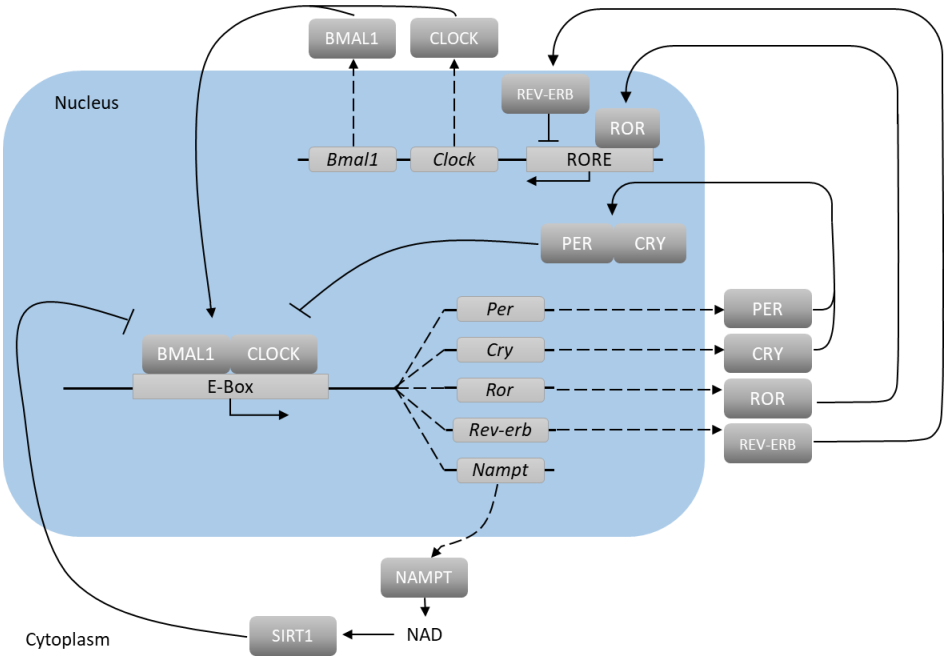

Figure 2. Circadian rhythm mechanism core*. BMAL1: brain and muscle Arnt-like protein-1; CLOCK: circadian locomotor output cycles kaput; PER: Period; CRY: Cryptochrome; ROR: retinoic acid orphan receptor; REV-ERB: orphan nuclear hormone receptor reverse-erb; NAMPT: nicotinamide phosphoribosyltransferase; SIRT1: sirtuin 1; NAD: nicotinamide adenine dinucleotide.*

orphan receptor (RORα and β), which constitute a short feedback loop controlling Bmal1 transcription through its repression and activation, respectively. Peroxisome proliferator–activated receptor alpha (PPARα) and the coactivator peroxisome proliferators–activated receptor gamma coactivator 1-alpha (PGC1α) are other nuclear receptors which also stimulate Bmal1 transcription [5,32,35].

Post-transcriptional control of circadian machinery mediated by phosphorylation and acetylation has also been reported. Casein kinases (CK1ε and CK1δ) are involved in this autoregulatory loop, targeting PER proteins for degradation [36] and adenosine monophosphate kinase (AMPK), which targets CRY proteins also for degradation [37]. CK1ε also phosphorylates and partially activates BMAL1. Furthermore, CLOCK has been shown to exhibit histone acetyltransferase activity, revealing that also chromatin remodeling is crucial

for the central clock mechanism [38]. In this regard, CLOCK acetylates several proteins of the core clock apparatus, thus allowing cycles of acetylation and deacetylation. The latter activity also involves sirtuin 1 (SIRT1), which in turn regulates circadian rhythms by deacetylating both histones and non-histone proteins, such as BMAL1 and PER2. In addition, the association between SIRT1 and CLOCK and its recruitment to CLOCK-BMAL1 dimer at circadian promoters has been reported, pointing to SIRT1 as an enzymatic rheostat of CLOCK function, which would thus transduce signals originating from cellular metabolites to the circadian machinery [39].

Besides circadian rhythms, mammals also exhibit circannual rhythms that that approximate to a year. This seasonal rhythm is not as well studied as the circadian rhythm. However, it is known that there is a very close relationship with the circadian clock genes, which also control this circannual or seasonal rhythm. In peripheral organs, circannual rhythmicity is not self-maintained and requires maintenance by photoperiod signals. These signals are recognised in the RHT in the form of light/dark cycles and transmitted to the pineal gland by multi-synaptic neuronal pathways. The pineal gland integrates these photoperiod signals and regulates the production of melatonin, which is rhythmically produced at night [42].

The controlling center of seasonal changes is the pars tuberalis (PT) located in the pituitary gland, in charge of translating photoperiodic signals to adapt metabolism and behavior to the season of the year [40]. This structure, together with the median eminence (ME), the portal system, the pars distalis (PD) and the third ventricle, constitute the main anatomical structures of the seasonal control in animals [41]. On the one hand, PT-specific cells are known as calendar cells as they are associated with the signal transduction of the light-dark cycle [41]. These cells present the highest amount of melatonin receptors MT1 and also have immunoreactivity for beta-subunit of thyrotropin (TSHβ) and their expression is regulated by photoperiod and melatonin signals [42,43]. On the other hand, TSHβ is also expressed by PD cells but its regulation is different

between both cell lines since this expression is controlled by active thyroid hormone (TH), triiodothyronine (T3) or TSH-releasing hormone (TRH) being included in the regulatory mechanisms of the hypothalamic-pituitary-thyroid axis (TPH) [43,44]. Thus, PT translates photic information through the neurohormone melatonin, which encodes the duration of the night since it is produced only during the night, starting its secretion early in the evening and ceasing at dawn, showing a peak in blood at the height of the dark phase[45–47]. Melatonin receptors have been shown to be coupled to different G proteins that inhibit the accumulation of cyclic adenosine monophosphate (cAMP) [48–50]. Along with this mechanism, intracellular signaling is also mediated by calcium and potassium channels, phospholipase C, adenylate cyclase and guanylate cyclase [49,50]. Thus, melatonin can regulate many physiological factors in humans, and those include immunity, metabolism, and body temperature.

Another important component of the seasonal machinery is the eyes-absent 3 (EYA3) protein, which presents an expression linked to light-dark cycles in PT and melatonin showed the ability to suppress its expression in PT [51]. EYA3 has its peak of highest expression 12 hours after the onset of the dark phase, independent of the duration of daylight hours [52]. Thus, during a short photoperiod EYA3 should peak even at night since during this photoperiod there are more hours of darkness, having the melatonin peak for longer and with greater intensity, but Eya3 expression is blunted by melatonin repression [51,53]. In contrast, when the light phase is longer during the long photoperiod and melatonin levels are low and last for less time, Eya3 expression peaks during the early morning. At that time EYA3 induction is exacerbated since melatonin levels are minimal and there is no repression of Eya3 expression [51]. In addition, the regulation of T3 by photoperiodic changes through the expression of the deiodinase gene has been reported [54]. Melatonin-mediated expression of EYA3 promotes a molecular pathway involving the circadian transcription factor thyrotroph embryonic factor (TEF) and a nuclear

complex formed by EYA3 and Six (sine oculis)-binding homeodomain factor 1 (SIX1). This nuclear complex, EYA3-SIX1, activates TSHβ expression, in turn potentiated by TEF [51,55]. Subsequently, TSHβ binds to its specific receptor (TSHR) in the ependymal layer of the infundibular recess, promoting significant up-regulation of type II iodothyronine deiodinase (Dio2), which catalyses the conversion of thyroxine (T4) to bioactive T3, and to the repression of type III iodothyronine deiodinase (Dio3), which converts T3 to the inactive form rT3 or T4 to inactive diiodothyronine (T2). In contrast, short-day photoperiod induces Dio3 expression and inhibits the Dio2 gene [56,57]. Therefore, the EYA3 regulatory mechanism, which confines EYA3 to the long photoperiod via external photic signals, and the fact that hypothalamic T3 is regulated in a photoperiod-dependent manner, increasing TH levels during long photoperiod exposure, allows linking this protein and hormones to summer phenotype.

Moreover, chromogranin A (CHGA), whose expression is specific for PT thyrotrophs, has been identified as a winter marker [58–60]. In this structure there is a complex and dynamic binary coding between EYA3 and CHGA that is closely related to changes in the length of light-dark cycles[60]. Although a relationship between the changes in EYA3 and CHGA in seasonal changes in PT has not been established, this binary coding between the two proteins has been demonstrated in different studies [58–60]. Thus, EYA3, CHGA and their ratio are postulated as an excellent biomarker of seasonality in PT tissue.

1.3. Relationship between circadian and seasonal rhythms

The biological rhythms are closely interrelated among themselves since the light-dark cycle and its variations between seasons are the external driver of the hypothalamus for the central clock and pituitary via melatonin signals. Several studies confirm that the PT is a melatonin-dependent peripheral clock, which exhibits circadian rhythmicity and expresses clock genes [3,55]. Thus, increased nocturnal melatonin concentrations promote increased CRY mRNA levels, whereas PER expression is downregulated. Therefore, the relationship between

PER and CRY, which follow the onset and end of light respectively, indicates changes in the length of daylight hours in the light-dark cycle, closely related to melatonin oscillations. Consequently, the interval phase between PER and CRY during the short photoperiod is shorter than in response to the long photoperiod [61,62]. Furthermore, the results of a study conducted by Dardente et al. suggest a regulation of EYA3 by the circadian system via binding of CLOCK and BMAL1 to conserved E-boxes in the EYA3 gene promoter [51]. In this context, the peak of CRY transcription during twilight promoted by melatonin signaling represses the CLOCK-BMAL1 dimer until dawn, which is the time when EYA3 transcription occurs [51,62,63]. Moreover, modulation of TSHβ via E-box elements by the CLOCK-BMAL1 dimer in mice has been reported [51,64].

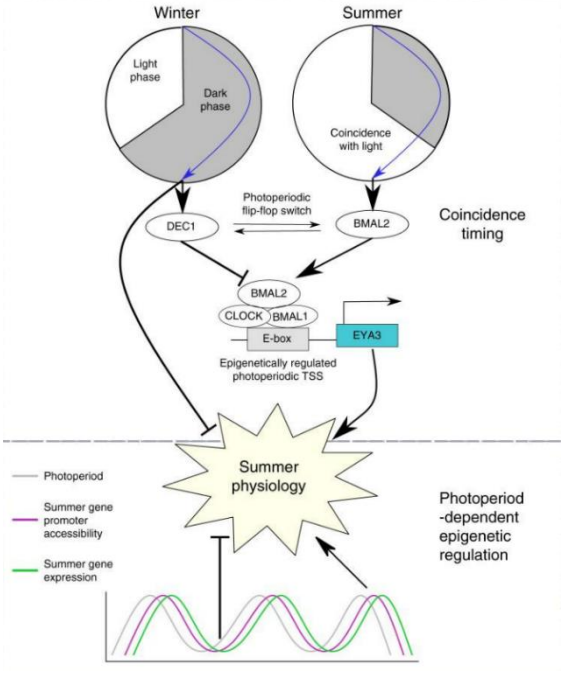

Figure 3.Interaction between circadian clock genes and photoperiod machinery*. BMAL1: brain and muscle Arnt-like protein-1; CLOCK: circadian locomotor output cycles kaput; BMAL2: brain and muscle Arnt-like protein-2; DEC1: Deleted in esophageal cancer 1; EYA3: the eyes-absent 3. Figure adapted from Wood SH, et al. 2020.*

In addition, brain and muscle Arnt-like protein-2 (BMAL2) has recently been reported to act as a coactivator of EYA3 with CLOCK:BMAL1 dimmer, probably in a complex through PAS-B domain interaction [65]. Within the PT, BMAL2 and EYA3 have a role as photoperiod coactivators. Although the regulation of BMAL2 is not clear, the results of this study indicate the presence of E-boxes in BMAL2. It has also been reported that the prolonged presence of melatonin causes transcription of repressor genes 12 hours after the onset of the dark phase. Among these repressor genes, we show that the circadian repressor deleted in esophageal cancer 1 (DEC1) blocks EYA3 expression via BMAL2 [65]. Therefore, DEC1 and BMAL2 act as a circadian switch for photoperiodic timing (Figure 3).

In conclusion, this evidence shows how the two machineries of biological rhythms are closely related by the light-dark cycle and its transduction in hypothalamus and pituitary. Therefore, although the molecular mechanisms of the relationship between the central clock and the calendar genes are not exactly known, the importance of circadian rhythms on seasonal rhythms through photic signals, but also of the timing, phase, and amplitude of the induced changes in the signals, is evident.

1.4. Metabolism and its rhythmic regulation

The circadian clock controls many key points of metabolic pathways to modulate them throughout the day according to photic signals. Specifically, this modulation can be achieved through circadian control of its rate-limiting steps and proteins, such as nuclear receptors and nutrient sensors, and by regulating metabolite levels [66].

The control of the activity or expression of rate-limiting enzyme is one of the mechanisms through which many metabolic pathways can be regulated in a circadian manner [67]. For example, the regulation of the nicotinamide adenine dinucleotide (NAD) salvage pathway is controlled by the circadian clock. The expression of the rate-limiting enzyme of this pathway, nicotinamide

phosphoribosyltransferase (NAMPT), oscillates in a circadian manner, which produces a circadian rhythmicity in NAD+ levels [68–70]. Similarly, the activity of the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR), which is the rate-limiting enzyme in cholesterol biosynthesis, shows a peak level at night and lower levels during the light hours, is another example of the control of a metabolic pathway by the circadian clock [71]. This finding is useful in the treatment of hypercholesterolemia and the administration of cholesterol-lowering drugs such as statins, that act by inhibiting HMGCR, which produce maximum effects when are administered in the night [31]. Comparably, the enzyme aminolevulinate synthase 1 (ALAS1), the rate-limiting enzyme in heme biosynthesis, regulates the cellular heme levels in a circadian manner since its expression is controlled by circadian rhythm [72]. These are some examples of how the metabolism is orchestrated in a circadian manner, in order to achieve the best efficiency at every moment of the day.

Due to the central role of the essential metabolic cofactor NAD+ in numerous metabolic processes, this is an attractive candidate as an integrator of circadian rhythms and nutrient sensing pathways through SIRT1 functionality. SIRT1 is a NAD+-dependent deacetylase, key in metabolism since deacetylates several proteins that participate in metabolic pathways and regulates gene expression by histone deacetylation [73]. Therefore, the NAD+-dependence of SIRT1 directly links cellular energy metabolism and deacetylation of target proteins. Circadian oscillations in NAD+ entail the rhythmicity in the activity of this enzyme, which, as was previously mentioned, operating as a rheostat of the circadian machinery. In addition, SIRT1 also regulates several metabolic proteins. For example, SIRT1 regulates cholesterol metabolism through activation of the hepatic X receptor (LXR), which regulates cholesterol metabolism [74]. Deacetylase also regulates the expression of several gluconeogenic genes through activation of Forkhead box O1 (FOXO1) and PGC1α, which, in turn, is a regulator of circadian rhythms [75,76]. PGC1α also regulates lipid metabolism and the gene expression of key components of

mitochondrial biogenesis, and it has been pointed out as a critical metabolic regulator in many vital organs, including white and brown adipose tissue, skeletal muscle, heart, liver, and kidney [77]. Therefore, it seems that PGC1α might be a very important integrator of metabolic status and circadian function.

In addition to SIRT1, the enzyme AMPK is activated when the cellular energy status is low. When the AMP/ATP ratio is high, this enzyme activates catabolic process to obtain ATP and deactivates ATP-consuming processes. As previously mentioned, AMPK modulates the circadian cycle via phosphorylation of CRY1 and CK1ε. In addition, the activity of AMPK was found to be rhythmic in the mouse liver, hypothalamus and in mouse fibroblasts [37,78]. Interestingly, AMPK activation also leads to an increase in NAD+ levels [79] and modulate circadian gene expression indirectly via SIRT1 activation.

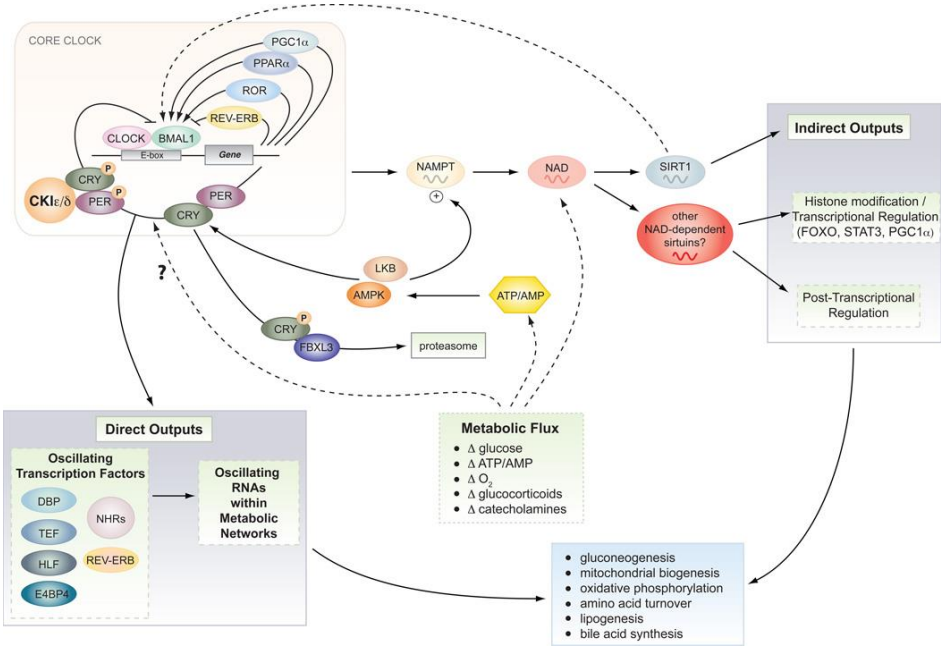

Figure 4. Direct and indirect outputs of the core clock mechanism*. Cascade of signaling pathways by which the central clock interfaces with metabolism, a set of feedback loops that synchronize various metabolic processes. The clock also receives reciprocal information from nutrient signaling pathways (including SIRT1 and AMPK) to couple circadian cycles to metabolic flux, especially in peripheral tissues. BMAL1: brain and muscle Arnt-like protein-1; CLOCK: circadian locomotor output cycles kaput; PER: Period; CRY: Cryptochrome; ROR: retinoic acid orphan receptor; REV-ERB: orphan nuclear hormone receptor reverse-erb; NAMPT: nicotinamide phosphoribosyltransferase; SIRT1: sirtuin 1; NAD: nicotinamide adenine dinucleotide. Figure adapted from Bass J, et al. 2013.*

Other mechanism involved in the regulation of the metabolism by the circadian clock is controlling the nuclear receptors. As previously mentioned, CLOCK and BMAL1 control the expression of the nuclear receptors RORα, REV-ERBα and PPARα, which in turn regulate the transcription of BMAL1. In addition, REV-ERBα also regulates lipid metabolism and adipogenesis and PPARα controls fatty acid oxidation and apolipoprotein synthesis, which would demonstrate a very close relation between nuclear receptors, metabolism, and circadian clock. Moreover, the rhythmic and tissue-specific expression of other nuclear receptors, including other PPAR family members (most notably γ and δ) and estrogenic-related receptor (ERR) family members (α, β, γ) could be related to the observed daily oscillations in glucose and lipid metabolism [80]. In fact, several studies have been demonstrated the importance of BMAL1 in glucose metabolism where it has been observed that a downregulation of insulin secretion is led by a bmal1 deletion [81,82].

The physical association of PER proteins (PER 1–3) with different nuclear receptors has also been demonstrated, pointing to another mode of regulation of nuclear receptor function by the circadian clock. Hence, PER2 interacts with many nuclear receptors including PPARγ, estrogenic receptor-α (ERα), PPARα, REV-ERBα, hepatocyte nuclear factor 4 alpha (HNF4α), thyroid hormone receptor-α (TRα), nuclear receptor related 1 protein (NURR1) and RORα among others, and PER3 with PPARγ. These interactions besides modulates the expression of core clock genes, such as BMAL1, influences several metabolic pathways, such as lipid and glycogen metabolism. In this sense, PER2 inhibits, in white adipose tissue, the recruitment of PPARγ to its target promoters [83] and PER3, binding to PPARγ target sites, inhibits PPARγ activity and blocks adipogenesis [84]. In addition, its interaction with REV-ERBα and other nuclear receptors is decisive for glycogen metabolism [85]. All these pathways are summarizing in figure 4. Furthermore, mouse plasma levels of hundreds of metabolites, including phospholipids, amino acids, and urea-cycle metabolites, have been demonstrated to exhibit circadian oscillations, which are

independent of the age, sex, or genetic background of the mice. These variations are so precise that allow to determinate the biological time by measuring the concentrations of these metabolites [86]. These observations support that the circadian clock regulates metabolism at various levels and, therefore, disruptions in circadian rhythms could manifest in metabolic disorders [87].

1.5. Biological rhythm disruption and its consequences in metabolism

1.5.1. Light-dark cycle disturbance

In modern societies, an important factor that disrupts biological rhythms is artificial light as we are continuously subjected to it. Mentioned previously, one of the most important Zeitgeber is the light, which is able to synchronize biological rhythms. Hence, changes in the light-dark cycles have been linked to altered gene expressions and protein synthesis, promoting the development of various diseases. In this context, recent studies have evaluated the impact of several degrees of light exposure, including constant dim light during the dark phase, different bright intensities, length of the light phases or alternating light exposure in the dark phase [88]. The described health outcomes associated with artificial light include behavioral and mood disorders, impact on the neurological, reproductive, and immune system alterations and even changes in the microbiota [89,90]. Furthermore, disorders in lipid metabolism and in colon functionality in rats by circadian alteration via changes in day length have been reported [91,92]. Moreover, light-dark cycle shifts have effects on behavioral and feeding patterns and on body weight gain in male Fischer 344 rats [93,94].

Exposure to artificial light during the night profoundly affects melatonin synthesis. In fact, suppression of melatonin at an exposure of more than 100 lux has been reported [95]. Recently, Verma et al. (2019) demonstrated in

animal models that continuous light exposure (24 h/500 lux/10 days) caused oxidative stress, decreasing the antioxidant defense system. These alterations were attenuated when the animals were supplemented with melatonin (10 mg/kg), demonstrating the key role of this molecule in maintaining health status [96]. Furthermore, dysregulation of melatonin production has been linked to tumor growth. In this regard, both dim and bright light are capable of altering the circadian rhythm of melatonin, stimulating cancer development [97]. Interestingly, exposure to bright light during the day can advance the circadian rhythm of melatonin, enhancing the cholinergic tone of the parasympathetic nervous system and promoting the release of digestive juices and gastrointestinal motility [98]. In this sense, bright light seems to counteract diarrhoea and constipation in humans [99]. On the other hand, the use of dim light during the day suppresses the digestion of the dinner, producing a poor absorption of the carbohydrates contained in food [100].

Regarding the influence of light and dark cycles on feeding patterns, a recent study observed that changes in these cycles produce an alteration of diurnal rhythms in food intake, altering gastric vagal mechanosensitive and responsiveness to food-related stimuli in C57BL/6 mice exposed to a rotating light cycle for 8 weeks and fed standard chow diet [101]. These disruptions could be associated to gastrointestinal motility disorders since the gastric vagal afferent mechanosensitive plays an important role in vago-vagal reflexes, controlling gastrointestinal function. In addition, it was also observed an increase in the size and number of meals during the light phase. Interestingly, although the total food daily intake was not altered, these changes in the dietary timing produced an increase in the body weight gain. In addition, disruption of the light-dark cycle by social jetlag has shown in rats an increase in consumption of fat- and carbohydrate-rich foods when combined with cafeteria (CAF) diet, altering feeding patterns [102].

Furthermore, it has been reported that a long-term exposure to an altered light-dark cycle has a very important effect on increasing body weight gain

[92,93,103]. However, the first days of an abrupt change of photoperiod could be causing environmental stress, a situation that is related to a lower body weight gain, which would explain the difference in results [104,105]. In this context, it has been reported that reversal of the light/dark cycle showed a decrease in body weight compared to the control group in rats [94].

A population group especially exposed to disturbance of light-dark cycle are the shift-workers. Thus, it has been described that night-shift workers suffer alterations in their natural light–dark schedule, thus promoting the disruption of their circadian rhythms [106]. In this study conducted in 2020, researchers concluded that disturbance in circadian rhythm were reflected by blood pressure and the cortisol levels. In this sense, cortisol level significantly increased before and after the shift. Regarding blood pressure, a decrease was observed during the morning but not in the afternoon or evening compared to daily workers.

1.5.2. Nutrition, food, and dietary Patterns

Finally, another relevant factor contributing to the alteration of biological rhythms is dietary patterns, including the timing, number of meals, restrictions, and composition of the diet. In fact, it has been postulated that quantitative and qualitative changes in dietary patterns in Westernized societies may be causing alterations in biological rhythms [107]. In this regard, it has been described that dietary pattern may cause a mismatch between the central and peripheral clocks, leading to the development of metabolic disorders [108]. Hence, mice fed a high-fat diet showed alterations in the expression of circadian rhythm genes, nuclear receptors that regulate clock transcription factors, and clock-controlled genes involved in fuel utilization in the hypothalamus, liver, and adipose tissue. Moreover, changes in the rhythm of locomotor activity were also observed [8].

In addition, the frequency and timing of meals regulated circadian rhythms and energy metabolism in liver and muscle of rats [109]. Furthermore, the intake of

sugars, lipids or other nutrients at different times of the day is known to have a different impact on the body [8,110–113]. In this regard, a study has reported the importance of the timing pattern of food intake on liver gene transcription in wild-type mice [110,112]. Changes in brown adipose tissue and skeletal muscle metabolism dependent on feeding time have also been reported in Wistar rats [114]. In addition, it was observed in humans that late food consumption is associated with an increase in body fat [115]. Thus, not only the composition of our food can modulate the peripheral clocks, but the timing of our food intake is now gaining importance.

In the organism exists food-anticipatory processes that follow circadian oscillations, but these processes are altered by the meal timing, thus generating this desynchrony. Noteworthy, it seems that the regulation of food anticipatory processes is independent of the SCN although the mechanisms by which are controlled remains unclear [116]. In this framework it has been described that different timing in the meals cause changes in the behavior and physiology, adapting to these patterns and modifying the anticipatory responses [117]. For example, after few days of restricted feeding, eating only during few hours in the middle of the light period, animals adjust their anticipatory activity to those hours [118]. However, when the restricted feeding is done at night this alteration clashes with the central clock [119]. In this line, studies performed in rats submitted to restricted feeding during rest phase observed the existence of a misalignment between the SCN and the liver, uncoupling key gene clocks such as *Bmal1*, *Clock* and *Per1* from *Ppar* in liver, thus contributing to metabolic disorders [120].

It has been seen that skipping breakfast is associated with a higher prevalence of obesity and impairs postprandial fasting lipid levels and insulin sensitivity in humans [121,122]. In addition, modification of the time of feeding itself can greatly affect body weight. In this sense, late lunch is associated with a lower tendency of reducing body weight while earlier lunch favors it. In addition, late dinner (two hours before sleeping) leads to decrease of glucose tolerance [121].

Not only the timing of meals but the quantitative and qualitative composition of the diets also has an impact on the biological rhythms. In this sense, epidemiological studies performed in adults showed that diets with a higher proportion of carbohydrates and less fat in breakfast and lunch correlates with lower incidence of type 2 diabetes (T2D). Furthermore, consumption of carbohydrate in dinner after 20:00 produces higher body mass index values [123]. Other studies performed in adults under caloric restriction (1500 kcal /day) showed that eating a breakfast rich in carbohydrates and proteins seems to be beneficial for weight maintenance after weight loss, enhancing weight loss, satiety and suppression of the hormone ghrelin, which stimulates the appetite [124]. Interestingly, in a 12-week intervention study in humans, where either a high-calorie breakfast or a high-calorie dinner was given, was observed that those with a high-calorie breakfast gained less weight during the intervention compared to those with a hypercaloric dinner, thus, the feeding patterns affect the circadian regulation metabolism in a time-of-day-dependent manner [125].

In animal studies, it has been described those mice fed high-fat diet suffer an alteration of their normal circadian rhythms, increasing the consumption of food and the meal size in the light period while decreasing it in the night-time. This fact dampened the diurnal rhythm of food intake, reducing the amplitude of the circadian expression profile of *Bmal1*, *Clock*, and *Per2* in liver and fat adipose tissue [8,101]. In addition, these authors observed that mice presented altered levels of leptin and insulin, as well as expression of peripheral clock genes, including *Ror* and *Ppar*. Hypothalamic neuropeptides, such as agouti-related protein (AgRP) and Neuropeptide Y (NPY), and factors involved in lipid metabolism, including sterol regulatory element-binding protein 1c (SREBP1c) and acetyl coenzyme A carboxylase (ACC), were also altered [8,101].

Considering all the existing evidence, some authors have suggested that the effects of the diet on the clock genes are reversible and that nutritional challenges may act resynchronizing these clocks [9]. In this sense, in addition to

macronutrients, the capability of several bioactive compounds such as polyphenols, as previously mentioned, to act in the modulation of clock genes is currently under study by the scientific community.

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1.6. Phenolic compounds and Biological Rhythms

This section is developed in **manuscript 1: "Phenolic compounds and Biological Rhythms: Who takes the lead?"**, this review discusses the current state of research on the interaction between biological rhythms and the effect of polyphenols in three main sections.

1. **Phenolic compounds:**

   This group of chemicals are one of the most heterogeneous groups of plant secondary metabolites. They have been shown to exert several beneficial effects for the treatment and/or prevention of different chronic diseases such as diabetes, cardiovascular disease, and neurodegenerative diseases.

2. **Biological rhythms:**

   Biological processes present rhythms to adjust the functionality of the organisms to the environmental changes such as light, food availability or predator activity. Thus, circadian, and seasonal rhythms enable organisms to optimize the metabolism and energy utilisation to sustain life adapting their behaviour and physiology to the appropriate time of the day or of the year respectively.

3. **Phenolic compounds and biological rhythms modulation:**

   Recently, the time of the day and the season of the year in which these bioactive compounds are consumed have emerged as another factor that may significantly impact on their bioavailability, metabolisms, and bioactivities. Moreover, this interaction between phenolic compounds and biological rhythms could be bidirectional so that the modulation of biological rhythms by some of these compounds has been recently reported. In fact, according to xenohormesis hypothesis phenolic compounds would be able to act as chemical cues allowing mammals to

favourably adapt to changing environmental conditions. Therefore, this section is discussed in two subsections.

a. **Biological rhythm impact on phenolic compounds activity.**
b. **Phenolic compounds effects on biological rhythms.**

Manuscript 1

Phenolic compounds and Biological Rhythms: Who takes the lead?

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ABSTRACT

Background: Phenolic compounds are one of the most heterogeneous group of plant secondary metabolites with over 50,000 diverse molecules identified so far. Despite the low bioavailability of native forms, they have been shown to exert several beneficial effects against chronic diseases such as diabetes, cardiovascular disease, and neurodegenerative diseases. There are different factors that may affect their absorption and distribution in tissues, but the exact mechanisms remain unclear. Recently, the time of the day and the season of the year in which they are consumed have emerged as another factor that may significantly impact on their metabolism and bioactivities.

Scope and approach: This review emphasizes the importance of the interaction between phenolic compounds and biological rhythms and its impact on the bioactivities of these metabolites. This may have implications for the food industry as food rich in phenolic compounds may exert different effects depending on the time of consumption.

Key findings and conclusions: Phenolic compounds broad activity could be explained by their extensive transformation, including metabolization in the colon by the gut microbiota, which leads to the production of multitude of different metabolites. Biological rhythms play a significant role in this metabolism affecting their bioactivities and, at the same time, phenolic compounds may exert their effects by promoting homeostasis at a basal signalling level through interactions with the biological clock system. This is in accordance with the xenohormesis hypothesis, which explains that chemical cues synthetized by plants are able to allow animals to favourably adapt to changing environmental conditions.

1. Phenolic compounds

Dietary phenolic compounds have received tremendous scientific and consumers attention due to their roles in human health. These compounds are bioactive molecules obtained from plant-based foods such as fruits, nuts, seeds, leaves and roots, plant-derived beverages such as tea or wine, and algal-derived products (Del Rio et al., 2013; Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). They are an important class of plant secondary metabolites which play crucial physiological roles generally involved in the attraction of pollinators, the execution of structural functions, the protection against ultraviolet (UV) radiation and the defence of plants against microbial invasion and herbivores (Cutrim & Cortez, 2018; Sharma et al., 2019). Under abiotic stress conditions, such as drought, heavy metal, salinity, high/low temperature, and UV radiations, plants increase phenolic compounds synthesis, especially polyphenols, as a defence mechanism to cope with environmental unfavourable conditions (Sharma et al., 2019). This complex large group of phytochemicals is chemically characterized by the presence of at least one hydroxyl group in the structure bonded directly to an aromatic ring and can be classified in flavonoids (isoflavones, flavones, flavonols, flavanols, flavanones and anthocyanidins) and non-flavonoids (phenolic alcohols, phenolic acids, stilbenes and lignans) (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Structurally phenolic compounds are very heterogeneous, and over 50,000 diverse molecules have been identified so far (Ziaullah & Rupasinghe, 2015)).

The study of these compounds may be challenging due to their

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heterogenicity and variety, and the complexity of their matrices, and because they are easily oxidized. However, it became an emerging field of interest for the scientific community due to their strong antioxidant properties and capacity to neutralize free radicals. Indeed, they have demonstrated to be the main contributor for antioxidant effects in fruits, even superior to that of the vitamin C (Tsao, 2010). In the last years different studies have demonstrated that phenolic compounds are also able to exert different effects at molecular level including the modulation of nuclear receptors, miRNAs and enzymatic activity as well as epigenetic modifications affecting different signalling pathways (Pan, Lai, Wu, & Ho, 2013). In this regard, they are also of interest for different industry sectors such as food and nutraceutical. Moreover, when added to foods, their antioxidants properties delay the formation of toxic oxidation products, maintain nutritional quality, and extend the shelf-life of products (Grobelna, Anna; Kalisz et al., 2020; Grobelna et al, 2019a, 2019b; Kalisz et al., 2020; Shahidi & Yeo, 2018). In addition, these compounds can be also of interest for agriculture, in which some can be used as allelochemicals (Mousavi, Karami, Haghighi, Alizadeh, & Maggi, 2021) and biostimulants (Mazrou, 2019) as well as other chemicals such as azadirachtin, a limonoid terpenoid found in *Azadirachta indica*, which has shown to be potent insecticide in ecological agriculture (Kharwar et al., 2020).

Different beneficial effects of phenolic compounds consumption have been shown in several epidemiological studies, including anticancer, anti-inflammatory, antibacterial activities, analgesic, anti-allergic, and anti-Alzheimer's properties, which may help in the treatment and/or prevention of different chronic diseases such as diabetes, cardiovascular disease, and neurodegenerative diseases (García-Conesa & Larrosa, 2020; Luca et al., 2020; Shahidi & Yeo, 2018; Tsao, 2010). In addition, recent studies have shown that phenolic compounds from *Moringa oleifera* which protect this plant against stress factors, may have a potential pharmacological use due to their anti-inflammatory and anti-hypertensive effects in both experimental models and clinical trials (Oldoni et al., 2021; Ożarowski et al., 2021). These findings reveal the effects of phenolics to promote health and the relevance of studying their role in the metabolism. Nevertheless, despite this broad activity the phenolic compounds present in food have shown a low bioavailability (Manach et al., 2004). Indeed, different studies have revealed that phenolics bioavailability is affected by different external and internal host factors such as dose, length of the treatment, sex and age (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). Host health status has also recently pointed out as other factor influencing the bioavailability of phenolic compounds (Margalef, Pons, Iglesias-Carres, Bravo, et al., 2017; Margalef, Pons, Iglesias-Carres, Quiñones, et al., 2017). Due to this low bioavailability, only a small part of the ingested phenolics is absorbed by the small intestine (5–10%) while the rest

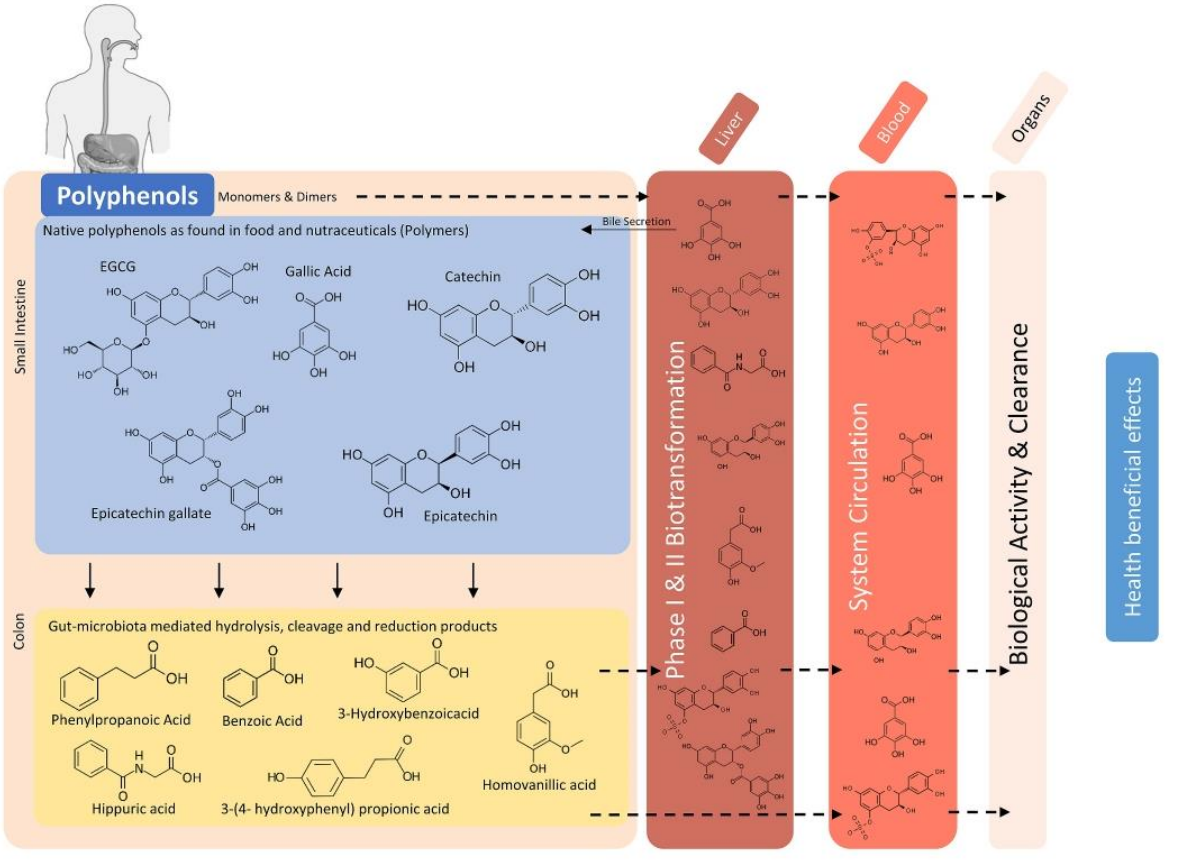

Fig. 1. Metabolism of a grape seed proanthocyanidin extract (GSPE). Tissue and systemic distribution of the main polyphenol metabolites identified by HPLC-MS/MS in Wistar rats after oral administration of GSPE (1 g/kg). Part of the native polyphenols found in the extract are absorbed at small intestine level and distributed to different tissues including the liver. Other native polyphenols reach the colon where they are metabolized by the gut microbiota, via deglycosylation, dehydroxylation and demethylation reactions, and absorbed for their distribution. In the liver, polyphenols metabolites undergo further biotransformation by glucuronidation, sulfation and/or methylation reactions. In addition, these metabolites may be transferred back into the intestine through the enterohepatic cycle. Adapted from (Guerrero et al., 2013; Margalef et al., 2015a, 2015b).

(90–95%) reach the colon. In the small intestine they are transformed by conjugation reactions including glucuronidation, sulfation and/or methylation and absorbed by the enterocytes. In the colon they are metabolized by the gut microbiota (via deglycosylation, dehydroxylation and demethylation reactions) and absorbed by the colonocytes (Ozdal et al., 2016). The absorbed phenolic compounds and their derived metabolites are transported to the liver by the portal circulation and they are further metabolized by the above-mentioned conjugation reactions. In addition, these metabolites may be transferred back into the intestine through the enterohepatic cycle (Van Duynhoven et al., 2011). Finally, the absorbed phenolic metabolites are transported through the systemic circulation to the different tissues and organs (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013). This complex absorption and metabolism process is illustrated in Fig. 1, taking as example the case of a grape seed proanthocyanidin extract (GSPE) in which our group have extensive experience. Therefore, phenolic compounds are subject to an extensive transformation leading to the production of several different metabolites, which explains the large inter-individual variability in response to their consumption.

In addition to the factors mentioned above, biological rhythms have been shown to influence the activity of bioactive compounds, including phenolic compounds (Arola-Arnal et al., 2019). In this context, the time of the day or the season of the year should be taken into account when investigating the bioactivities of these compounds. This has led to the development of a new research area called chrononutrition. Moreover, it has been proposed that animals are able to favourably adapt to changing environmental conditions through the consumption of chemical cues synthetized by plants, which is known as xenohormesis (Howitz & Sinclair, 2008). According to this hypothesis, plants experiencing mild stress activate their own cellular defences by synthetizing phytochemicals such as phenolic compounds that, when they are incorporated through the diet, will allow mammals to adjust their metabolism in anticipation of adverse conditions promoting their survival. Thus, phenolic compounds and especially polyphenols may provide physiological and metabolic signals facilitating the adaptation to the environment (Baur & Sinclair, 2008). Hence, we hypothesize that phenolic compounds may exert their beneficial effects promoting homeostasis maintenance at a basal signalling level, including interactions with the biological clock system.

2. Biological rhythms

Biological rhythms are periodic variations in the intensity of biological processes and vital functions of living organisms caused by different factors such as the rotation of the Earth around its axis (circadian cycle) and its translation around de sun (seasonal cycle). They allow for the adjustment of the organisms to environmental changes such as food availability or predator activity. Thus, circadian and seasonal rhythms enable organisms to optimize the metabolism and energy utilization to sustain life adapting their behaviour and physiology to the appropriate time of the day or of the year respectively. In this sense, physiological and behavioural systems of mammals such as sleep-wake cycle, cardiovascular activity, endocrine system, blood pressure, body temperature, renal activity, gastrointestinal tract activity and hepatic metabolism are regulated to a greater or lower extent by circadian rhythms (Claustrat, Brun, & Chazot, 2005; Levi & Schibler, 2007; Ohdo, 2003). These time-of-day-dependent rhythms are due to changes in energy expenditure and nutrient utilization that occur in cycles of 24 h (McGinnis & Young, 2016). The central clock regulatory system is located in the hypothalamic suprachiasmatic nucleus (SCN), which contains 10,000–15,000 neurons. This master clock can work autonomously, without any external input, but can be reprogrammed by external environmental cues named *zeitgebers* such as light, the most important one, which is perceived by the retina through the retinohypothalamic tract (Reppert & Weaver, 2002). Central clock provides signals, via the autonomic nervous system or circulating humoral factors including melatonin and cortisol (Dibner, Schibler, & Albrecht, 2009), to several peripheral oscillators to maintain rhythmicity and to ensure temporally coordinated physiology. These peripheral clocks are present in almost all other mammalian tissues, including liver, muscle, pancreas, and adipose tissue, where they maintain circadian rhythms and regulate tissue-specific gene expression and functionality. Peripheral oscillators are also regulated by behavioural signals such as physical activity and, most notably, fasting/feeding states (Levi & Schibler, 2007). Therefore, although circadian clock regulates multiple metabolic pathways, metabolites availability and feeding behaviour can also influence rhythms, being two systems that are reciprocally regulated. Thus, food entrains the liver clock, whereas light acts through the brain clock to control feeding time. In mammals, the circadian machinery is an intracellular mechanism sharing the same molecular components in SCN neurons and peripheral cells. The central feature of this molecular rhythmicity is the transcription–translation autoregulatory feedback loop which cycles with a periodicity of 24 h (Ohdo, 2003). The transcriptional activators of this feedback system are the circadian locomotor output cycles kaput (CLOCK) and the brain and muscle Arnt-like protein-1 (BMAL1). CLOCK and BMAL1 form a heterodimer that can activate the transcription upon binding to E-box (5′-CACGTG -3′) and E-box-like sequences found in the promoters of circadian-responsive genes. Its target factors Period (PER) and Cryptochrome (CRY) are rhythmically accumulated in the cytoplasm, oligomerize after reaching an appropriate concentration and translocate to the nucleus where interact with *Clock* and *Bmal1* inhibiting their own transcription. All the above mentioned clock elements exhibit a 24 h transcriptional oscillation in SCN cells and peripheral tissues, except for *Clock* that has been shown not to oscillate in the SCN (Dunlap, 1999; Lowrey & Takahashi, 2004; Reppert & Weaver, 2002; Young & Kay, 2001).

These clock genes are also responsible for circannual rhythms modulation. In peripheral organs, circannual rhythmicity is not self-sustained and requires maintenance by photoperiod signals. These signals are recognised in the retina and transmitted to the pineal gland by multi-synaptic neuronal pathways. The pineal gland integrates these photoperiod signals and regulates the production of melatonin, which is rhythmically produced at night (Korf, 2018). Thus, melatonin signals encode night length. Importantly, melatonin can regulate many physiological factors in humans, and those include immunity, metabolism and body temperature. Thus, melatonin controls the rhythmicity and seasonality of body physiology (Claustrat et al., 2005).

Biological rhythms may impact on the physiology and metabolism of the organism by through different mechanisms including: 1) modulation of the expression of enzymes involved in the rate-limiting steps of metabolic pathways, 2) interaction of the clock machinery with proteins such as nuclear receptors and nutrient sensors, and 3) regulation of metabolites levels (Sahar & Sassone-Corsi, 2012). However, this rhythmicity can be desynchronized from the central circadian clock by different factors, including environmental conditions (light-dark cycles), lifestyle and behavioural choices (work schedules, eating patterns, and social jet lag) or the presence of diseases. A large body of studies has extensively shown that this chronic circadian misalignment leads to serious health consequences, increasing the risk of developing obesity, metabolic and cardiovascular diseases (CVD), cancer or diabetes (Cannizzaro et al., 2020; Guerrero-Vargas, Espitia-Bautista, Buijs, & Escobar, 2018; Johnston, Ordovás, Scheer, & Turek, 2016; Kecklund & Axelsson, 2016). In this sense, many of them have been focused on evaluating the effect of circadian disruption on shift workers, which frequently experience circadian misalignment due to alterations of sleep-wake and fast-feeding timings.

Especially remarkable, the changes in dietary habits observed in last decades have also a high influence on the onset of these disruptions, as hypercaloric diets rich in free sugars and high-fat content are related with the development of metabolic diseases and related comorbidities. Taking into consideration all this evidence that link disruption of biological rhythms and health diseases, in their recent review Zimmet et al.

(2019) introduced the term "circadian syndrome", defining it as the underlying aetiological factor of metabolic syndrome. These authors describe this circadian syndrome as the joint of the metabolic syndrome cluster and their comorbidities including sleep apnea, depression and non-alcoholic fatty liver disease (Zimmet et al., 2019).

3. Phenolic compounds and biological rhythms modulation

The bioactivity of compounds is usually determined without taking into account the time of their administration. However, as discussed above, it seems evident that biological rhythms must be considered as many physiological and metabolic processes present time-dependent oscillations. Therefore, the time of the day or the season of the year in which dietary compounds are consumed must be a main determinant of the effect of diet on health (Johnston et al., 2016). This concept led to the development of the chrononutrition field, but unfortunately, there are not many studies evaluating the effects of the timing of the administration of bioactive components on their bioavailability and efficacy. Those that have been carried out so far are mainly focused on the study of foods or nutraceuticals rich in melatonin, an important component of the internal clock, or its precursors tryptophan and/or serotonin. For example, in a study using tryptophan enriched infant milk formulas to improve the development of the wake-sleep rhythms, the infants receiving the enriched formula during dark time showed improvements in the sleep parameters studied, in comparison with those receiving the enriched formula during light time or receiving standard infant commercial milk. The urinary metabolites of serotonin suggest that the observed improvements were due to an increased use of serotonin to melatonin synthesis (Aparicio et al., 2007). In other studies, the consumption twice a day as the lunch and dinner desserts of either a diet enriched with Jerte Valley cherries and a Jerte Valley cherry-based nutraceutical product, both rich in melatonin, serotonin, and tryptophan (González-Gómez et al., 2009), improved the antioxidant status of young, middle-aged, and elderly individuals (Garrido et al., 2010; Garrido, González-Gómez et al., 2013). The timing of the meal was critical for achieving the beneficial effects of these dietary interventions since serotonin and melatonin have opposite circadian rhythms. Thus, consumption of cherries at lunchtime allowed for an increase in the diurnal circulating serotonin concentration which indirectly also led to an increase in the nocturnal circulating melatonin concentration by enhancing the amount of serotonin available to be converted into melatonin at night (Garrido, Terrón, & Rodríguez, 2013). Moreover, this is a bidirectional interaction, and not only the biological rhythms may affect dietary components metabolism and functionality but also the other way around. Indeed, in the case of phenolic compounds, it has been shown that they are able to impact on the clock system, leading to changes in gene expression.

3.1. Biological rhythms impact on phenolic compounds activity

As discussed before, biological rhythms may significantly impact on dietary compounds bioavailability and activities. In the case of phenolic composition it has been shown that its bioavailability follows endogenous circadian rhythms inducing changes in its antioxidant properties (Soengas, Cartea, Velasco, & Francisco, 2018). Moreover, their effectivity has been suggested to be closely related to the circadian rhythm of many physiological processes. Regarding glucose homeostasis it is known that glucose metabolism is tightly regulated in a diurnal rhythm based on the external light-dark cycle (Challet, 2013; Johnston, 2014) and internal feeding-fasting cycles (Al-Naimi, Hampton, Richard, Tzung, & Morgan, 2004; Kalsbeek, La Fleur, & Fliers, 2014). Thus, the misalignment of this feed-fasted state could be related to the predisposition to suffer from certain pathologies such as T2D or CVD, which have been observed especially in shift-working people (Al-Naimi et al., 2004; Pan, Schernhammer, Sun, & Hu, 2011). Several studies have reported the diurnal and/or nocturnal effect of phenolic compounds on specific physiological contexts. In this regard, our group has reported the ability of GSPE on the modulation of the circadian rhythm of liver clock-core and clock-controlled genes, which were dependent on the administration time (ZT0, at the beginning of the light phase *vs.* ZT12, at the beginning of the dark phase) (Ribas-Latre et al., 2015). In particular, oral administration of GSPE to male Wistar rats led to increased ratio of hepatic acetylated BMAL1 only at ZT12. In addition, this research pointed out that nicotinamide phosphoribosyl transferase (NAMPT), the rate limiting enzyme in nicotinamide adenine dinucleotide -NAD-biosynthesis) and NAD were important molecular targets of GSPE in the liver. In this regard, GSPE was able to modulate the levels of NAMPT and NAD in opposite ways depending on its administration time (reducing their levels in the diurnal treatment and increasing their levels in the nocturnal treatment). In consequence, this polyphenolic extract could act as a hepatic adaptation factor through the improvement of the energetic profile as well as through the increase of mitochondrial functional and oxidation at night, when rats are active. Finally, it was concluded that BMAL1 acetylation pattern, which was dependent on the GSPE administration time, could explain the *Nampt* overexpression and the following NAD peak in the liver, only when the polyphenol extract was administered at night. Moreover, in another study, GSPE showed different effects depending on the administration time in male Wistar rats. In this study, when administrated at ZT0 (light on), GSPE was able to delay the diurnal decrease in melatonin levels and altered the oscillations of some metabolites in plasma as well as *Bmal1* and *Nampt* expression pattern in the hypothalamus. However, when administrated at ZT12 (light off) no effects were observed (Ribas-Latre, Del Bas, Baselga-Escudero, Casanova, Arola-Arnal, Salvadó, Arola, et al., 2015). In addition, acute resveratrol (RSV) administration in male Wistar rats synchronized with a 12-h dark-light cycle, showed opposite effects on lipoperoxidation tissue level depending of the time of the day, being pro-oxidant when administered during light span and antioxidant when administered during the night-time (Gadacha et al., 2009). More concisely, this research evaluated the dosing-time dependency of acute RSV administration on lipoperoxidation levels in several male rat tissues (heart, liver, and kidney). A powerful antioxidant effect in these tissues was found when RSV was administered during the active phase (at dark). However, when administered during the rest phase (at light), RSV exerted pro-oxidant effects in the three organs, but in absence of any harmful effect at the tested dose. Authors indicated that this opposite effect of RSV depending on the administration time could be due to circadian changes in basal level of superoxide dismutase (SOD) isoforms and after resveratrol treatment in several organs. On the other hand, it has been demonstrated that a single-dose of a polyphenol-rich extract from the algae *Fucus vesiculosus* is able to slightly reduce the elevated postprandial blood glucose responses in the evening in healthy women (Murray, Dordevic, Ryan, & Bonham, 2019). Another study in young men evaluated the effects of timing of an acute catechin-rich green tea ingestion on postprandial glucose metabolism (Takahashi et al., 2019). The main finding of this study was that the acute ingestion of catechin-rich green tea reduced postprandial plasma glucose concentrations, in the evening but not in the morning.

In addition to circadian rhythms, circannual rhythms may also influence phenolics bioavailability and functionality. In this context, our group has recently shown that exposure of Fischer 344 rats to distinct photoperiods influences the bioavailability of red grape polyphenols (Iglesias-Carres et al., 2019). Moreover, hypothalamic leptin sensitivity was influenced by polyphenol-rich seasonal fruits (cherry and grape), in a photoperiod-dependent manner in Fischer 344 rats (Ibars et al., 2018). Thus, a significant increased hypothalamic gene expression of leptin receptor isoform B (*Obrb*) was observed only when cherries were consumed in short-day photoperiod. In addition, hypothalamic pro-opiomelanocortin (*Pomc*) expression, which is under leptin regulation, was significantly increased by grapes and cherries intake only when consumed in the SD photoperiod, indicating a clear photoperiod effect. Furthermore, consumption of polyphenol-rich fruit out of season

led to metabolism disruption, including lipid and glucose homeostasis, in both standard and cafeteria diet-fed rats (Mariné-Casadó et al., 2019) and causes changes in the white adipose tissue gene expression and in its morphology to a phenotype prone to fat accumulation (Gibert-Ramos, Crescenti, & Salvadó, 2018). Thus, cherry consumption for example, which is a fruit characteristic of long photoperiod, led to increased glycemia and insulinemia when consumed in short photoperiod by obese rats, and led to enhanced whole-body lipid utilization, resembling the substrate oxidation pattern observed in the animals exposed to 18 h of light. Taken all together, the most optimum phenolics profile in plant products should be in accordance with their seasonality and may also vary depending on the time of the day. Hence, their consumption in the wrong time may contribute to misleading signals promoting the development of metabolic diseases such as metabolic syndrome and obesity (Arola-Arnal et al., 2019). Further studies are needed to elucidate the mechanisms involved and the connection between specific phenolic compounds from seasonal foods and metabolism according to the period of the year.

On the other hand, gut microbiota has been postulated as one of the main elements in maintaining biological rhythms. Indeed, germ-free mice exhibited impaired circadian clock gene expression, despite the existence of light and dark signals (Leone et al., 2015). Moreover, diurnal and seasonal oscillations in gut microbes have been described (Carey, Walters, & Knight, 2013; R. M. Voigt, Summa, et al., 2016). In addition, mutations of the circadian protein CLOCK promotes intestinal dysbiosis (Robin M. Voigt, Summa, et al., 2016). Hence, the interaction between phenolic compounds and biological rhythms may be at least partially mediated by the gut microbiota. Thus, intestinal microbiota oscillations due to biological rhythms may affect to phenolic compounds metabolism and transformation in the colon and, therefore, leads to the production of different derived metabolites with different functionalities. Indeed, there have been studies that have shown evidence of this interaction between polyphenols-gut microbiota and circadian rhythms (Guo, Ho, Zhang, Cao, Wang, & Shao et al., 2019; Guo, Song, Ho, Zhang, Zhang, & Cao, et al., 2019; Man, Xia, Daiber, & Li, 2020).

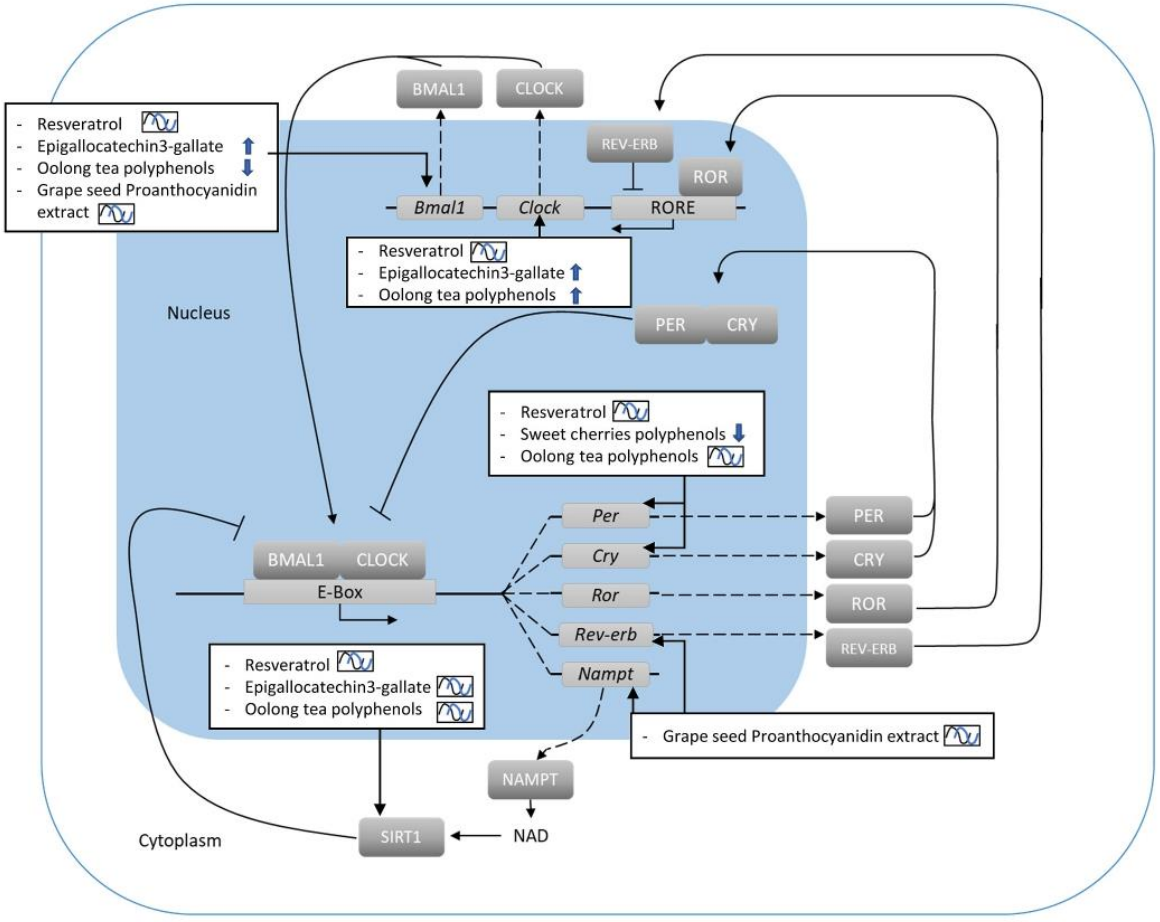

Fig. 2. Phenolic modulation of circadian intracellular machinery. This mechanism, which shares the same components in suprachiasmatic nucleus (SCN) neurons and peripheral cells, is characterized by the transcription-translation autoregulatory feedback loop in 24 h. The circadian locomotor output cycles kaput (CLOCK) and the brain and muscle Arnt-like protein-1 (BMAL1) are the major components. CLOCK-BMAL1 heterodimer can active the transcription of circadian-responsive genes upon binding to E-box sequences found in the promoters. Its target factors Period (PER) and Cryptochrome (CRY) are rhythmically accumulated in the cytoplasm, where oligomerize once reached an appropriate concentration and migrate into the nucleus interacting with Clock and Bmal1 inhibiting their own transcription. Multitude phenolic compounds or products have shown to regulate the pattern of the circadian rhythmicity acting in specific points of the intracellular machinery.

3.2. Phenolic compounds effects on biological rhythms

As mentioned above, dietary compounds may influence biological rhythms by modulating clock genes expression. In fact, nutritional challenges have been shown to affect the clock system (Eckel-Mahan et al., 2013). In this regard, in addition to macronutrients, the capability of several bioactive compounds to act in the modulation of clock genes is currently under study by the scientific community.

Indeed, phenolic compounds can function as *zeitgebers* for this molecular clock machinery regulating these genes expression (Fig. 2). This was first evidenced in 2008 (Oike & Kobori, 2008) by an *in vitro* study with RSV, which was able to regulate circadian clock genes in Rat-1 fibroblast cells. These findings support the potential interaction between phenolics functionality and circadian rhythms, which is of increasing research interest. In this sense, and regarding *in vitro* studies, several approaches have been done to disrupt circadian oscillations of clock genes by applying oxidative stress (H_2O_2, acrylamide) or metabolic (free fatty acids) stimuli on human hepatoma carcinoma cells (HepG2) as well as on primary hepatocytes. In such situations, the subsequent application of several types of phenolic compounds (e.g. RSV, Cichoric acid (CA), Green tea polyphenols) satisfactorily readdressed the misled circadian rhythm (R. Guo et al., 2018; Qi et al., 2018; Tan et al., 2019). Interestingly, these effects were linked to others beneficial effects of phenolic compounds including antioxidant, anti-inflammatory or anti-steatosis effects, among others. Additionally, the role of the central master clock in mediating the effects of phenolics has been investigated by knocking-down the regulator genes *Bmal1* and *Cry* using small interfering RNA (siRNA). This silencing, partially abrogated the beneficial effects of phenolic compounds, strengthening in consequence the pivotal role of the clock machinery in mediating these effects (R. Guo et al., 2018; Qi et al., 2018; Tan et al., 2019). For instance, CA regulated the circadian rhythm expressions of clock genes and, *Bmal1* silencing inhibited the effects of CA on lipid drop accumulation by downregulation of the p-Akt/Akt pathway and elevation of fatty acid synthase and acetyl coenzyme A carboxylase protein and mRNA levels and in HepG2 cells (R. Guo et al., 2018). A similar approach was used for studying the beneficial role of RSV on acrylamide oxidative damage. This study showed that RSV-mediated activation of Nrf2/NQO-1, which are essential antioxidant cell modulators, disappeared after knocking down *Bmal1*, indicating that RSV is able to restore the cellular redox status in a *Bmal1*-dependent manner (R. Guo et al., 2018). In consequence, these studies have contributed to a better understanding of the interaction between phenolic compounds and circadian clock and its influence on oxidative stress, inflammation response as well as metabolic disorders. Furthermore, other authors have shown that some biological processes that follow circadian oscillations, such as triacylglycerol secretion (Del Bas et al., 2008) and microRNA expression (Arola-Arnal & Bladé, 2011), are strongly modulated by phenolic products such as GSPE (Arola-Arnal & Bladé, 2011; Del Bas et al., 2008), cocoa proanthocyanidin extract (CPE) and pure epigallocatechin gallate isolated from green tea (EGCG) (Arola-Arnal & Bladé, 2011). Interestingly, GSPE is also able to mimic melatonin action by upregulating BMAL1 expression, and consequently it is able to modulate the circadian rhythm of clock genes in HepG2 cells (Ribas-Latre, Del Bas, Baselga-Escudero, Casanova, Arola-Arnal, Salvadó, Bladé, et al., 2015). Hence, although GSPE did not affect the percentage of rhythmicity of any studied clock gene (*Bmal1*, *Clock*, *Rorα*, *REV-Erbα*, *Per2* and *Cry1*), it increased the amplitude of REV-ERBα. On the other hand, GSPE significantly shifted the acrophase of both the core clock and clock-controlled genes. Additionally, the altered gene expression of *Bmal1* and *Rorα* clearly suggested both genes as targets of GSPE. Finally, it was also shown that the molecular mechanisms by which melatonin and GSPE induced the overexpression of *Bmal1* were different, being dependent on the melatonin receptor 1 (MT1) in the case of melatonin but independent from this receptor in the case of GSPE. In this regard, by using an elegant approach, Shinozaji et al. (Shinozaki et al., 2017) showed a remarkable effect of several flavonoids on circadian rhythms. For this purpose, they tested the modifications in the circadian clock amplitude, period, and phase induced by the addition of polyphenols (transiently or continuously) in embryonic fibroblasts from PER2:LUCIFERASE (PER2:LUC) mice. They discovered that the transient treatment with some flavonoids induced a phase delay of the PER2:LUC rhythm at the down slope phase. On the other hand, the continuous application of nobiletin and tangeretin, which are polymethoxy flavonoids, increased the amplitude and lengthened the period of the PER2:LUC rhythm. Interestingly, this nobiletin-induced phase delay was dependent on ERK activity, because the co-treatment with the ERK inhibitor U0126 abrogated such nobiletin effect.

The influence of phenolic compounds on the circadian rhythm modulation has also been addressed *in vivo*. For example, it has been shown that there is a significant coordination between circadian clock machinery and hepatic metabolism (Zhou et al., 2014). In particular, it was shown that CLOCK/BMAL1 regulates circadian change of mouse hepatic insulin sensitivity by SIRT1. Importantly, RSV abolished the negative effect of constant darkness on SIRT-1 activity, as well as on glucose and insulin intolerance (Zhou et al., 2014). This suggests that RSV could be an interesting option for combating metabolic disorders under circadian misalignment conditions thorough modulating the circadian regulation of SIRT1. Additionally, it has also been shown in the adipose tissue that RSV reverses the alteration induced by high-fat diet (HFD) feeding in the expression of *Rev-Erbα* in male Wistar rats (Miranda et al., 2013). Furthermore, another study showed that RSV was able to restore the circadian rhythmic disorder of lipid metabolism induced by HFD in male C57BL/6 mice. Specifically, RSV modulated the expression rhythm of clock genes (*Clock*, *Bmal1* and *Per2*) and clock-controlled lipid metabolism related genes (*Sirt1*, *Pparγ*, *Srebp-1c*, *Acc1* and *Fas*). In consequence, RSV was able to restore the circadian misalignment due to HFD (Sun et al., 2015). Similar studies have been done with other phenolic compounds like EGCG (Mi et al., 2017). Other studies have addressed the effect of GSPE on molecular clock machinery in a healthy *vs.* obesity scenario. In this regard, the oral administration of GSPE for 21 days to healthy male Wistar rats induced the overexpression of core clock genes in a dose-dependent manner. Additionally, the acetylated level of BMAL1, which is a SIRT1 target, also increased in a dose dependent manner in the liver and mesenteric white adipose tissue (WAT) of these animals (Ribas-Latre et al., 2015). In the same study, the administration of GSPE to cafeteria diet-fed obese rats was able to counteract the perturbations induced by obesity in the clock genes mainly in the liver and gut, but surprisingly the polyphenol rich extract was less effective in normalizing the clock gene disruption in WAT. This reinforces the concept that the clock machinery is also an important target for phenolic compounds in its already known protective effect against obesity (Ribas-Latre et al., 2015).

4. Conclusions

Biological rhythms must be considered when studying phenolic compounds as they have been demonstrated to significantly impact on their bioavailability and functionality. Moreover, despite to the complex absorption and metabolism process to which dietary phenolic compounds are subjected, leading to the production of thousands of different metabolites, they have been shown to exert several beneficial effects. This broad range of activity may be due to their capability to promote homeostasis at a basal signalling level through interactions with the biological clock system. Indeed, a multitude of studies has shown that circadian and circannual rhythms may affect their absorption and metabolism, impacting their bioactivity and functionality. Moreover, dietary phenolic compounds have been proven to exert many beneficial metabolic and physiological effects in different experimental studies by modulating genes related to the clock machinery and associated signalling pathways. The studies discussed here support the xenohormesis hypothesis, which postulates that stress signalling molecules produced

by plants, such as phenolic compounds, allows mammals to better cope with the imbalances produced throughout their life. Further studies are needed in order to elucidate the specific mechanisms involved in this interesting two-way phenolic compounds-biological rhythms interaction.

CRediT authorship contribution statement

JAR, JSR, FIB, GA, MS, AA, MM, MJS, LA, CTF and BM: writing. JAR, CTF and BM: reviewing & editing; JSR: artwork.

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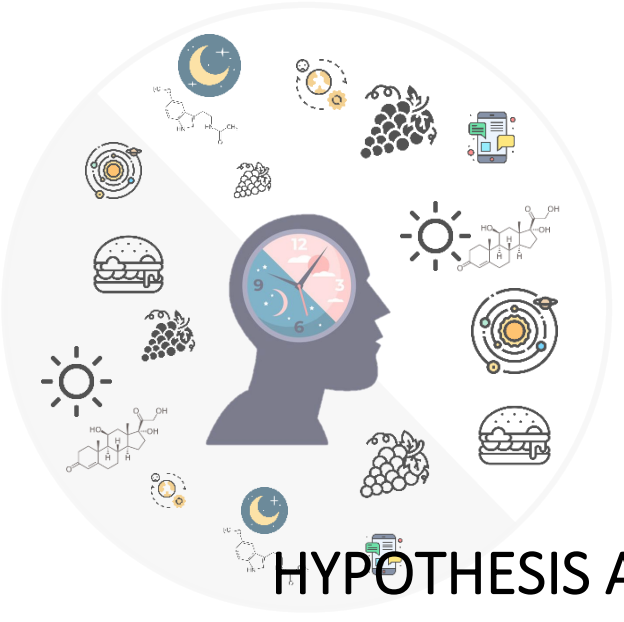

HYPOTHESIS AND OBJECTIVE

UNIVERSITAT ROVIRA I VIRGILI
INTERACTION BETWEEN CIRCADIAN RHYTHM AND GRAPE SEED PROANTHOCYANIDINS IN HEALTHY AND OBESE RATS: POTENTIAL ROLE AS A CENTRAL AND PERIPHERICAL CLOCK SYNCHRONIZER
Jorge Ricardo Soliz Rueda

Hypothesis and objectives

Biological rhythms play an important role in the physiological and metabolic adaptation of the organism to external environmental changes such as the rotation of the Earth around its axis or its translation around the Sun. This adaptation to the time of day (circadian rhythm) or year (seasonal rhythm) allows the optimization of the metabolism and energy expenditure. However, in the last century, remarkable lifestyle changes are occurring in modern occidental societies. These modifications include variations in dietary patterns, predominating the consumption of more calorie-dense food, and alterations of the natural light/dark phases caused by the common phenomenon known as “social jetlag”, which produces discrepancy between work and free days, social and biological time or changes in the frequency and timing of meals, and by a wide use of new technologies in the dark part of the day. All these alterations cause a misalignment between external cues and internal clock and can lead to the development of some typical comorbidities of metabolic syndrome such as obesity, cardiovascular diseases, type 2 diabetes, among others. All these metabolic disturbances in addition to other conditions such as fatigue, sleep disturbance, depression or decreased cognitive ability, resulting also of biological rhythm alterations, make up what is known as the "Circadian Syndrome".

In addition, phenolic compounds are important food chemicals present in fruits and vegetables, which exert beneficial effects on disorders related to metabolic syndrome. Proanthocyanidins (PACs) are a class of polyphenols constituted by polymers of flavanols and its gallate derivatives and our group has recently demonstrated that their bioavailability and bioactivity depend on the timing and season in which they are consumed. Nevertheless, the association between phenolic compounds and biological rhythms seems to be bidirectional since some of them have demonstrated their activity as modulators of circadian and seasonal rhythms. In this regard, our group have demonstrated that PACs can

modulate central and peripheral biological rhythms, both in healthy animals under jet-lag conditions and in cafeteria (CAF)-diet obese rats, and the interaction of phenolic compounds with the clock system has been recently pointed out as a potential mechanism involved in their beneficial effects [**Manuscript 1**].

Therefore, we hypothesized that beneficial effects of PACs can be mediated by their interaction with the clock system, acting as synchronizers or *zeitgebers* and reprograming the biological rhythms in a disturbance state.

Then, the aim of this thesis was to elucidate whether PACs can synchronize biological rhythms and investigate if these phenolic compounds can restore biological rhythms in different situations of disruption such as changes in light-dark cycles or in feeding patterns. In addition, the time-of-day-dependent effects of PACs were investigated. As a strategy to address this general objective, it is proposed to use a grape seed proanthocyanidin-rich extract (GSPE) since the beneficial effects of this extract on different key aspects of metabolic syndrome, including their targets and mechanisms of action, have been extensively investigated by our group.

To achieve the general objective, the following specific objectives have been proposed:

- **To evaluate the PACs modulation of circadian rhythm and metabolism in photoperiod-sensitive Fischer 344 rats under CAF diet-induced disruption**. (*Chapter 1*).

  The photoperiod-sensitive Fischer 344 rats are an appropriate animal model with which to study biological rhythms. However, the circadian metabolism oscillations of these animals are not broadly studied. Moreover, since calorie-dense food, in addition to alter the metabolism and cause metabolic diseases, disrupt circadian oscillations, it is proposed the study of biological rhythms of F344 rats fed CAF diet. The use of this diet, high in fat and sugar, is considered of relevance since

rats fed CAF diet is considered a robust model of human metabolic syndrome. However, the effects of CAF-diet in biological rhythms have been less studied than other obesogenic diets such as high fat-diet, and more in CAF diet-fed photoperiod-sensitive Fischer 344 rats.

Therefore, it was necessary a proper study using healthy, and CAF diet fed Fischer 344 rats to completely elucidate their circadian rhythms in healthy and metabolic disease conditions. In order to assess this objective two goals were proposed:

- To evaluate the circadian rhythms in healthy Fischer 344 rats.

  In order to better study the biological rhythms of these animals and considering that circadian rhythmicity can also be modified by changes in food intake pattern, F344 rats were fed a standard chow diet [**Manuscript 2**].
- To elucidate the circadian rhythms in CAF diet induced obese F344 rats and to investigate the PACs effects on their circadian metabolism rhythms.

  F344 rats were fed CAF diet and supplemented daily with vehicle or GSPE, which were administered before their active or resting phases to also investigate the effects of PACs supplementation time **[Manuscript 3].**

- **To investigate the effect of PACs on circadian rhythms in a jetlag condition in healthy and CAF-induce obese F344 rats** (*Chapter 2*).

  Current lifestyles, artificial light, and shift work, among others, lead to disruptions in the normal metabolic function due to alterations in the light-dark cycle. In addition, the interaction between metabolic diseases and circadian rhythms is bidirectional so that also circadian misalignment has been identified as a risk factor for developing metabolic disorders. Moreover, the potential of these phenolic compounds to prevent the alteration caused by an abrupt change of

photoperiod was addressed. In order to assess this objective two goals were proposed:

- To investigate the effects of PACs on circadian modulators and metabolism in healthy and CAF-induced obese F344 rats subjected to a sudden change of light/dark cycle **[Manuscript 4]** and **[Manuscript 5].**
- To elucidate the effect of PACs on circadian physiological rhythms and cardiovascular risk factors in CAF-induced obese F344 rats subjected to a sudden change of light/dark cycle **[Manuscript 6].**

The research performed in this thesis has been supported by the Spanish Ministry of Science and Innovation (grant number: AGL2016-77105-R). This thesis has been carried out at the Nutrigenomics Research Group of the Universitat Rovira i Virgili with a grant for the recruitment of predoctoral research staff for the training of PhDs (FPI) (BES-2017-080919) awarded by the Spanish Ministry of Science and Innovation and FSE "El FSE invierte en tu futuro". An international phase was carried out at the Department of Anatomy and Neuroscience from APC microbiome Ireland and the University College Cork (Cork, Ireland) under the supervision of Dr Harriët Schellekens from 9th April to 29th September 2021.

EXPERIMENTAL DESIGNS

UNIVERSITAT ROVIRA I VIRGILI
INTERACTION BETWEEN CIRCADIAN RHYTHM AND GRAPE SEED PROANTHOCYANIDINS IN HEALTHY AND OBESE RATS: POTENTIAL ROLE AS A CENTRAL AND PERIPHERICAL CLOCK SYNCHRONIZER
Jorge Ricardo Soliz Rueda

Experimental designs

Different experimental designs were used to assess the main hypothesis and reach the experimental objectives previously described in this thesis.

1. **To evaluate the PACs modulation of circadian rhythm and metabolism in photoperiod-sensitive Fischer 344 rats under CAF diet-induced disruption .**

In order to better study the biological rhythms of these animals and considering that circadian rhythmicity can also be modified by changes in food intake pattern, F344 rats were fed a standard chow diet. For this purpose, thirty-two 12-week-old male Fischer rats were fed a standard chow diet and supplemented daily with a low dose of sugar (158 mg/Kg, equivalent to 2 g in humans) at 8 a.m. and 8 p.m. (ZT0 and ZT12, respectively) for 4 weeks. At the end of the experimental period, animals were sacrificed at ZT1, ZT7, ZT13 and ZT19. Body weight gain and other cardiometabolic risk factors changed depending on the time in which the sugar was administered (Figure 1).

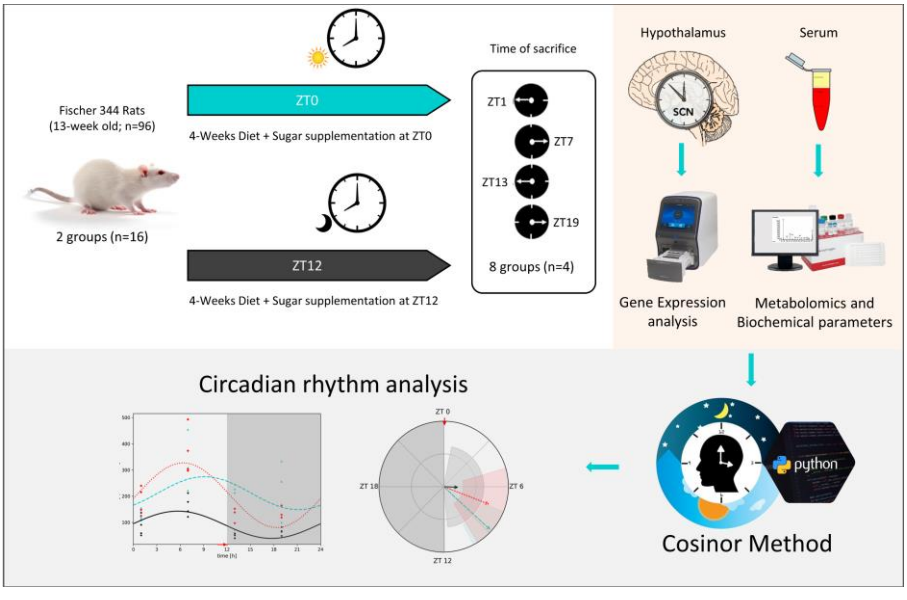

Figure 1.Experimental design to fine-tune the analysis of 4 time points in 24 hours in order to study the circadian rhythms of central clock and metabolic profiles. *Rats were supplemented sugar at ZT0 and ZT12 and sacrificed in 4 different time points in order to analyze the oscillation of various parameters and evaluate the metabolic profile and circadian rhythm. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg GSPE.*

Subsequently, in order to investigate the effects that GSPE on the modulation of the altered circadian rhythm by diet composition, we evaluated the effects of the cafeteria diet on circadian rhythm and how GSPE can reverse the disturbance on the central clock and cardiovascular factors caused by the CAF diet. For that, sixty-four 12-week-old male Fischer rats were housed under standard laboratory conditions (temperature 22 ºC, 12 h light/dark cycle) divided into 6 groups: 2 groups were fed a standard chow diet and 4 groups were fed a CAF for 5 weeks. Then, a daily oral administration of GSPE (25mg/kg body weight) or vehicle were administered at 8 a.m. and 8 p.m. (ZT0 and ZT12, respectively) to CAF groups for 4 weeks. Animals were sacrificed at ZT1, ZT7, ZT13 and ZT19 in order to analyze the circadian rhythm. Tissues and serum samples were collected and frozen until further analysis (Figure 2).

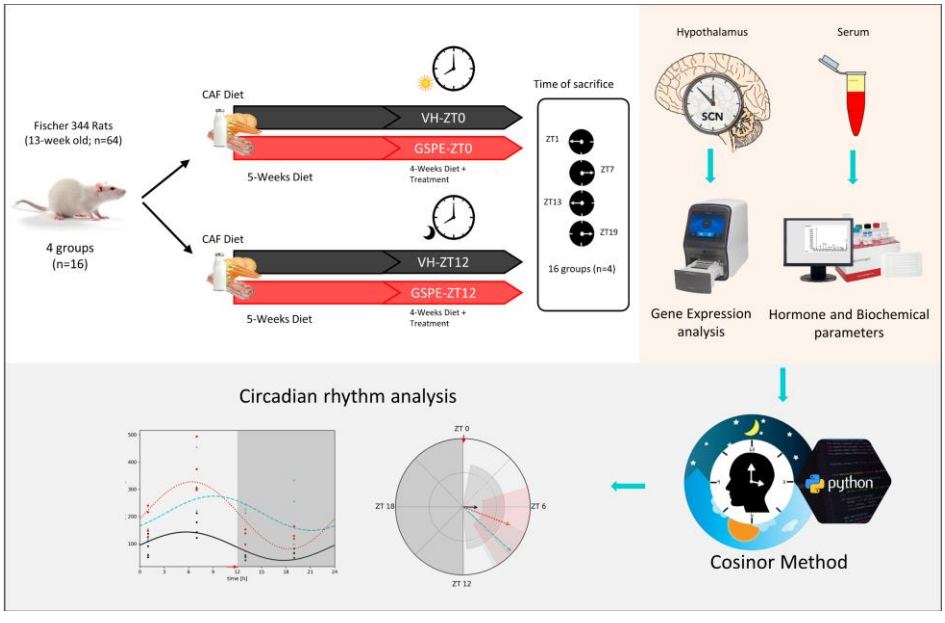

Figure 2. Experimental design to evaluate GSPE effects on Circadian Rhythms. *CAF diet-induced obese rats were treated with GSPE or vehicle at ZT0 and ZT12 and sacrificed in 4 different time points in order to analyze the oscillation of various parameters and evaluate the metabolic profile and circadian rhythm. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg GSPE.*

2. To investigate the effect of PACs on circadian rhythms in a jetlag condition in healthy and CAF-induce obese F344 rats .

To investigate the effects of PACs on circadian modulators and metabolism in healthy and CAF-induced obese F344 rats subjected to a sudden change of light/dark cycle. Thus, the aim of this study was to evaluate the effect of GSPE in healthy and obese rats when were abruptly switched from a standard (12 h light/day, L12) to a long (18 h light/day, L18) or short (6 h light/day, L6) photoperiod. Forty-eight rats were fed standard or CAF diet for 6 weeks under L12 conditions. After this time, the animals were switched to L18 or L6 and administered vehicle or GSPE (25mg/kg) for 1 week. Saphenous blood was drawn at ZT3 and ZT15 for serum hormone analysis. In addition, indirect calorimetry was performed one week before the photoperiod change and one day before the end of the experiment (Figure 3). After the week of change, the animals were sacrificed, and the tissues and final serum were analyzed to evaluate the GSPE effect on biological rhythms (Figure 4).

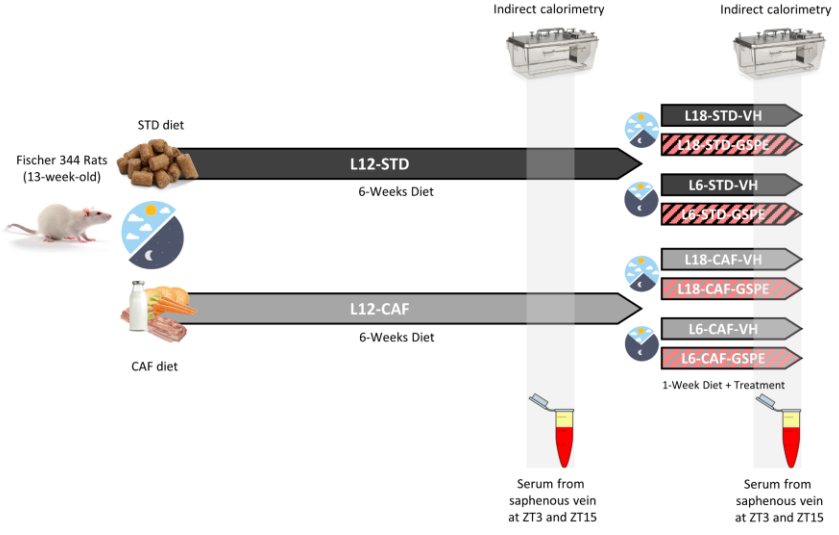

Figure 3. Experimental design to evaluate the effect of GSPE after an abrupt disturbance of photoperiod. *STD-fed and CAF-fed rats were switched to a new light-dark cycle to disrupt their central clock and evaluate the effects of GSPE on circadian rhythm. STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg GSPE; L12, normal photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

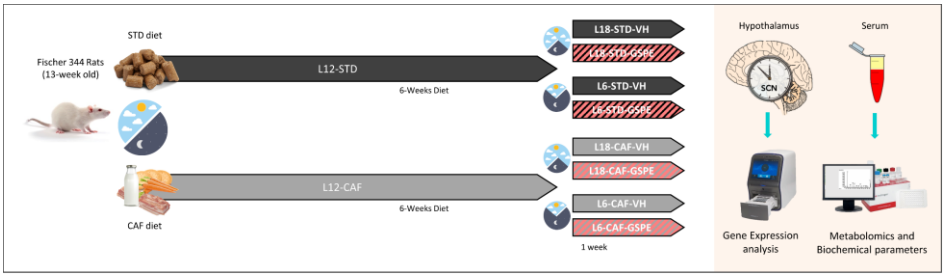

Figure 4. Experimental design to evaluate the effect of GSPE after an abrupt disturbance of photoperiod. *STD-fed and CAF-fed rats were switched to a new light-dark cycle to disrupt their central clock and evaluate the effects of GSPE on SCN and metabolic profile. STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg GSPE; L12, normal photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

To elucidate the effect of PACs on circadian physiological rhythms and cardiovascular risk factors in CAF-induced obese F344 rats subjected to a sudden. Activity, body temperature and blood pressure values were recorded by radiotelemetry in CAF-fed rats for 6 weeks under L12 photoperiod. After this time, animals were transferred to L18 or L6 photoperiod and administered vehicle (VH) or GSPE (25mg/Kg) for 1 week (Figure 5).

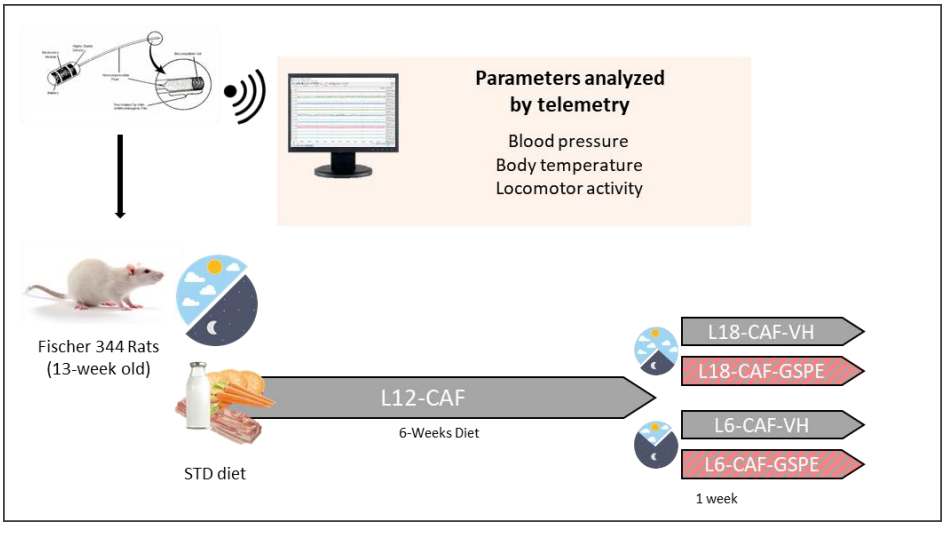

Figure 5. Experimental design to evaluate the effect of GSPE after an abrupt disturbance of photoperiod. *CAF, Cafeteria diet fed rats; VH, CAF-diet rats administered vehicle; CAF-GSPE, CAF-diet rats administered 25 mg/kg GSPE; L12, normal photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

RESULTS

UNIVERSITAT ROVIRA I VIRGILI
INTERACTION BETWEEN CIRCADIAN RHYTHM AND GRAPE SEED PROANTHOCYANIDINS IN HEALTHY AND OBESE RATS: POTENTIAL ROLE AS A CENTRAL AND PERIPHERICAL CLOCK SYNCHRONIZER
Jorge Ricardo Soliz Rueda

Chapter 1

To evaluate the PACs modulation of circadian rhythm and metabolism in photoperiod-sensitive Fischer 344 rats under CAF diet-induced disruption

Manuscript 2

Objective: to evaluate the circadian rhythms in healthy Fischer 344 rats.

Administration time of a low dose of sugar causes changes in the central clock and alters the metabolism in healthy rats

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Keywords: biological rhythms, clock genes, cosinor, chrononutrition, zeitgebers.

Abbreviations: SCN: Hypothalamic suprachiasmatic nucleus; RHT: Retinohypothalamic tract; STD: Standard chow diet; BW: Body weight; SCM: sweetened condensed milk; EI: electronic impact; T3: Triiodothyronine; T4: Thyroxine; NEFAs: non-esterified free fatty acids; SEM: standard error of mean; AUC: under the curve; sPLS-DA: the sparse Partial Least Squares Discriminant Analysis; PCA: principal component analysis; SIRT: several NAD-dependent deacetylases.

ABSTRACT

Circadian rhythm is an endogenously generated natural cycle that acts as a pacemaker of different mechanisms in the organism, and whose alteration increases the metabolic risk. The circadian rhythm is mainly reset by light, but it is also regulated by behavioral cues such as the timing of food intake. The aim of this study was to investigate whether the timing of daily chronic administration of a low dose of sugar is determinant in causing changes in the central clock and modifying the circadian rhythm of metabolism in healthy rats. For this purpose, thirty-two 12-week-old male Fischer rats were fed a standard diet and supplemented daily with a low dose of sugar (158 mg/Kg, equivalent to 2 g in humans) at 8 a.m. and 8 p.m. (ZT0 and ZT12, respectively) for 4 weeks. At the end of the experimental period, animals were sacrificed at ZT1, ZT7, ZT13 and ZT19. Body weight gain and other cardiometabolic risk factors changed depending on the time in which the sugar was administered. Increased body weight gain and a higher cardiometabolic risks were observed when sugar was administered at the beginning of the light phase (rats resting period). In addition, clock and food intake signaling genes changed depending on the time of administration. The hypothalamic genes expression of *Nampt, Bmal1, Rev-erbα* and *Cart* showed the most prominent change in their circadian expression pattern. These results show that the metabolic effects of a low dose of sugar are strongly depending on the administration time.

Significance Statement

It has recently been reported that the alteration of the circadian rhythm can promote metabolic disorders and lead to comorbidities typical of the metabolic syndrome such as obesity, cardiovascular problems, or type 2 diabetes. In this context, changes in dietary patterns result of the modern lifestyle (shift work, artificial light, etc.) play a crucial role in the dysregulation of circadian rhythms. Not only the food composition but also the time at which we intake it can disturb or modulate the circadian rhythm. In the present study, we report how the time of intaking a minimal amount of sugar can change the metabolic profile

and the central clock in a time-of -day-dependent manner in healthy rats, having an impact on body weight gain.

Author contributions: L.A., F.I.B., and B.M. designed research; J.R. S-R. and R. L-F-S. performed research; J.R. S-R. and R. L-F-S. analyzed data; J.R. S-R. wrote the paper; C. T-F. and B.M. contributed to the conceptualization, review, and editing; and L. A., H. S. and B.M. contributed to the conceptualization, supervision, writing, project administration, and funding acquisition.

Competing Interest Statement: The authors declare no competing interest.

INTRODUCTION

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

The control center of this rhythm is located in the hypothalamic suprachiasmatic nucleus (SCN) which maintains the rhythms of processes autonomously. At molecular level, the central clock is regulated by self-regulatory transcriptional-translation feedback loops of the clock genes (11). Thus, the heterodimers of CLOCK (circadian locomotor kaput protein production cycles) and BMAL1 (brain and muscle ARNT 1, also known as ARNTL) act as positive regulators to promote the transcription of period (PER1 and PER2) and cryptochrome (CRY1 and CRY2) proteins. PER and CRY proteins form a complex in the nucleus that acts as a negative regulator during the day, interacting with CLOCK/BMAL1 and inhibiting their own transcription. The subsequent degradation of PER and CRY proteins leads to the restart of the cycle with a period of approximately 24 hours. CLOCK/BMAL1 also activate the circadian expression of the nuclear receptors RORα, RORβ, ERV-ERBα and REV-

ERBβ, which have an important role in stabilizing the central clock and their expression drives the transcription of BMAL1 and CLOCK (12–14). Finally, the CLOCK/BMAL1 heterodimer enhances the transcription of metabolic genes, such as nicotinamide phosphoribosyltransferase (*Nampt*) and Rev-erbα (15–18) which are involved in the modulation of appetite and energy metabolism pathways in the arcuate nucleus (Arc) of the hypothalamus (19–23). This region consists of two main neuronal populations: Neuropeptide Y—agouti-related protein (NPY-AGRP) neurons and pro-opiomelanocortin—cocaine-amphetamine-regulated transcript (POMC-CART) neurons. NPY-AGRP orexigenic neurons respond to ghrelin signals promoting food intake and decreasing energy expenditure. On the other hand, POMC-CART anorexigenic neurons are activated by leptin promoting satiety and increasing energy expenditure in response to feeding (19,24).

External cues called zeitgebers, such as light or food, play a crucial role in reprogramming or altering, the circadian rhythm (13,25). Light is perceived by the retina via the retinohypothalamic tract (RHT) and reset the central clock, but the expression of clock genes in the other peripheral tissues as liver (26,27), are also regulated by diet composition. Hence, mice fed a high-fat diet showed alterations in the expression of circadian rhythm genes, nuclear receptors that regulate clock transcription factors, and clock-controlled genes involved in fuel utilization in the hypothalamus, liver, and adipose tissue. Moreover, changes in the rhythm of locomotor activity were also observed (28). In addition, the frequency and timing of meals regulated circadian rhythms and energy metabolism in liver and muscle of rats (27). Furthermore, the intake of sugars, lipids, or other nutrients at different times of the day is known to have a different impact on the body (28–32). In this regard, a study has reported the importance of the timing pattern of food intake on liver gene transcription in wild-type mice (29,30). Changes in brown adipose tissue and skeletal muscle metabolism dependent on feeding time have also been reported in Wistar rats (33). In addition, it was observed in humans that late food consumption is

associated with an increase in body fat (34). Thus, not only the composition of our food can modulate the peripheral clocks, but the timing of our food intake is now gaining importance.

Therefore, the aim of the present study is to know whether the time of the day of chronic supplementation of a low dose of sugar, equivalent to 2 g in humans (half teaspoon), is determinant in causing changes in the central clock and modifying the circadian rhythm of metabolism in healthy rats. For this purpose, thirty-two rats were fed with standard chow (STD) diet and given a low dose of sugar at 8 a.m. and 8 p.m. (ZT0 and ZT12). The central clock, body weight (BW) gain and the main biochemical parameters and hormones in serum were analyzed by obtaining samples at 4 different times in a 24-hour period, we also analyzed genes of food intake signaling.

Results

Differential effects of timing of sugar administration on body weight gain, leptin levels and appetite gene expression

BW gain increased unequally according to the time of sugar administration (Fig. 1A). An increase in BW gain was observed in animals administered at ZT0 compared to rats supplemented at ZT12. This difference in BW gain was significant during the last three weeks of the experiment. In addition, the area under the curve (AUC) of BW gain confirmed this higher BW gain of the rats administered sugar at ZT0 (Fig. 1A). No significant changes in food intake were observed, although there was a slight tendency for rats supplemented with sugar at ZT0 compared to ZT2 to show a higher cumulative food intake during the supplementation period (Fig. 1A). Nevertheless, since food intake values were recorded for 24-hours period, differences between groups in food intake throughout the 12-hours of light phase and/or 12-hours of dark phase cannot be ruled out.

Serum leptin levels and appetite gene expression in hypothalamus were studied and their circadian rhythm were analyzed by the cosinor method. A tendency

to show circadian rhythm could be detected (p=0.054) for serum leptin concentration in rats supplemented at ZT12, showing acrophase 3 hours before turning off the light (Fig. 1B) (Supplementary 1). In the case of ZT0 supplemented rats, no circadian rhythm was detected, the leptin concentration in serum was similar throughout the four time points. Furthermore, higher leptin concentration was observed at the ZT1 time point when the dose was administered at morning compared to the ZT12 group by Mann-Whitney test.

Regarding appetite signaling genes, the anorexigenic gene *Cart* (Fig. 1C) presented circadian rhythm for the two groups (p=0.009 and p=0.022 respectively for ZT0 and ZT12), however, both groups displayed opposite circadian rhythm, exhibiting reverse acrophase ZT18 for ZT0 group vs ZT7 for ZT12 group (p<0,001) (Supplementary 3 and 4). In addition, there was a decrease in *Cart* gene expression in the ZT0 group at time point ZT7 compared to the ZT12 group. During the dark phase, a significant increase was observed at time points ZT13 and ZT19 in the ZT0 groups compared to ZT12. No differences were found between groups ZT0 and ZT12 neither for the anorexigenic gene *Pomc* nor for the orexigenic genes *Npy* and *Argp* (Supplementary 5).

Differential effects of timing of sugar administration on biochemical parameters and insulin levels

No differences were found by Mann-Whitney test between ZT0 and ZT12 at each time point. Nevertheless, circadian rhythm of serum glucose levels was only detected (p=0.032) in the group that received the dose in ZT12 (Fig. 2A). However, in the case of insulin levels, the circadian rhythm was detected in both ZT0 and ZT12 groups (p=0.031 and p=0.048 respectively for ZT0 and ZT12) (Fig. 2B). Nonetheless, this parameter displayed an opposite rhythm depending on timing administration, occurring the acrophase always 6 hours after receiving the sugar dose, at ZT18 and ZT6 for the ZT12 and ZT0 groups, respectively. Likewise, a higher insulin concentration was found at point ZT7 when sugar was

given at ZT0, while a tendency to decrease and a significant decrement was seen in this same group for points ZT13 and ZT19 respectively compared to the groups administered at ZT12.

Other biochemical parameters such as cholesterol, triglycerides and NEFAs (Fig. 2C-E) were also studied. No rhythms were detected for any ZT0 or ZT12 group in serum cholesterol and NEFAs. However, both a decrease in cholesterol for a single time point (ZT7) and a tendency to decrease the concentration of NEFAs for time point ZT19 were observed in the ZT0 group. In the case of triglycerides, circadian rhythm was found in both groups, with a tendency to have a lower amplitude in the case of the ZT0-supplemented group (25.79 vs 52.06). In the time points ZT7 and ZT19 a tendency to decrease serum triglyceride concentration in the ZT0 group compared to the ZT12 group was observed.

Differential effects of timing of sugar administration on serum hormones

Serum corticosterone concentration (Fig. 3A) showed a clear circadian rhythm in both groups (p=0.02 and p=0.001 respectively for ZT0 and ZT12). However, and although no differences were found between the ZT0 and ZT12 groups at each time point by Mann-Whitney test, a tendency to decrease the acrophase when the sugar was administered in the morning respect to the night was observed. While the circadian rhythm of the group receiving the sugar dose in the morning peaked around ZT11 (Supplementary 1), just before lighting off, the ZT12 corticosterone peak appeared two hours after lighting off, around ZT14 (p=0.089) (Supplementary 2). Regarding glucocorticoid receptors, no circadian rhythm was detected in either group for *Nr3c1* or *Nr3c2* expression in hypothalamus. Despite this, the circadian rhythm models of these receptors showed reverse acrophases. In addition, we could observe a lower expression of the *Nr3c1* gene during the dark phase at ZT13 (p=0.021) and a tendency to decrease its expression at ZT19 (p=0.083) in ZT0 groups compared to ZT12 animals. In addition, in the case of *Nr3c2*, a significant decrease in its expression was observed at ZT7 (p=0.02) when rats were administered at the morning.

Serum melatonin levels displayed circadian rhythm for both groups (p=0.037 and p=0.011 respectively for ZT0 and ZT12 groups) (Fig. 3B). The circadian rhythm obtained had a normal behavior, showing the highest melatonin concentration during the dark phase and the lowest during the light phase. However, serum testosterone levels and its circadian rhythm was different depending on the timing of sugar administration (Fig 3C). A significant testosterone rhythm was detected in rats that received sugar at ZT12 (p=0.039) while no rhythm was observed in those supplemented at ZT0 (Supplementary 1). In addition, the model obtained from the testosterone concentrations of the rats receiving sugar at ZT0 showed a marked acrophase shift (p=0.024), with acrophase estimated close to ZT14 for this group and around ZT21 for the group receiving sugar at night (Supplementary 2). However, no significant differences were found for serum testosterone concentration between both experimental groups at the different time points. Nonetheless, a slight increase in testosterone levels was observed at ZT13 when the rats received sugar at ZT0 compared to ZT12 group.

The two main thyroid hormones were also studied (Supplementary 6). Regarding serum T3 levels, no significant circadian rhythm was detected for both supplementation conditions (Supplementary 1). However, modeling showed a higher T3 concentration in rats that received sugar at ZT0 compared to rats supplemented at ZT12. Furthermore, serum T3 showed significantly different levels at the different time points depending on the time of administration where a significantly higher concentration of this hormone was observed at ZT1 and ZT7 and a downward trend at ZT19 when rats received sugar at ZT0 instead ZT12 (Supplementary 6A). In the case of serum T4, no significant circadian rhythms were found for any condition, although a tendency to display rhythm was observed in the animals administered the sugar in the morning. Nevertheless, the models showed an opposite behavior between groups in T4 concentration in the 24-h period, peaking in dark for ZT0 group and in light period for ZT12 group (Supplementary 1 and 2). In addition, a tendency

to decrease at ZT13 and increase at ZT19 were observed in the animals administered at ZT0 compared to ZT12 group by Mann-Whitney test. Respect to the T3-to-T4 ratio circadian rhythm was detected ($p=0.013$) only for the group supplemented with sugar at ZT12. The ZT0 group did not display circadian rhythm, bur the model showed a significant acrophase shift compared to ZT12-supplemented rats ($p<0.001$) (Supplementary 1 and 2). Furthermore, when the ratio was compared between groups in each time point an increase in the T3-to-T4 ratio was observed in ZT7 and ZT13 for rats supplemented with sugar at ZT0 (Fig. 3D).

Circadian rhythm patterns of hypothalamic clock genes were altered differently according to the time of supplementation of a low dose of sugar

Hypothalamic clock genes were analyzed to know the central clock rhythmicity of the animals administered sugar in the different conditions. These genes control the central clock, synchronizing external signals from the environment with the peripheral clocks of the organism. The Cosinor method was also used to detect and model the circadian rhythms of the expression of different clock genes. The results showed important differences in the circadian expression patterns of hypothalamic clock genes in the groups supplemented at different times (Fig. 4). The supplementation at ZT0 resulted in a significant decrease in *Bmal1* expression ($p=0.043$) at ZT19 and a tendency ($p=0.083$) to decrease at ZT13 by Mann-Whitney test (Fig. 4A). Regarding the circadian rhythm of this gene, a rhythmicity was detected for both groups ($p<0.01$) by Cosinor analysis. Nevertheless, differences in the acrophases ($p<0.001$) were observed between both groups (Supplementary 3 and 4). In ZT0 group the expression of *Bmal1* peaked at the beginning of the light phase (around ZT2), while the group supplemented at ZT12 showed the acrophase before the end of the dark phase (around ZT22). Significant changes were also observed in *Rev-erbα* gene expression (Fig. 4B). In this case, a significant decrease in expression was observed at ZT13 ($p=0.021$) and ZT19 ($p=0.021$) in the ZT0-supplemented group

compared to ZT12- supplemented group by Mann-Whitney test. These differences translated into a change in the circadian pattern of the expression of this gene. Although the expression of this gene displayed circadian rhythm in the animals administered at ZT0 and ZT12, both acrophase and oscillation amplitude showed differences between the groups (p=0.008 and p=0.010, respectively) (Supplementary 4).

Nevertheless, the genes that showed more differences in their expression depending on timing of sugar administration were *Nampt* and *Rorα*. Regarding *Nampt* gene expression no significant circadian rhythm was detected in either group (Fig. 4C). However, the peak of expression of this gene was different depending on the sugar administration time (p=0.024). While for rats administered at ZT0 the acrophase was observed at ZT23, for rats supplemented at ZT12 it was found at ZT15 (Supplementary 3). In addition, a decrease in the expression of this gene was observed at ZT13 when rats were supplemented with sugar at ZT0 by Mann-Whitney test. Other finding related to this gene was that a greater variability of its expression in the following hours upon the dose, at ZT1 and ZT7 and ZT13 and ZT19 in rats supplemented at ZT0 and ZT12 respectively. For *Rorα* expression, only was detected circadian rhythm for the group supplemented at ZT12 (p=0.018) (Fig. 4D and Supplementary 3). The circadian rhythm model of *Rorα* in rats that received sugar at ZT0 showed a trend to have a lower expression of the gene over a 24-hour period. In addition, a significantly lower expression was observed at ZT1 (p=0.043) and ZT13 (p=0.021) when the dose was administered at ZT0 compared to ZT12-supplemented rats (Fig. 4D).

Timing of sugar supplementation changed both metabolomics profile and the number of metabolites with circadian rhythm

Overall effects of timing of supplementation on the serum metabolomic profile were analyzed by principal component analysis (PCA) and the sparse Partial Least Squares Discriminant Analysis (sPLS-DA) (Fig. 5C). A total of 66

metabolites were identified and integrated in serum. No clustering by dose time was observed using PCA, however, in sPLS-DA samples did cluster by time of dose. The heat map analysis also revealed a change in the serum metabolomic profile according to the timing of sugar administration (Fig. 5A), clearly differentiating the 4 groups that received the sugar in the morning from the 4 groups that were supplemented in the dark period. In addition, an oscillation of the metabolites according to the time of sacrifice could be observed, indicating that there is a circadian rhythm, or at least a diurnal rhythm, in a large part of the metabolites, especially in the groups treated at ZT12. Statistical analysis between the different groups found 22 metabolites whose concentration was significantly different due to time of sugar administration and time of slaughter. For example, diurnal sugar supplementation caused a strong significant increase in the concentration of heptanoic acid, 3-Hydroxyisovaleric Acid, Glycolic Acid and Dodecanoic Acid (Supplementary 7). This last metabolite is involved in lipolysis. Other metabolites involved in energy metabolism such as pyruvic acid and 3-Phosphoglyceric Acid in glycolysis and citric acid in the Krebs cycle were also affected (Fig. 5D). Moreover, metabolites were analyzed with the Cosinor method to know their circadian pattern (Supplementary 8). A significant difference in the circadian profile of serum metabolomics was detected according to the time of sugar supplementation (Fig. 5B). While circadian rhythm was only detected in 6 metabolites ($p<0.05$) when the sugar dose was administered in the morning, for the ZT12 treated groups circadian rhythm was detected in 17 metabolites (Fig. 5F). Many metabolites with circadian rhythm participate in important energy metabolism processes such as lipolysis (Dodecanoic Acid, Palmitic Acid, 2-Hydroxy Butyric Acid and Oleic Acid). All these metabolites showed circadian oscillations when sugar was administered in ZT12, but no rhythms were detected in any of them when sugar was administered in the morning. Other processes such as the Krebs cycle and glycolysis also showed or not rhythms according to the time of sugar administration, thus citric acid and Oxalic Acids presented circadian rhythm only

if the dose was administered at ZT0, while Glycerol-1-phosphate, Malic Acid, Fumaric Acid presented rhythm only when the animals were supplemented at ZT12. Circadian rhythm was also observed in 12 amino acids, from all of them Alanine showed rhythmicity in both groups, Taurine only in the ZT0 group and the remaining 10 amino acids displayed circadian oscillation only when the sugar was supplemented at ZT12.

Discussion

In recent years, the time of food intake has been reported to have an important impact on the circadian rhythm of the organism, causing changes in behavior, metabolism, and some physiological processes (10,29,32). These effects of food intake patterns on circadian rhythms have been also related to alterations on the metabolism (29,30). Hence, alterations in food intake time pattern can lead to the development of overweight or obesity, a condition associated with diabetes, cardiovascular diseases, dyslipidemia, among others (8,35), increasing the risk of suffering metabolic syndrome (36). In this study, as expected, a differential effect depending on timing of sugar administration was observed on serum glucose and a loss of circadian rhythm pattern was showed by the animal sugar administered at ZT0. In addition, a more pronounced drop in serum glucose levels were observed in ZT12 animals during the dark phase, coinciding with the highest expression of the core clock gene *Bmal1*.These changes in glucose oscillation depending on the timing of sugar administration was also accompanied by changes in insulin circadian rhythms. In fact, although this hormone displayed circadian rhythm in both groups of animals, insulin oscillation along the day was depending on the timing of sugar administration. While the acrophase of ZT0 group appeared in the light phase, as the acrophase of *Bmal1* expression, ZT12 group displayed the insulin and *Bmal1* acrophases in the dark phase. Regarding to the lipid biochemical parameters, no major changes were found between groups, although a little increase in serum cholesterol and triglycerides at some time point when supplementation was at

ZT12 led to a better circadian profile of these parameters in these rats compared to animals supplemented at ZT0.

In addition, the results of this study confirmed the importance of timing of intake in metabolism since health rats that received a low dose of sugar during the morning, showed a higher BW gain compared to rats that received sugar in the dark phase. It has been described that chocolate intake, a hypercaloric and palatable meal, at the beginning of the light phase showed greater weight gain than rats that ate chocolate at the beginning of the dark phase in rats (37). Moreover, differential effect on BW gain depending on timing intake have been also reported in a 12-week clinical trial since participants who consumed a high-calorie meal before the rest phase, dinner versus breakfast, gained more BW during the intervention (38). In our study the amount of sugar administered was low, but also the animals supplemented at the beginning of their resting period, the rats are nocturnal animals, showed higher BW gain. This amount of sugar, using a translation of animal to human doses (39) and estimating the daily intake for a 70 kg human, corresponds to an intake of 2.3 g/day (half teaspoon). Therefore, although experimental results obtained of our study cannot be directly translatable to humans, the fact that only 158 mg/kg of sugar promotes differences in body weight gain depending on timing of its administration, suggests that in humans the daily ingestion of a minimum amount of sugar and specifically the timing of this intake should not be underestimated.

The most important controller of the circadian rhythm is the central clock, this pacemaker is in hypothalamus and maintains the synchronization of the peripheral clocks with the environment and its oscillators through hormone signals and neural connections (25). *Nampt* is an important clock gene and hypothalamic *Nampt* expression is related to energetic metabolism and food intake control through the regulation of the signaling intake genes, and genes related to mitochondrial function (19–21). NAMPT is the rate-limiting enzyme in the biosynthesis of NAD through its salvage pathway (40). Therefore, this metabolite exhibits circadian rhythm due to the level oscillation in NAMPT. NAD

homeostasis is related to the free radicals-mediated production of reactive oxygen species, which has been linked to innumerable pathologies, including metabolic diseases (41). Moreover, this metabolite activates several NAD-dependent deacetylases (SIRT), controlling the activity of many cellular proteins.

Nampt expression is mediated by the CLOCK/BMAL1 heterodimer (42,43). However, in this study hypothalamic circadian rhythm for this gene was not detected for any group. Nevertheless, an estimation of the rhythm with Cosinor method showed clear differences between both groups, indicating that the timing of sugar administration would be also determinant in the expression of this gene. Specifically, the results showed remarkably changes in the expression of *Nampt* between groups, peaking in the dark phase in the ZT12 group and in the morning in the animals administered at ZT0. Therefore, a reverse pattern with displacement of acrophases depending on timing of supplementation was observed. Regarding the lack of hypothalamic *Nampt* rhythmic expression, it has been previously reported using hypothalamic neurons that presented a functional circadian clock, displaying circadian rhythmicity for core clock genes as *Bmal1* (20). According to this, our results also showed rhythmicity for *Bmal1* both in ZT0 and ZT12 animals, although a delayed in the acrophase of ZT0 animals compared to ZT12 rats was observed. The changes observed in Bmal1 expression could explain the differences observed for *Nampt* expression between groups and could be promoting the changes in the rhythms of other metabolites and hormones, according to the defined role of *Bmal1* as a master regulator of molecular clock system (44).

In addition to the key role of NAD in the synchronization of metabolism, it has been reported that NAMPT administration promoted, in the arcuate nucleus of rats, increased food intake and decreased *Cart* expression (45). Our results showed that *Cart* expression is lower during the dark phase in rats supplemented with sugar at ZT12, while *Nampt* showed higher expression in this phase than in the light period. On the contrary, for rats supplemented at

ZT0 an upregulation of *Cart* was observed during the dark phase while *Nampt* was lower during most of this phase, and in any case lower than rats supplemented with sugar at ZT12. In addition, although no significant circadian rhythms were obtained, circadian rhythm models of *Pomc* expression showed an acrophase located in the dark phase of rats supplemented with sugar at ZT0 and a higher peak expression than rats supplemented at ZT12. In addition, although no significant changes in food intake were found, a significant change in leptin was observed. This hormone, secreted by fat cells, exhibit important functions in the regulation of food intake and metabolism, and leptin resistance may promote the development of obesity. In humans, leptin shows a marked circadian rhythm with a peak two hours before the onset of the activity phase, and an alteration in its secretion promotes the loss of the circadian rhythm of metabolism and feeding (46,47). In our study, circadian rhythm was detected in serum leptin concentrations in the ZT12 group, with a peak 3 hours before the activity phase, whereas no circadian rhythm was detected in the rats that received sugar in the morning. Thus, the change in the circadian rhythms of the anorexigenic genes *Cart* and *Pomc* and their differences in the time of maximum expression depending on timing of sugar administration, together with the circadian changes of leptin, suggest differences in the energetic metabolism between groups.

Regarding *Rev-erbα*, an important change was observed in the animals supplemented at ZT12 compared with ZT0-supplemented group. The expression of this gene has been related to the maintenance of the central clock and, as well as *Nampt*, the regulation of energy metabolism (22). It has been observed that the absence of expression of this gene is associated in male mice with an increase in BW (23). Our results showed a decrease in the expression of this gene during the dark phase in ZT0-supplemented rats, which gained more BW than ZT12-supplemented rats. These results could indicate a rhythmic disruption of metabolism via *Nampt* and *Rev-erbα* expression by sugar intake outside of the normal feeding cycle. According to this ZT0-supplemented group

also presented a decreased *Cart* expression in the light period, which could result in an increased food intake during its resting phase.

In this context, the metabolic profile of rats was evaluated by serum metabolomics. Different studies show how metabolism is circadian regulated by the biological clocks and feeding time patterns (48–51). Thus, for example, in addition to the direct involvement of *Nampt*, pathways such as lipolysis or glycolysis and Krebs cycle are altered by changes in feeding patterns that modify the clock system (48–51). In our study the fact that ZT0-supplemented rats showed only 6 metabolites with circadian rhythm, while rats supplemented at ZT12 showed 17 metabolites with rhythm, indicated a change in overall metabolic profile of the rats depending on the timing of administration. Furthermore, it has been reported that T3/T4 ratio has circadian rhythm that is determined by feeding patterns, which is lost when there is a change in these patterns (52,53). According to this, in addition to the metabolic alterations found in ZT0 group, a loss of oscillation of the T3/T4 ratio was observed in these animals, while it is maintained in the ZT12 group.

Glucocorticoid receptors are important regulators of metabolic rhythmicity in the central nervous system. These regulate glucocorticoid effects, such as cortisol in humans or corticosterone in rodents, via the hypothalamic-pituitary-adrenal (HPA) axis (54–56). In addition, the circadian maintenance of corticosterone release appears to be regulated through the action of the clock system on the expression of glucocorticoid receptors such as *Nr3c1* (54,57–59). More specifically, it is known that there is a regulation between *Nr3c1* and *Rev-erbα* that affects the expression of both receptors (60). Therefore, disruption of the normal rhythmicity of their expression could lead to diseases of glucocorticoid sensitivity or resistance (61,62). The results of this study showed differences in the circadian pattern of serum corticosterone concentration, which may have resulted from the changes on *Nr3c1* and *Nr3c2* receptors and differently shaped clock genes produced by sugar supplementation at different times of the day.

In conclusion, daily supplementation with a low dose of sugar in healthy rats showed differential effects depending on the timing of sugar administration. Remarkable metabolic and circadian differences were observed when sugar was administered at the beginning of the light phase, time when rats rest. This supplementation at different timings resulted in changes of expression circadian patterns from core clock genes such as *Bmal1* or *Rev-erbα* and major alterations in genes such as *Nampt* and *Cart* or glucocorticoid receptor genes. In addition, to the circadian disruption of these genes, the result showed important changes on metabolism since a loss of rhythmicity of hormones, metabolites and an increase of body weight gain was observed in rats administered the sugar in the morning.

Materials and Methods

Animal Procedures

The animals used were 12-week-old male Fischer 344 rats from Charles Rivar Labratories (Barcelona, Spain) housed in pairs under standard laboratory conditions at 22°C and 12 hours light-dark cycle. Rats were weighted and randomly divided into two groups (n=16 per group) and fed ad libitum with STD diet (2.90 kcal·g-1; A04, Panlab, Barcelona, Spain) for 2 weeks. After this acclimation time, a low dose (diluted 1/5) of a low fat sweetened condensed milk (SCM) (60,8% glucose, 8,9% protein, 0,2% fat) was administered by syringe to the animals at 8 a.m. and 8 p.m. (ZT0 and ZT12, respectively), for 4 weeks. This amount of SCM consisted of 158 mg of sugar per kg of rat, which is equivalent to 2,5 g in humans (half teaspoon). To avoid the effect of our intervention at different times to give the dose, all thirty-two rats were in the same room, and we made sure that all rats approached the end of the box at two supplementation times. In order to obtain four different time points in a period of 24 hours to analyze circadian rhythms, animals were sacrificed at 4 different times (n=4): ZT1, ZT7, ZT13 and ZT19 (Supplementary 9). BW and food intake were recorded weekly during the whole experimental procedure. After the experimental period, animals were deprived of food for 3 hours and killed

by decapitation. Blood was collected, and serum was obtained by centrifugation, the samples were stored at -80°C until analyses. The tissue samples were rapidly removed after death, frozen in liquid nitrogen, and stored at -80°C until further analyses.

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

Gene Expression Analysis

The total RNA, containing the microRNA, 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 supplementary 10 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as a percentage of the ZT0 group at ZT1 group using the $2^{-\Delta\Delta Ct}$ method with PPIA gene as an endogens control, as reported by Schmittgen and Livak (63).

Hormone Analysis

The serum hormones concentrations were measured by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ). Serum samples were thawed at 4 °C. 50 µL of serum were mixed with 250 µL of methanol containing the internal standard (2 ng/mL). Then, the mixture was vortexed and centrifuged for 5 minutes at 4 ºC and 15000 rpm. The supernatant was transferred to a new tube and mixed with 700 µL of 0,1 % formic acid in water. The sample was loaded to an SPE previously conditioned with methanol and 0,1 % formic acid in water. The cartridge was washed with 0,1 % formic acid in water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45 ºC 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 x 2.1 mm) from Agilent Technologies. For these measures, the Eurecat research center (Reus, Spain) worked with us to carry out the technique.

Serum Analysis

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

Metabolomic analysis

Metabolomic analysis in the 96 rat serum samples was performed at Centre for Omic Sciences (COS, Tarragona, Spain) using gas chromatography coupled to Quadrupole Time-of-Flight Mass Spectrometry (GC-qTOF). The extraction was performed by adding 400 µL of methanol:water (8:2) containing internal

standard mixture to serum samples (approx. 100 µL). Then, the samples were mixed, incubated at 4ºC for 10 min, centrifuged at 15,000 rpm 4ºC for 10 min and supernatant was evaporated to dryness before compound derivatization (methoximation and silylation). The derivatized compounds were analyzed by GC-qTOF (model 7200 of Agilent, USA). The chromatographic separation was based on Fiehn Method (64) using a J&W Scientific HP5-MS (30 m x 0.25 mm i.d., 0.25 µm film capillary column and helium as carrier gas using an oven program from 60 to 325ºC. Ionization was done by electronic impact (EI), with electron energy of 70eV and operating in full Scan mode. Identification of metabolites was performed using commercial standards and by matching their EI mass spectrum and retention time to metabolomic Fiehn library (from Agilent) which contains more than 1.400 metabolites. After putative identification of metabolites, these were semi-quantified in terms of internal standard response ratio.

Statistical Analysis

BW gain and cumulative food intake data were represented as means ± 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. The correlation coefficient was calculated with Pearson coefficient test. Theses statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

In order to analyze the circadian rhythms, we used Cosinor-based rhythmometry method. For this, a script was developed by J.R. S-R using PyCharm software (v.2018.2.4, JetBrains s.r.o., Prague, Czech Republic) with

Python version 3.7.4 and circadian-rhythm estimated was plotted using CosinorPy package (v.1.1) [126]. We considered the presence of circadian rhythm when the model of each gene expressions fit the cosine curves ($P < 0.05$). In addition, this method allowed for obtaining rhythmicity parameters such as the MESOR (circadian rhythm-adjusted mean), amplitude (the difference between the peak and the mean value of a wave) or acrophase (the time at which the peak of a rhythm occurs) of the circadian rhythm.

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FIGURES

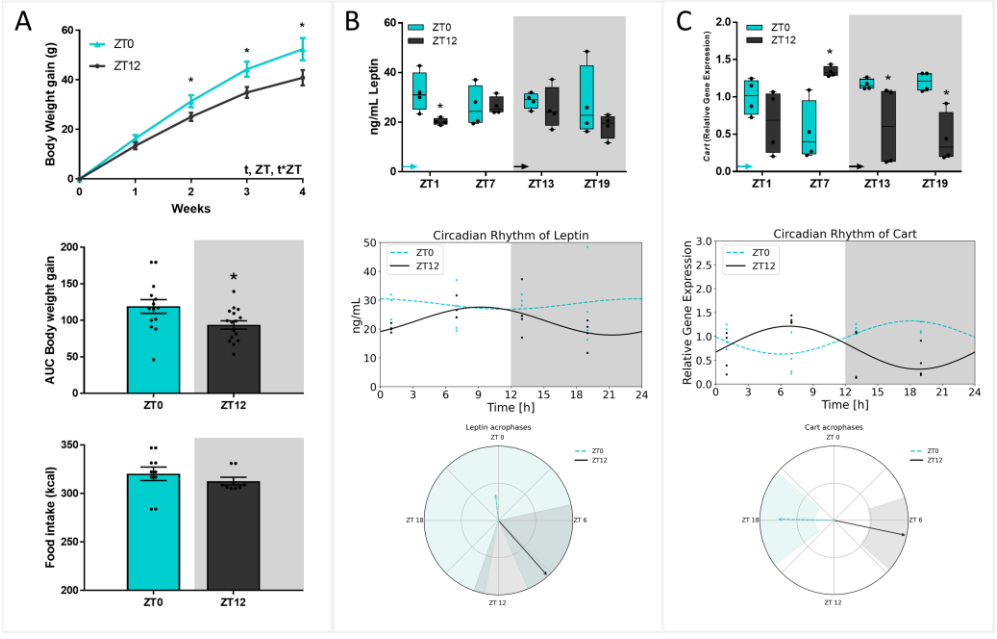


Figure 1. Body weight gain (g), food intake (kcal) leptin and Cart expression. *(A) Body weight gain in grams in the four weeks of the experiment. AUC of body weight in the four weeks of the experiment. Cumulative food intake in kcal in the four weeks of the experiment. ZT, time of supplementation effect; t, time effect; t*ZT, interaction between ZT and t. Repeated measured-ANOVA was used followed by Student´s t test between ZT0 vs ZT12 (p ≤ 0.05). (B) Leptin (ng/mL). Data shown as Median and Interquartile Range. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.5). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.1). Circadian rhythms estimated and Acrophases with their amplitude represented of leptin for ZT0 and ZT12. (C) Cart gene expression. Data shown as Median and Interquartile Range. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.5). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.1). Circadian rhythms estimated and Acrophases with their amplitude represented of Cart for ZT0 and ZT12.*

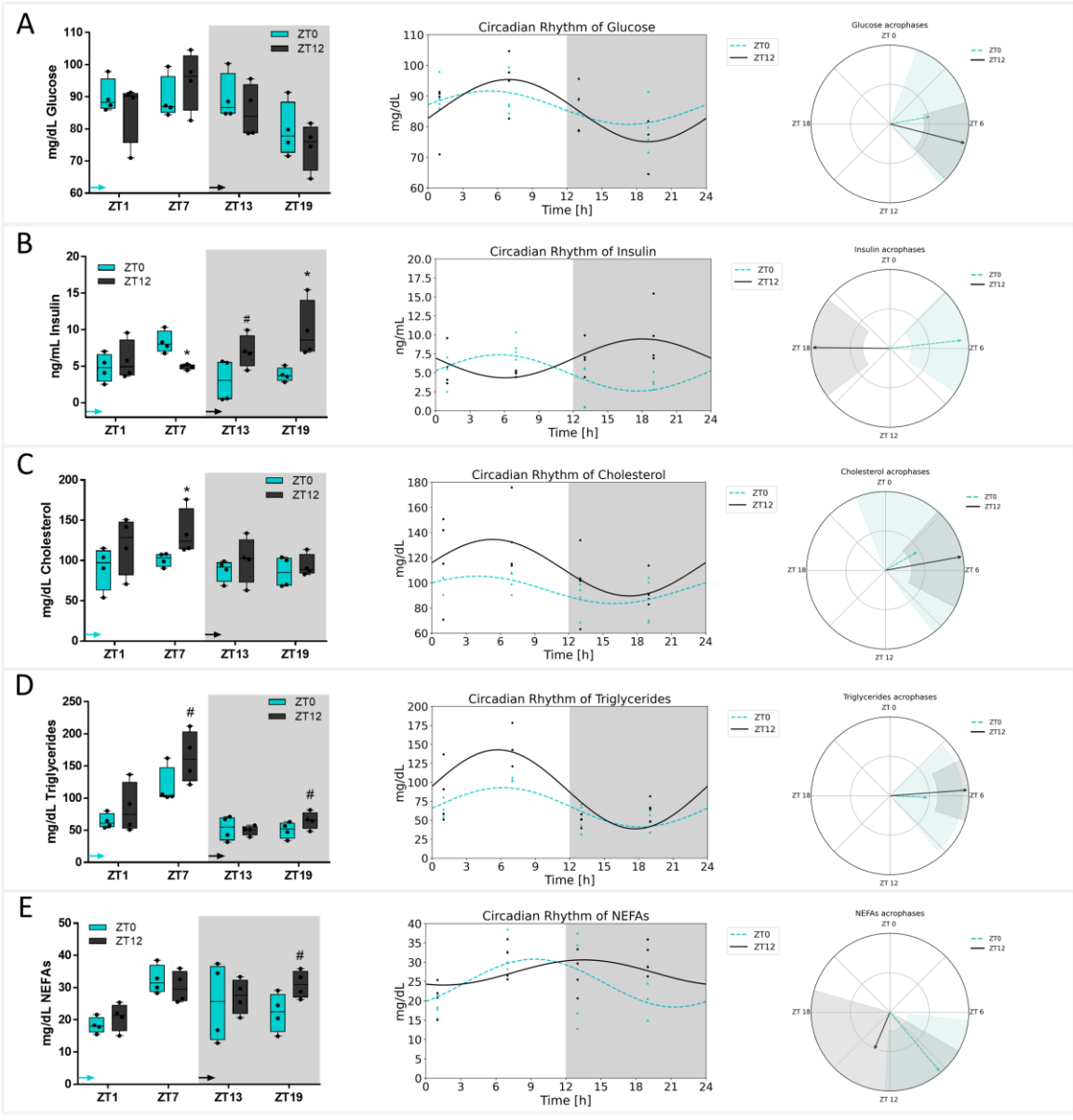


Figure 2. Serum parameters. *(A) Data shown as Median and Interquartile Range of Serum Glucose (mg/dL). Circadian rhythms estimated and Acrophases with their amplitude represented of Glucose for ZT0 and ZT12. (B) Data shown as Median and Interquartile Range of Insulin (ng/mL). Circadian rhythms estimated and Acrophases with their amplitude represented of Insulin for ZT0 and ZT12. (C) Data shown as Median and Interquartile Range of Serum Cholesterol (mg/dL). Circadian rhythms estimated and Acrophases with their amplitude represented of Cholesterol for ZT0 and ZT12. (D) Data shown as Median and Interquartile Range of Serum Triglycerides (mg/dL). Circadian rhythms estimated and Acrophases with their amplitude represented of Triglycerides for ZT0 and ZT12. (E) Data shown as Median and Interquartile Range of NEFAs (mg/dL). Circadian rhythms estimated and Acrophases with their amplitude represented of NEFAs for ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.5). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.1).*

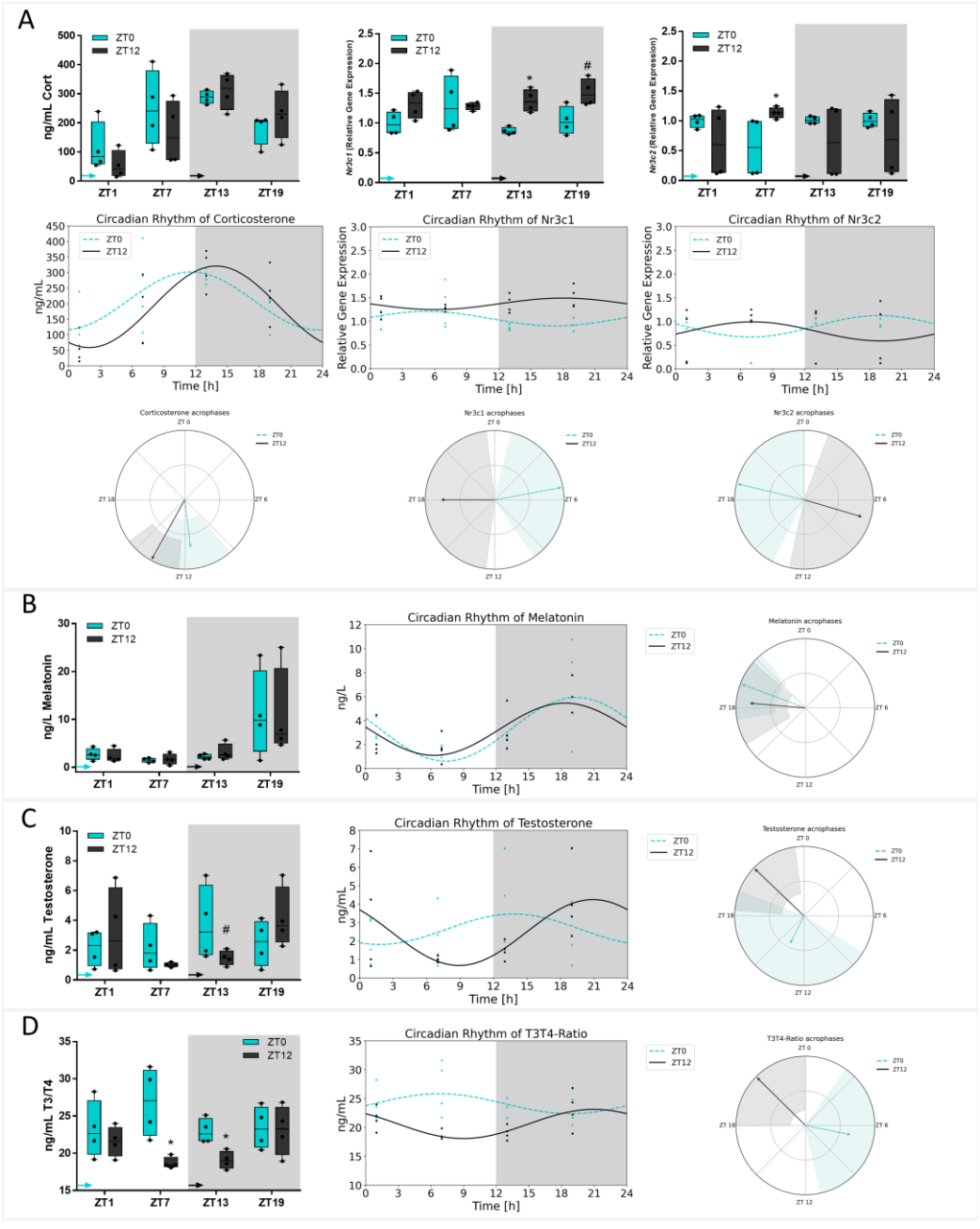


Figure 3. Estimated circadian rhythms of hormones. *(A) Data shown as Median and Interquartile Range of Corticosterone (ng/mL), Nr3c1 and Nr3c2 expression. Circadian rhythms estimated and Acrophases with their amplitude represented of Corticosterone (ng/mL), Nr3c1 and Nr3c2 expression for ZT0 and ZT12. (B) Data shown as Median and Interquartile Range of Melatonin (ng/L). Circadian rhythms estimated and Acrophases with their amplitude represented of Melatonin for ZT0 and ZT12. (C) Data shown as Median and Interquartile Range of Testosterone (ng/mL). Circadian rhythms estimated and Acrophases with their amplitude represented of Testosterone for ZT0 and ZT12. (D) Data shown as Median and Interquartile Range of T3-to-T4 ratio. Circadian rhythms estimated and Acrophases with their amplitude represented of T3-to-T4 ratio for ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.5). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.1).*

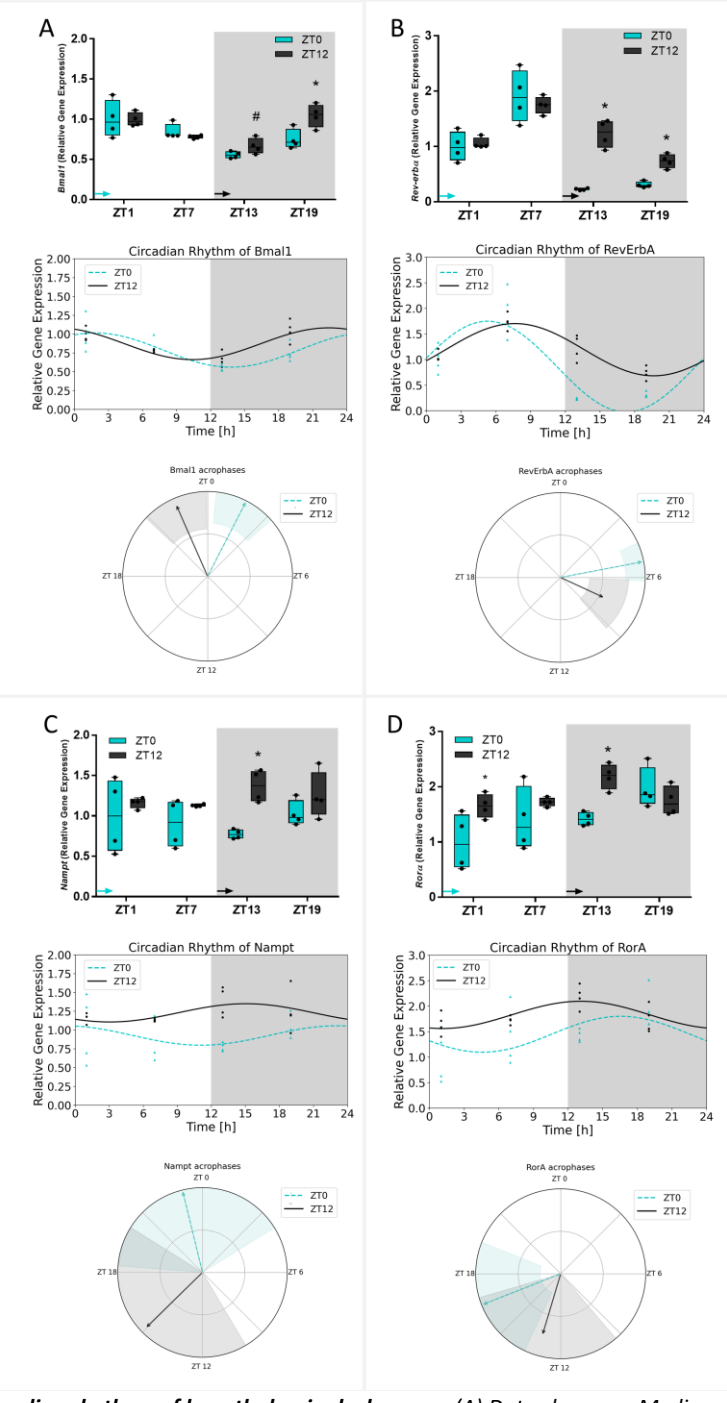


Figure 4. Estimated circadian rhythms of hypothalamic clock genes. *(A) Data shown as Median and Interquartile Range of Bmal1 (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of Bmal1 for ZT0 and ZT12. (B) Data shown as Median and Interquartile Range of Rev-erbα (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of Rev-erbα for ZT0 and ZT12. (C) Data shown as Median and Interquartile Range of Nampt (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of Nampt for ZT0 and ZT12. (D) Data shown as Median and Interquartile Range of Rorα (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of Rorα for ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.5). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney p < 0.1).*

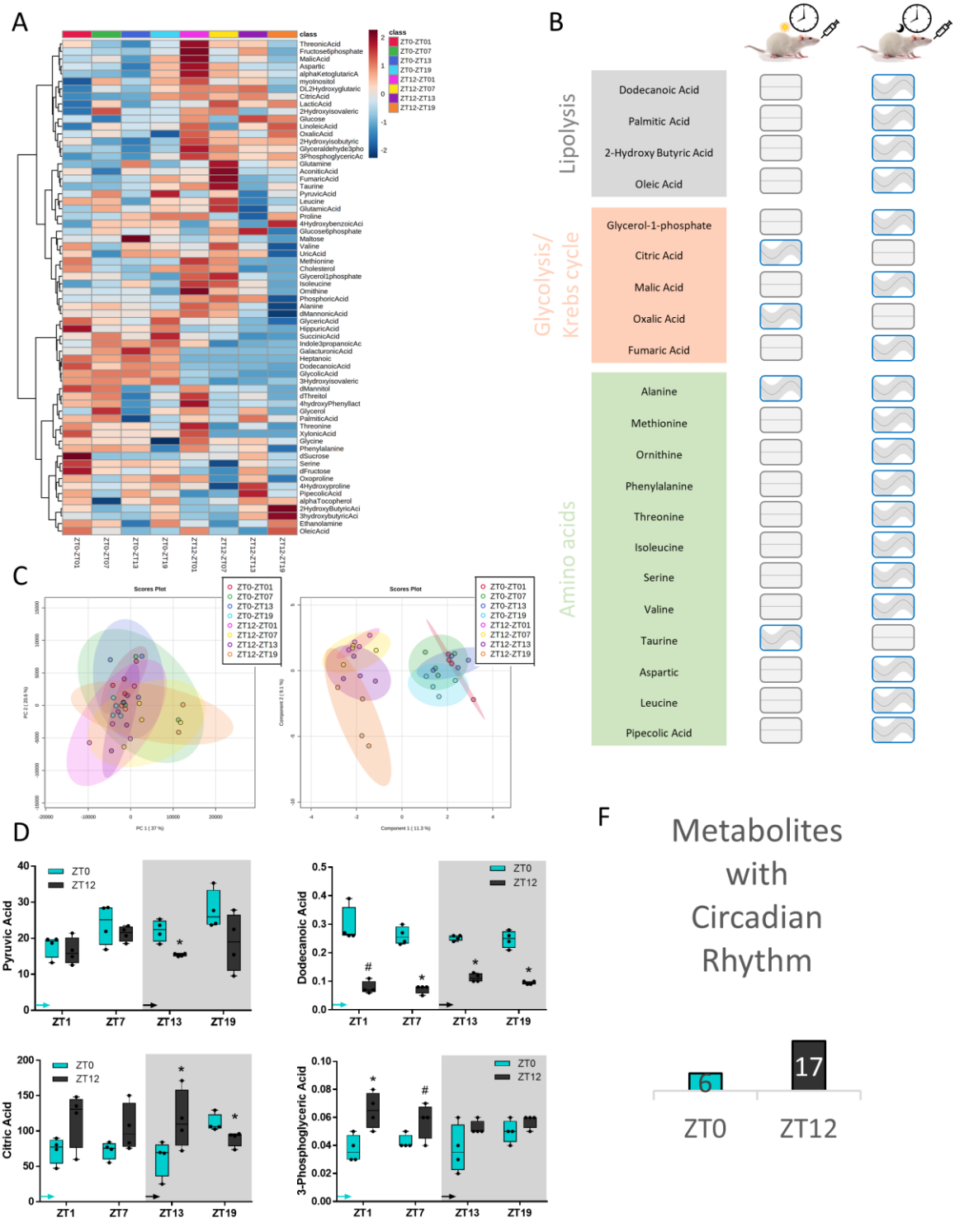


Figure 5. Metabolomics analysis for groups supplemented at ZT0 and ZT12. *A) Heatmap of abundance of Serum metabolites per experimental group. B) General overview of metabolites and their rhythmicity. C) Relative metabolomic composition throughout principal component analysis (PCA) and the sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of serum samples for each group. F) Metabolites with circadian rhythm.*

SUPPLEMENTARY

***Supplementary 1. Circadian parameters of biochemical parameters and hormones*.**

Parameter	Group	Period (h)	p	MESOR	Amplitude	p(amplitude)	Acrophase[h]	p(acrophase)
Glucose	ZT0	24	0.102	86.17	5.43	0.019*	5.32	0.002**
	ZT12	24	0.032*	85.22	10.15	0.004**	6.96	0.000***
Insulin	ZT0	24	0.031*	4.98	2.39	0.002**	5.57	0.000***
	ZT12	24	0.048*	6.90	2.56	0.005**	18.04	0.000***
Leptin	ZT0	24	0.859	28.64	1.81	0.579	23.57	0.001**
	ZT12	24	0.054#	22.72	4.84	0.007**	9.23	0.000***
Cholesterol	ZT0	24	0.128	94.38	10.89	0.027*	4.00	0.020*
	ZT12	24	0.101	111.98	22.48	0.019*	5.32	0.001**
Triglycerides	ZT0	24	0.005**	67.01	25.79	0.000***	6.18	0.000***
	ZT12	24	0.007**	90.71	52.06	0.000***	5.71	0.000***
NEFAs	ZT0	24	0.158	24.79	5.96	0.038*	9.10	0.000***
	ZT12	24	0.498	27.57	2.76	0.224	13.61	0.000***
Corticosterone	ZT0	24	0.020*	208.56	93.16	0.001**	11.55	0.000***
	ZT12	24	0.001**	189.69	131.05	0.000***	13.94	0.000***
Melatonin	ZT0	24	0.037*	3.27	2.66	0.003**	19.37	0.000***
	ZT12	24	0.011*	3.27	2.18	0.000***	18.33	0.000***

Testosterone	ZT0	24	0.436	2.64	0.82	0.183	13.78	0.000***
	ZT12	24	0.039*	2.46	1.78	0.004**	20.91	0.000***
T3	ZT0	24	0.700	1.24	0.08	0.392	8.22	0.065#
	ZT12	24	0.537	0.98	0.07	0.253	15.16	0.000***
T4	ZT0	24	0.092#	51.03	3.85	0.016*	20.11	0.000***
	ZT12	24	0.183	48.69	5.19	0.049*	10.02	0.000***
T3-to-T4 ratio	ZT0	24	0.403	24.12	1.73	0.162	6.77	0.013*
	ZT12	24	0.013*	20.61	2.52	0.000***	20.98	0.000***

*Acrophase is the time at which the peak of a rhythm occurs ([h]. hours); Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The values are the estimation of circadian parameters obtained by Cosinor method. * Circadian rhythm and parameters significantly detected (Cosinor analysis. p<0.05). # Indicates tendency (Cosinor analysis. p<0.1).*

Supplementary 2. Comparison of circadian parameters of biochemical parameters and hormones between groups.

test	p	p(d_amplitude)	p(d_acrophase)
Glucose	0.040*	0.261	0.479
Insulin	0.005**	0.891	0.000***
Leptin	0.149	0.413	0.015*
Cholesterol	0.023*	0.293	0.668
Triglycerides	0.000***	0.090#	0.788
NEFAs	0.213	0.385	0.240
Corticosterone	0.000***	0.337	0.089#
Melatonin	0.008**	0.657	0.517
Testosterone	0.106	0.272	0.024#
T3	0.037*	0.933	0.224
T4	0.114	0.664	0.000***
T3-to-T4 ratio	0.006**	0.579	0.000***

Acrophase is the time at which the peak of a rhythm occurs ([h]. hours); Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The first column (p) indicates if there are significant differences between two circadian rhythms. The circadian parameter values are the differences between two groups of each circadian parameter. * The effect of diet within ZT0 vs ZT12 (comparison Cosinor analysis. $p<0.05$)

Supplementary 3. Circadian parameters of hypothalamic genes.

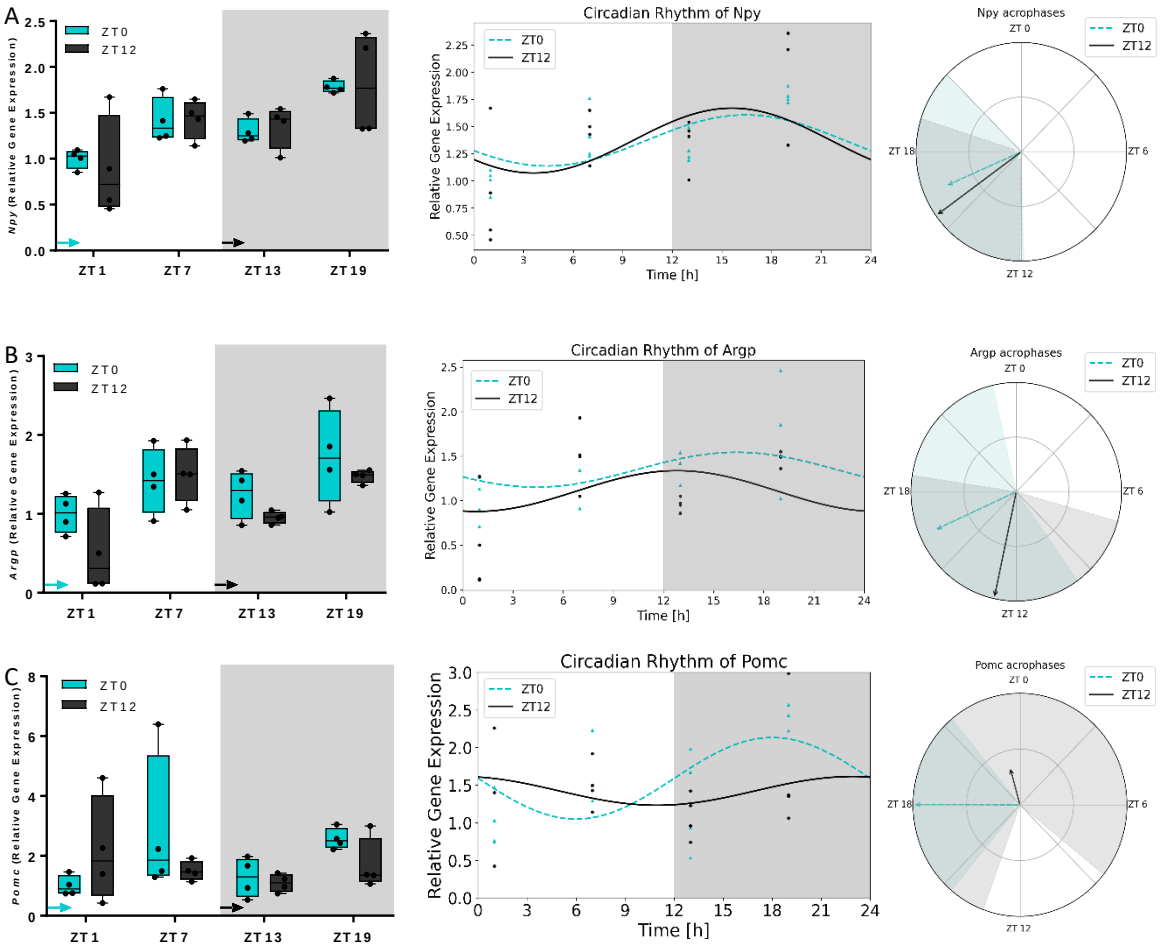
Parameter	Group	Period (h)	p	MESOR	Amplitude	p(amplitude)	Acrophase[h]	p(acrophase)
Bmal1	ZT0	24	0.002**	0.79	0.23	0.000***	1.81	0.025*
	ZT12	24	0.000***	0.87	0.21	0.000***	22.42	0.000***
Rev-erbα	ZT0	24	0.000***	0.86	0.89	0.000***	5.28	0.000***
	ZT12	24	0.000***	1.19	0.51	0.000***	7.65	0.000***
Nampt	ZT0	24	0.448	0.93	0.13	0.191	23.09	0.000***
	ZT12	24	0.191	1.23	0.12	0.052#	15.04	0.000***
Rorα	ZT0	24	0.170	1.45	0.35	0.044*	16.57	0.000***
	ZT12	24	0.018*	1.83	0.27	0.001**	13.11	0.000***
Pomc	ZT0	24	0.174	1.59	0.54	0.044*	18.01	0.000***
	ZT12	24	0.738	1.42	0.19	0.431	22.96	0.000***
Cart	ZT0	24	0.009**	0.98	0.35	0.000***	18.08	0.000***
	ZT12	24	0.022*	0.76	0.45	0.001**	6.79	0.000***
Npy	ZT0	24	0.109	1.37	0.23	0.021*	16.43	0.000***
	ZT12	24	0.262	1.37	0.30	0.085#	15.61	0.000***
Argp	ZT0	24	0.517	1.35	0.20	0.239	16.38	0.000***
	ZT12	24	0.489	1.11	0.23	0.219	12.81	0.000***
Nr3c1	ZT0	24	0.390	1.06	0.15	0.154	5.30	0.048*
	ZT12	24	0.224	1.37	0.12	0.067#	18.01	0.000***
Nr3c2	ZT0	24	0.116	0.90	0.23	0.024*	18.89	0.000***
	ZT12	24	0.592	0.79	0.20	0.296	7.09	0.052#

Acrophase is the time at which the peak of a rhythm occurs ([h]. hours); Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The values are the estimation of circadian parameters obtained by Cosinor method. * Circadian rhythm and parameters significantly detected (Cosinor analysis. $p<0.05$). # Indicates tendency (Cosinor analysis. $p<0.1$)

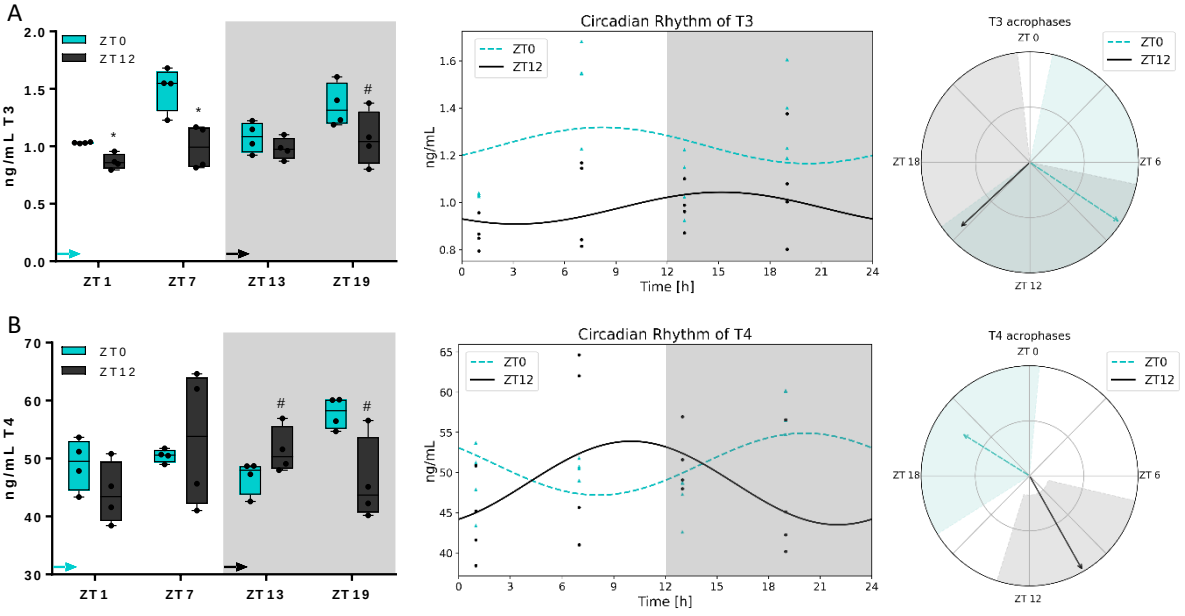
Supplementary 4. Comparison of circadian parameters of hypothalamic genes between groups

Genes	p	p(d_amplitude)	p(d_acrophase)
Bmal1	0.000***	0.790	0.000***
Rev-erbα	0.000***	0.010*	0.008**
Nampt	0.014*	0.948	0.024*
Rorα	0.012*	0.659	0.156
Pomc	0.364	0.333	0.341
Cart	0.002**	0.539	0.000***
Npy	0.247	0.751	0.781
Argp	0.464	0.893	0.431
Nr3c1	0.017*	0.800	0.000***
Nr3c2	0.511	0.908	0.002**

Acrophase is the time at which the peak of a rhythm occurs ([h]. hours); Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The first column (p) indicates if there are significant differences between two circadian rhythms. The circadian parameter values are the differences between two groups of each circadian parameter. * The effect of diet within ZT0 vs ZT12 (comparison Cosinor analysis. $p<0.05$)



Supplementary 5. Estimated circadian rhythms of hypothalamic food intake genes. (A) Data shown as Median and Interquartile Range of *Npy* (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of *Npy* for ZT0 and ZT12. (B) Data shown as Median and Interquartile Range of *Argp* (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of *Argp* for ZT0 and ZT12. (C) Data shown as Median and Interquartile Range of *Pomc* (Relative gene expression). Circadian rhythms estimated and Acrophases with their amplitude represented of *Pomc* for ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 by Mann-Whitney $p < 0.5$. # Indicates tendency between ZT0 vs ZT12 by Mann-Whitney $p < 0.1$.



Supplementary 6. Estimated circadian rhythms of hormones. (A) Data shown as Median and Interquartile Range of T3 (ng/mL). Circadian rhythms estimated and Acrophases with their amplitude represented of T3 for ZT0 and ZT12. (B) Data shown as Median and Interquartile Range of T4 (ng/mL). Circadian rhythms estimated and Acrophases with their amplitude represented of melatonin for ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 by Mann-Whitney $p < 0.5$. # Indicates tendency between ZT0 vs ZT12 by Mann-Whitney $p < 0.1$.

Supplementary 7. Serum metabolites (Arbitrary units) in ZT0 and ZT12- supplemented rats.

METABOLITES	ZT0-ZT1 (n=4)	ZT12-ZT1 (n=4)	ZT0-ZT7 (n=4)	ZT12-ZT7 (n=4)	ZT0-ZT13 (n=4)	ZT12-ZT13 (n=4)	ZT0-ZT19 (n=4)	ZT12-ZT19 (n=4)	P-VALUE	FDR
	MEDIAN (IQR)	MEDIAN (IQR)	MEDIAN (IQR)	MEDIAN (IQR)	MEDIAN (IQR)	MEDIAN (IQR)	MEDIAN (IQR)	MEDIAN (IQR)		
Heptanoic	0.455 (0.4425-0.4825)	0.025 (0.02-0.03)*	0.49 (0.435-0.53)	0.025 (0.02-0.03)*	0.455 (0.4325-0.515)	0.025 (0.02-0.0375)*	0.47 (0.4325-0.515)	0.025 (0.02-0.0375)*	0.001	0.002
3HydroxyisovalericAcid	0.495 (0.42-0.5925)	0.27 (0.245-0.31)*	0.54 (0.4575-0.5775)	0.26 (0.2225-0.29)*	0.485 (0.4425-0.5125)	0.27 (0.2075-0.3175)*	0.555 (0.5425-0.5675)	0.295 (0.29-0.435)*	0.001	0.005
GlycolicAcid	0.12 (0.11-0.13)	0.08 (0.07-0.09)*	0.13 (0.1125-0.14)	0.095 (0.0825-0.1075)*	0.125 (0.12-0.1375)	0.08 (0.08-0.095)*	0.115 (0.11-0.1275)	0.085 (0.0725-0.0975)*	0.001	0.007
DodecanoicAcid	0.265 (0.26-0.36)	0.07 (0.0625-0.1)#	0.255 (0.2325-0.2925)	0.08 (0.0575-0.08)*	0.255 (0.2425-0.26)	0.11 (0.1-0.1275)*	0.25 (0.2175-0.275)	0.095 (0.09-0.1)*	0.003	0.009
PhosphoricAcid	60.835 (57.3225-76.6925)	78.89 (70.1975-94.535)*	68.405 (57.6725-75.785)	87.025 (78.8775-95.48)*	67.39 (63.4875-70.16)	82.44 (72.025-89.93)*	62.255 (60.02-69.44)	53.57 (46.6125-59.7025)*	0.004	0.011
Alanine	15.62 (14.6725-16.605)	18.015 (16.9775-19.33)*	16.46 (15.2325-17.5)	17.38 (15.915-17.885)	15.545 (14.44-15.825)	14.34 (12.7475-16.2775)	14.3 (13.8375-14.9125)	13.295 (11.875-15.735)	0.007	0.014
dThreitol	0.03 (0.03-0.03)	0.035 (0.03-0.04)	0.03 (0.03-0.0375)	0.025 (0.02-0.03)#	0.02 (0.02-0.02)	0.03 (0.0225-0.03)*	0.03 (0.0225-0.03)	0.02 (0.02-0.0275)	0.011	0.016
Methionine	3.58 (3.1375-3.6925)	3.72 (3.595-3.8525)	3.365 (3.1275-3.595)	3.305 (3.1425-3.6775)	3.23 (3.14-3.4025)	2.965 (2.8975-3.3025)	3.345 (3.1875-3.405)	3.01 (2.9475-3.0575)*	0.012	0.018
Ornithine	2.315 (1.945-3.525)	6.18 (3.6875-7.9825)*	1.875 (1.3725-3.735)	2.85 (2.2025-5.7475)	2.6 (1.74-3.1975)	2.84 (2.2825-3.24)	1.265 (1.1175-1.7425)	1.965 (1.6-2.405)*	0.014	0.02
3hydroxybutyricAcid-3hydroxyisobutyricAcid	17 (8.715-49.375)	13.645 (7.305-27.965)	17.085 (12.775-23.2175)	12.895 (9.0375-14.5775)#	25.85 (7.6375-44.2275)	33.295 (24.255-49.88)	33.025 (30.3575-38.8575)	54.91 (46.2725-69.645)*	0.015	0.023
dFructose	0.04 (0.0325-0.04)	0.025 (0.02-0.03)*	0.03 (0.03-0.03)	0.02 (0.02-0.0275)*	0.03 (0.0225-0.03)	0.03 (0.03-0.0375)	0.03 (0.03-0.03)	0.025 (0.02-0.03)	0.017	0.025
Phenylalanine	3.86 (3.12-3.8875)	3.855 (3.83-4.075)	3.59 (3.4275-4.3225)	3.92 (3.4325-4.28)	3.62 (3.27-3.7975)	3.28 (2.975-3.5925)	3.475 (3.36-3.875)	3.09 (2.7875-3.175)*	0.019	0.027
Threonine	8.02 (6.9225-8.975)	8.995 (8.0875-9.3775)	7.14 (6.095-8.215)	7.385 (6.485-8.81)	7.135 (6.7675-7.7725)	7.02 (5.2525-7.8425)	6.445 (6.0775-6.82)	5.46 (4.93-6.3125)	0.019	0.03
Glucose	38.655 (34.4425-42.92)	47.62 (40.2875-52.9125)	38.74 (37.12-40.8325)	46.53 (43.31-48.2125)*	39.425 (33.4825-42.84)	50.035 (47.805-53.99)*	38.975 (35.2925-43.3775)	47.225 (45.0425-50.3225)*	0.019	0.032
myoInositol	0.92 (0.675-1)	1.155 (0.9675-1.4175)#	0.975 (0.845-1.195)	0.91 (0.7775-1.3425)	0.915 (0.8775-0.96)	0.895 (0.8275-1.0825)	0.94 (0.92-1.1625)	0.69 (0.6025-0.74)*	0.022	0.034
3PhosphoglycericAcid	0.035 (0.03-0.0475)	0.065 (0.0525-0.0775)*	0.04 (0.04-0.0475)	0.06 (0.045-0.0675)#	0.035 (0.0225-0.055)	0.05 (0.05-0.0575)	0.05 (0.0425-0.0575)	0.06 (0.0525-0.06)	0.023	0.036
alphaKetoglutaricAcid	5.165 (4.3125-5.7475)	9.63 (7.71-12.3)*	6.84 (5.0375-7.3975)	7.375 (6.695-8.6325)	4.84 (3.7625-6.18)	7.925 (5.815-10.74)#	7.12 (6.2575-8.5)	5.865 (4.7125-6.9575)	0.025	0.039

PyruvicAcid	19.19 (14.6-19.7375)	15.79 (13.1475-20.2325)	25.155 (18.14-28.5175)	21.535 (19.0775-23.1825)	22.38 (19.075-24.89)	15.41 (14.9925-15.7525)*	25.92 (23.7875-33.4225)	18.955 (11.015-26.475)	0.033	0.041
CitricAcid	77.375 (53.8125-87.505)	130.5 (76.2175-145.0025)	75.635 (60.035-82.4525)	95.61 (77.5-139.6475)	69.225 (35.87-80.7025)	109.665 (79.345-158.0075)*	106.185 (103.4075-123.64)	93.515 (78.365-95.75)*	0.034	0.043
Isoleucine	4.125 (3.725-4.2475)	4.79 (4.2025-5.19)	3.775 (3.6775-4.5475)	4.725 (3.7425-5.0775)	3.73 (3.445-3.8575)	3.455 (3.0025-3.765)	3.715 (3.31-4.2025)	3.18 (2.745-3.66)	0.036	0.045
Glutamine	0.47 (0.395-0.5225)	0.45 (0.3725-0.52)	0.38 (0.1675-0.4575)	0.765 (0.6425-0.8425)*	0.535 (0.4325-0.9225)	0.365 (0.3425-0.6125)	0.375 (0.2725-0.3875)	0.51 (0.395-0.67)	0.036	0.048
dMannitol	0.025 (0.01-0.0775)	0.015 (0.01-0.065)	0.03 (0.0125-0.055)	0.015 (0.01-0.02)	0 (0-0.0075)	0.005 (0-0.0325)	0.01 (0.01-0.0175)	0.005 (0-0.01)#	0.046	0.05
Oxoproline	153.425 (138.9975-160.0675)	149.745 (120.31-191.0375)	145.155 (137.49-149.5425)	120.245 (109.9625-135.2075)	139.54 (122.745-180.3275)	148.87 (132.985-154.705)	162.37 (154.11-172.94)	144.545 (139.275-150.7075)	0.058	0.052
MalicAcid	0.95 (0.735-1.1725)	2.015 (1.2525-2.4775)	1.24 (0.885-1.31)	1.21 (1.0325-1.485)	0.985 (0.7375-1.09)	1.15 (0.8575-1.54)	1.275 (1.23-1.6275)	1.03 (0.7825-1.135)	0.062	0.055
Serine	9.73 (9.5375-11.145)	10.75 (10.3375-11.62)	8.61 (8.2425-9.915)	8.705 (8.055-9.6925)	9.08 (8.9025-9.3775)	8.745 (8.395-9.1175)	8.845 (8.4875-9.435)	8.035 (7.595-9.06)	0.062	0.057
Valine	8.74 (8.0175-9.095)	9.61 (8.7225-10.955)	8.105 (7.785-9.43)	9.415 (8.295-10.1225)	8.25 (7.7025-8.5125)	7.595 (6.765-7.9525)	7.75 (6.72-8.6675)	6.43 (5.6775-7.2575)	0.067	0.059
Fructose6phosphate	0.175 (0.155-0.2325)	0.445 (0.28-0.6625)	0.235 (0.125-0.3225)	0.295 (0.2125-0.3775)	0.17 (0.1325-0.26)	0.25 (0.2225-0.4725)	0.24 (0.2025-0.3075)	0.115 (0.0825-0.1475)	0.07	0.061
GalacturonicAcid	0.01 (0.01-0.0175)	0.01 (0.01-0.01)	0.015 (0.01-0.02)	0.01 (0.01-0.01)	0.02 (0.0125-0.02)	0.01 (0.01-0.01)	0.015 (0.01-0.02)	0.01 (0.01-0.01)	0.074	0.064
SuccinicAcid	0.2 (0.1825-0.2475)	0.195 (0.155-0.265)	0.23 (0.2125-0.3)	0.175 (0.1375-0.25)	0.23 (0.185-0.3425)	0.155 (0.125-0.1775)	0.26 (0.2425-0.3225)	0.15 (0.1175-0.205)	0.091	0.066
ThreonicAcid	1.045 (0.8-1.785)	1.67 (1.26-2.0575)	0.97 (0.675-1.1675)	1.205 (0.9525-1.63)	0.775 (0.59-0.99)	1.275 (0.9325-1.52)	1.025 (0.9575-1.2425)	0.93 (0.7925-1.1725)	0.097	0.068
GlycericAcid	1.075 (0.7725-1.5275)	0.85 (0.81-1.1675)	0.79 (0.725-1.0875)	0.59 (0.5025-1.2475)	1.13 (0.94-1.485)	0.76 (0.515-0.8925)	1.21 (0.7225-1.54)	0.525 (0.47-0.5875)	0.097	0.07
AconiticAcid	0.075 (0.0625-0.1025)	0.075 (0.055-0.11)	0.05 (0.0175-0.09)	0.11 (0.08-0.2225)	0.06 (0.0525-0.1275)	0.055 (0.0325-0.1)	0.075 (0.055-0.0875)	0.07 (0.0475-0.0775)	0.1	0.073
LacticAcid	75.32 (71.07-77.29)	76.315 (67.87-84.565)	82.055 (76.3625-84.89)	95.72 (81.6325-102.6375)	84.265 (78.3975-97.9475)	80.015 (78.9475-83.5425)	95.49 (89.3925-97.6425)	88.685 (75.6125-101.5025)	0.121	0.075
DL2HydroxyglutaricAcid	0.225 (0.1775-0.3325)	0.51 (0.41-0.625)	0.335 (0.24-0.3475)	0.37 (0.33-0.4925)	0.24 (0.2-0.3175)	0.405 (0.3425-0.505)	0.345 (0.315-0.375)	0.315 (0.27-0.345)	0.121	0.077
Glyceraldehyde3phosphate	0 (0-0.0075)	0.01 (0.01-0.01)	0 (0-0.0075)	0.01 (0.0025-0.01)	0 (0-0)	0.005 (0-0.01)	0 (0-0.0075)	0.005 (0-0.01)	0.125	0.08
Taurine	0.645 (0.395-0.73)	0.72 (0.28-2.3675)	0.745 (0.5575-0.88)	1.445 (1.215-1.615)	1.005 (0.84-1.605)	0.64 (0.58-0.9475)	0.99 (0.5575-1.2425)	0.785 (0.6325-1.26)	0.137	0.082
dSucrose	0.09 (0.0275-0.2725)	0.075 (0.0125-0.1825)	0.015 (0.0025-0.0275)	0.01 (0.01-0.1075)	0.015 (0.01-0.0275)	0.06 (0.03-0.1125)	0.015 (0.01-0.0275)	0.04 (0.025-0.085)	0.147	0.084

Proline	17.435 (14.6875-20.0775)	26.345 (21.4475-32.78)	16.01 (13.8275-20.54)	19.55 (13.7325-31.3975)	18.745 (16.4325-19.58)	19.93 (15.565-21.7)	24.61 (13.925-25.575)	19.765 (17.6775-21.725)	0.15	0.086
Aspartic	0.41 (0.355-0.435)	0.525 (0.425-0.625)	0.4 (0.295-0.5725)	0.36 (0.33-0.51)	0.37 (0.325-0.4)	0.33 (0.3075-0.465)	0.38 (0.3225-0.46)	0.345 (0.31-0.395)	0.152	0.089
2HydroxyisovalericAcid	0.33 (0.3-0.48)	0.43 (0.23-0.75)	0.475 (0.4-0.5275)	0.43 (0.335-0.555)	0.35 (0.2975-0.3575)	0.3 (0.26-0.3175)	0.335 (0.3225-0.355)	0.335 (0.2925-0.4225)	0.16	0.091
Glucose6phosphate	0.05 (0.025-0.06)	0.095 (0.06-0.1)	0.05 (0.0425-0.0725)	0.06 (0.0525-0.0675)	0.05 (0.0425-0.05)	0.06 (0.05-0.1)	0.045 (0.04-0.0725)	0.035 (0.0225-0.04)	0.173	0.093
Leucine	6.73 (5.7525-7.115)	7.73 (6.9475-8.5875)	6.08 (5.8175-7.2575)	7.455 (6.2025-8.0625)	6.425 (5.875-6.6375)	5.705 (5.12-6.2825)	6.19 (5.4275-6.78)	5.215 (4.6875-5.87)	0.182	0.095
Glycine	5.245 (5.0075-5.325)	5.55 (5.37-5.715)	5.205 (4.76-5.4325)	5.255 (4.72-5.3475)	5.17 (4.78-5.29)	5.195 (4.975-5.49)	5.095 (4.9525-5.4925)	4.87 (4.8125-5.0475)	0.183	0.098
HippuricAcid	0.58 (0.17-0.885)	0.165 (0.105-0.255)	0.225 (0.0775-0.575)	0.175 (0.12-0.35)	0.305 (0.2675-0.3425)	0.155 (0.115-0.18)	0.495 (0.24-0.525)	0.12 (0.05-0.22)	0.201	0.1
LinoleicAcid	1.125 (0.8925-1.8375)	1.44 (0.905-4.105)	1.565 (1.1275-1.935)	1.07 (0.925-1.17)	1.555 (0.705-2.2625)	1.62 (1.475-1.66)	1.26 (1.035-2.085)	2.45 (1.81-3.45)	0.221	0.102
4hydroxyPhenyllacticAcid	43.6 (41.935-44.59)	76.11 (45.3975-103.5075)	38.925 (29.4875-69.835)	28.425 (23.945-60.4975)	22.36 (15.08-31.605)	33.825 (27.475-38.5325)	36.15 (27.8125-46.3175)	30.375 (23.755-36.5975)	0.248	0.105
alphaTocopherol	0.05 (0.035-0.05)	0.05 (0.03-0.0925)	0.025 (0.02-0.03)	0.04 (0.0325-0.0475)	0.05 (0.0175-0.06)	0.045 (0.04-0.0575)	0.05 (0.035-0.0575)	0.05 (0.035-0.05)	0.284	0.107
Glycerol1phosphate	0.285 (0.1775-0.355)	0.6 (0.3325-0.965)	0.29 (0.1775-0.4325)	0.305 (0.24-0.7375)	0.245 (0.2-0.29)	0.26 (0.1775-0.35)	0.17 (0.17-0.2675)	0.16 (0.1225-0.1675)	0.308	0.109
PalmiticAcid	88.68 (83.675-107.1625)	84.72 (74.9575-112.295)	95.045 (92.7625-111.75)	77.825 (71.0425-87.1875)	101.7 (90.1075-108.3725)	103.21 (94.66-107.3575)	101.58 (91.0125-104.88)	99.665 (90.74-124.8275)	0.388	0.111
OxalicAcid	39.945 (28.78-48.6125)	44.145 (32.0275-52.2125)	45.025 (38.9825-47.9475)	39.995 (23.9-46.9325)	40.53 (27.575-49.6525)	27.29 (27.09-37.5925)	24.895 (17.5-38.755)	41.515 (30.895-53.4025)	0.472	0.114
dMannonicAcid	0.035 (0.03-0.04)	0.04 (0.03-0.05)	0.035 (0.0225-0.0475)	0.035 (0.0225-0.055)	0.03 (0.03-0.045)	0.03 (0.03-0.0375)	0.04 (0.0325-0.04)	0.025 (0.02-0.03)	0.48	0.116
XylonicAcid	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.0025-0.01)	0.005 (0-0.01)	0.01 (0.0025-0.01)	0.005 (0-0.01)	0.01 (0.0025-0.01)	0.005 (0-0.01)	0.558	0.118
GlutamicAcid	9.005 (7.5975-10.9675)	11.17 (8.82-11.4875)	10.84 (8.605-12.5575)	10.115 (9.24-11.6275)	9.13 (7.3725-11.1875)	10.235 (8.45-12.95)	10.265 (9.055-12.645)	9.66 (9.0825-10.47)	0.565	0.12
FumaricAcid	2.255 (1.4775-4.66)	3.555 (2.92-5.8925)	3.01 (1.7425-4.2175)	3.735 (2.5825-5.15)	2.205 (1.3225-3.23)	2.51 (2.14-2.85)	3.295 (2.325-3.35)	2.265 (1.4775-2.88)	0.574	0.123
PipecolicAcid	0.05 (0.04-0.0975)	0.05 (0.0325-0.06)	0.05 (0.0325-0.075)	0.05 (0.0425-0.0575)	0.06 (0.05-0.0775)	0.07 (0.0625-0.0775)	0.085 (0.0575-0.0975)	0.05 (0.05-0.065)	0.61	0.125
2HydroxyButyricAcid	0.92 (0.505-3.5925)	0.625 (0.6125-1.29)	0.855 (0.81-1.1175)	0.855 (0.72-1.035)	1.225 (0.545-2.595)	2.22 (1.5575-3.01)	2.315 (1.94-2.4425)	3.89 (3.1475-5.1875)	0.622	0.127
4HydroxybenzoicAcid	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.1 (0.1-0.1075)	0.1 (0.1-0.1075)	0.1 (0.1-0.1075)	0.1 (0.1-0.1)	0.1 (0.1-0.1075)	0.1 (0.0925-0.1075)	0.64	0.13

Cholesterol	12.805 (9.075-15.8675)	10.565 (6.5925-21.7375)	10.095 (8.24-12.3325)	12.525 (9.7775-14.8675)	11.705 (11.035-13.4175)	10.22 (10.0875-12.61)	11.825 (9.15-16.0525)	9.815 (7.4325-10.15)	0.709	0.132
Maltose	0.01 (0.01-0.025)	0.015 (0.0025-0.02)	0.01 (0.01-0.025)	0.015 (0.01-0.0275)	0.025 (0.0125-0.0375)	0.015 (0.0025-0.02)	0.01 (0.01-0.025)	0.01 (0.0025-0.0175)	0.715	0.134
Indole3propanoicAcid	1.095 (0.3075-1.32)	1.025 (0.8375-1.265)	1.32 (0.6925-1.91)	1.04 (0.925-1.29)	1.35 (0.9925-1.6775)	0.895 (0.6625-1.105)	1.135 (0.965-1.605)	0.735 (0.095-1.435)	0.733	0.136
UricAcid	0.37 (0.27-0.38)	0.32 (0.0775-0.5625)	0.29 (0.2725-0.3375)	0.46 (0.41-0.4875)	0.475 (0.3675-0.5975)	0.375 (0.19-0.4775)	0.37 (0.28-0.4375)	0.22 (0.2-0.45)	0.739	0.139
4Hydroxyproline	3.14 (2.2825-3.8475)	3.63 (3.0175-4.715)	2.995 (2.6925-3.1025)	3.465 (2.0575-6.095)	2.925 (2.18-4.135)	3.605 (2.565-4.3225)	3.17 (3.0625-3.465)	2.825 (2.59-3.255)	0.789	0.141
Glycerol	0.695 (0.56-0.8675)	0.76 (0.6025-1.0825)	0.885 (0.795-0.945)	0.695 (0.495-0.94)	0.94 (0.7375-0.9925)	0.795 (0.75-0.8475)	0.705 (0.6375-0.9525)	0.875 (0.79-0.9525)	0.887	0.143
Ethanolamine	0.375 (0.355-0.41)	0.34 (0.3225-0.395)	0.385 (0.3275-0.39)	0.415 (0.3775-0.445)	0.405 (0.37-0.44)	0.35 (0.3225-0.3775)	0.39 (0.33-0.4275)	0.375 (0.34-0.44)	0.917	0.145
OleicAcid	2.37 (1.5675-8.265)	2.955 (1.6825-7.145)	3.08 (2.495-3.9725)	2.5 (2.4475-3.145)	3.165 (1.495-5.4725)	4.42 (3.9275-4.935)	2.68 (1.9275-3.5675)	6.42 (3.695-7.7875)	0.918	0.148
2HydroxyisobutyricAcid	1.92 (1.5475-2.2175)	2.135 (1.925-2.5475)	2.41 (1.365-3.02)	2.075 (1.4675-2.48)	2.01 (1.83-2.1825)	1.94 (1.53-2.53)	2.175 (2.02-2.42)	2.09 (1.84-3.6075)	0.925	0.15

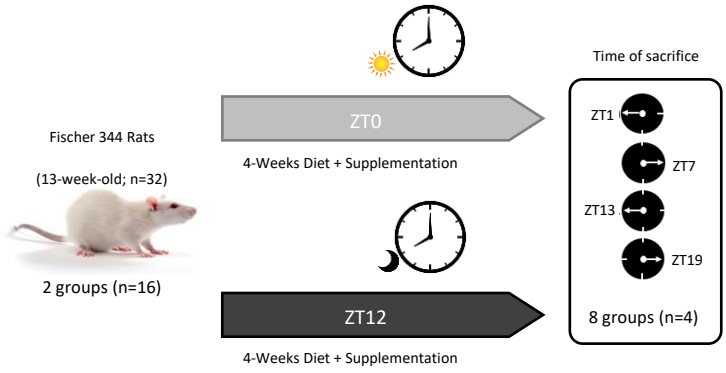
*Data shown as Median (Interquartile Range) (IQR) in Arbitrary units. P-value by Kruskal-Wallis' test; * indicates significant differences between ZT0 vs ZT12 by Mann-Whitney p < 0.5. # Indicates tendency between ZT0 vs ZT12 by Mann-Whitney p < 0.1.*

Supplementary 8. Circadian rhythm estimation of serum metabolites

Metabolite	ZT0 (p-value)	ZT12 (p-value)	Metabolite	ZT0 (p-value)	ZT12 (p-value)
2HydroxyButyricAcid	0.235	0.000***	Glycerol1phosphate	0.301	0.055#
2Hydroxyisobutyric Acid	0.937	0.801	Glycerol	0.142	0.438
2HydroxyisovalericAcid	0.048*	0.199	Glycine	0.825	0.278
3hydroxybutyricAcid	0.345	0.000***	GlycolicAcid	0.320	0.452
3HydroxyisovalericAcid	0.711	0.487	Heptanoic	0.882	0.808
3PhosphoglycericAcid	0.664	0.205	HippuricAcid	0.318	0.376
4HydroxybenzoicAcid	0.710	0.754	Indole3propanoicAcid	0.470	0.432
4hydroxyPhenyllacticAcid	0.099#	0.053#	Isoleucine	0.309	0.001**
4Hydroxyproline	0.815	0.516	LacticAcid	0.015*	0.735
AconiticAcid	0.703	0.263	Leucine	0.789	0.001**
Alanine	0.021*	0.003**	LinoleicAcid	0.859	0.106
alphaKetoglutaricAcid	0.718	0.353	MalicAcid	0.522	0.090#
alphaTocopherol	0.130	0.711	Maltose	0.443	0.510
Aspartic	0.722	0.096#	Methionine	0.391	0.001**
Cholesterol	0.562	0.121	myoInositol	0.896	0.081#
CitricAcid	0.070#	0.824	OleicAcid	0.355	0.079#
dFructose	0.007**	0.203	Ornithine	0.392	0.041*
DL2HydroxyglutaricAcid	0.774	0.245	OxalicAcid	0.084#	0.238
dMannitol	0.058#	0.442	Oxoproline	0.313	0.317
dMannonicAcid	0.921	0.207	PalmiticAcid	0.657	0.050*
DodecanoicAcid	0.627	0.011*	Phenylalanine	0.744	0.001**

dSucrose	0.161	0.869	PhosphoricAcid	0.774	0.003**
dThreitol	0.011*	0.278	PipecolicAcid	0.275	0.033*
Ethanolamine	0.595	0.717	Proline	0.334	0.191
Fructose6phosphate	0.914	0.172	PyruvicAcid	0.354	0.755
FumaricAcid	0.822	0.042*	Serine	0.244	0.024*
GalacturonicAcid	0.420	0.011*	SuccinicAcid	0.480	0.241
Glucose6phosphate	0.953	0.258	Taurine	0.048*	0.346
Glucose	0.993	0.438	ThreonicAcid	0.407	0.185
GlutamicAcid	1.000	0.867	Threonine	0.324	0.016*
Glutamine	0.982	0.189	UricAcid	0.095#	0.406
GlycericAcid	0.498	0.278	Valine	0.328	0.001**
Glyceraldehyde3phosphate	NA	NA	XylonicAcid	0.710	0.394

The values are the *estimation of circadian rhythm obtained by Cosinor method. * Circadian rhythm and parameters significantly detected (Cosinor analysis. p<0.05). # Indicates tendency (Cosinor analysis. p<0.1)*



Supplementary 9. Experimental design. *Rats were supplemented sugar at ZT0 and ZT12 and sacrificed in 4 different time points in order to analyze the oscillation of various parameters and evaluate the metabolic profile and circadian rhythm. At the end of the experimental period, animals were sacrificed at ZT1, ZT7, ZT13 and ZT19.*

Supplementary 10. Nucleotide sequences of primers used for PCR amplification in hypothalamus.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC
Rorα	CCCGATGTCTTCAAATCCTTAGG	TCAGTCAGATGCATAGAACACAAACTC
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG
Rev-erbα	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT
Pomc	CCTCAGAGAGCTGCCTTTCC	CCTGAGCGACTGTAGCAGAA
Cart	TTAACAACAATAAAGTTTGCGTTCC	CACACATACCAACACCATTCAAG
Npy	TGGCCAGATACTACTCCGCT	CTCAGGGCTGGATCTCTTGC
Argp	GCAGACCGAGCAGAAGATGT	GCGGTTCTGTGGATCTAGCA
Nr3c1	AGCATTACCACAGCTCACCC	CTGCATACAACACCTCGGGT
Nr3c2	TTCAGTATGCAGCCCTGTGG	CACTGGGAAACTGCCAAAGC
Ppia	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG

Manuscript 3

Objective: to elucidate the circadian rhythms in CAF-diet obese Fischer 344 rats and to investigate in these animals the PACs effects on their circadian metabolism rhythms.

Grape seed proanthocyanidin extract (GSPE) restores circadian rhythm of central clock and cardiovascular risk factors in CAF diet-induced obese rats

Jorge R. Soliz-Rueda[1], Raúl López-Fernández-Sobrino[1], Harriët Schellekens[2, 3], Francisca Isabel Bravo[1], Manuel Suárez[1], Miquel Mulero[1] and Begoña Muguerza[1]*

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Proanthocyanidins modulate central clock, restore circadian rhythmicity, and improve cardiometabolic risk factors in CAF diet-induced obese rats in a time-of-day-dependent manner

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Keywords: biological rhythms, clock genes, cosinor, chrononutrition, zeitgebers.

Abbreviations: SCN: Hypothalamic suprachiasmatic nucleus; RHT: Retinohypothalamic tract; STD: Standard chow diet; BW: Body weight; SCM: sweetened condensed milk; EI: electronic impact; T3: Triiodothyronine; T4: Thyroxine; NEFAs: non-esterified free fatty acids; SEM: standard error of mean; AUC: under the curve; sPLS-DA: the sparse Partial Least Squares Discriminant Analysis; PCA: principal component analysis; SIRT: several NAD-dependent deacetylases.

ABSTRACT

Circadian rhythms play an important role in the physiological and metabolic adaptation of the organism and their disruption increase the metabolic risk. Proanthocyanidins (PACs) are phenolic compounds which have demonstrated beneficial properties on metabolic disorders, although the bioactivity of these compounds may vary depending on the moment in which are administered. Different molecular mechanisms have been involved in their healthy properties, including modulation of circadian system. Therefore, the aim of this study was to evaluate whether the time of PACs administration may modulate their effects in an obesogenic context and if these phenolic compounds can restore the circadian disruption caused by a calorie-dense diet. Then, sixty-four Fisher rats were fed standard or cafeteria diet (CAF) for 5 weeks. After this, animals were daily administered a dose of 25mg/kg of a grape seed proanthocyanidin-rich extract (GSPE) or vehicle (VH) either at 8 a.m. or 8 p.m., (ZT0 and ZT12, respectively) for 4 weeks. Animals were sacrificed at different times, including ZT1, 7, 13 and 19. Results showed the detrimental effects of CAF diet on circadian rhythmicity of serum biochemical parameters and hormones and hypothalamic clock genes. GSPE administration improve the metabolic health of animals and restored the circadian rhythms of their biochemical parameters, hormones and clock and appetite signaling genes in a time-of-day-dependent manner, showing more effects when is administered ZT12. Notably melatonin rhythm, a key marker of the light-dark cycle, was restored by GSPE treatment at ZT12. In conclusion, PACs administration improved metabolic status in CAF-fed rats and restored circadian rhythm of central clock. Although further investigations are needed to elucidate the specific effect of PACs in these conditions, these results suggest that these phenolic compounds may modulate the circadian rhythm of the central clock contributing to the improvement of the metabolic profile, especially, when PACs were administered at night.

INTRODUCTION

The circadian rhythm regulates several metabolic and physiological processes to adapt the organism to the necessities required at each moment of the day to optimize the energy metabolism of the organisms. Thus, circadian rhythmicity has been reported for processes such as sleep-wake cycle, body temperature, blood pressure, liver metabolism, kidney activity, or gastrointestinal tract (1,2), and its disruption has been related to metabolic disorders (3–7). The main controller of this circadian rhythmicity is located in hypothalamic suprachiasmatic nucleus (SCN). This central clock oscillates autonomously by self-regulatory transcriptional-translation feedback loops (8). Thus, the heterodimers of CLOCK (circadian locomotor kaput protein production cycles) and BMAL1 (brain and muscle ARNT 1, also known as ARNTL) promote the up-regulation of transcription of period (PER1 and PER2) and cryptochrome (CRY1 and CRY2) proteins. PER and CRY proteins form a complex in the nucleus downregulating the CLOCK/BMAL1 heterodimer and its own transcription during the day. The subsequent degradation of PER and CRY proteins leads to the restart of the cycle with a period of approximately 24 hours. CLOCK/BMAL1 also activate the circadian expression of the nuclear receptors RORα, RORβ, ERV-ERBα and REV-ERBβ, which play an important role in stabilizing the central clock and since their expression drives the transcription of BMAL1 and CLOCK (9–11). Finally, the CLOCK/BMAL1 heterodimer stimulates the expression of metabolic clock genes, such as nicotinamide phosphoribosyltransferase (*Nampt*) and Rev-erbα (12–15), which are involved in the modulation of energy metabolism and appetite pathways in the arcuate nucleus (Arc) of the hypothalamus (16–20). This region is composed of two main neuronal populations: Neuropeptide Y—agouti-related protein (NPY-AGRP) neurons and pro-opiomelanocortin—cocaine-amphetamine-regulated transcript (POMC-CART) neurons. On the one hand, NPY-AGRP orexigenic neurons promote decreased energy expenditure and food intake in response to ghrelin. On the

other hand, anorexigenic POMC-CART neurons promote energy expenditure and satiety in response to feeding via leptin (16,21).

External cues known as zeitgebers, such as light, the most important one, or food, reprogram or alter the central and peripheral clocks (9,22). Central clock is reset by light perceived from the retina via retinohypothalamic tract (RHT) and triggers signals to keep the tissue peripheral clocks synchronized, which can, in turn, be regulated by other external signs as meal timing or food composition (23,24). However, in the last years, remarkable lifestyle changes are occurring in modern occidental societies such as variations in dietary patterns, predominating the consumption of more calorie-dense food, and alterations of the natural light/dark phases caused by the common phenomenon known as “social jetlag” (3–7). These misalignment between external cues and internal clock have been associated to the development of metabolic diseases (3–7). These metabolic disorders together with other comorbidities such as depression, sleep disturbances, cognitive dysfunction and steatohepatitis are part of the recently defined "Circadian Syndrome" (25).

The modulation and misaligning of biological rhythms by diet composition has been widely reported in experimental animals (23,24). Disturbances in the expression of circadian rhythm genes, nuclear receptors that regulate clock transcription factors, and clock-controlled genes involved in fuel utilization in the hypothalamus, liver, and adipose tissue have been reported in mice fed with high-fat diet (26). Moreover, loss of rhythmicity of hormones involved in the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) axes, such as corticosterone, thyroid-stimulating hormone (TSH) and testosterone, has been observed in both rodents and humans fed high fat diets (26–28). In addition, circadian disruptions in hepatic metabolites have been also reported in rats fed cafeteria (CAF) diet (29), This diet, which consists in highly palatable, energy dense and unhealthy human food, is considered a robust experimental model of human obesity and metabolic syndrome since (30).

Proanthocyanidins (PACs) are a class of polyphenols consisting of flavonoid polymers and their gallate derivatives, which are abundant in many foods, such as wine, tea, cocoa, and fruits, and whose consumption has been associated with multiple beneficial effects (31). PACs have demonstrated remarkable effects on metabolism, showing beneficial activities on different key aspects of metabolic syndrome (31–37). Different molecular mechanisms have been shown to be involved in the efficacy of PACs, including epigenetic modifications that have recently emerged as important mediators of their properties (38). In addition, different studies have demonstrated that PACs can act as a zeitgeber and modulate both central and peripheral clocks. In this sense, it has been reported that PACs are able to modulate central-clock genes and peripheral-clock genes in liver and gut of both healthy and CAF diet-induced obese rats (39,40). Furthermore, acute administration of PACs when the light turns on, at the zeitgebers time 0 (ZT0), modulate expression of clock genes in hypothalamus and plasma melatonin levels in healthy rats, while if these phenolic compounds were administered when the light turn off, at ZT12, modulated hepatic clock genes (41,42). In addition to this, modulatory effects on central and peripheral hepatic clocks have also been observed under jet lag conditions (41,42). Thus, the interaction of these phenolic compounds with the clock system has been recently suggested as other mechanism involved in their beneficial effects (43).

Therefore, the aim of the present study was to investigate whether the modulatory effects of PACs on the central clock in an obesogenic context depend on the time of the day when they are administered. Furthermore, we evaluated whether their beneficial effects on cardiometabolic risk factors could also depend on the time of phenolic consumption. For this purpose, CAF diet-induced obese rats were administered vehicle (VH) or PACs either at 8 a.m. or 8 p.m. (ZT0 and ZT12, respectively). Samples were obtained at 4 different time points in a 24-hour period, at 1, 7, 13 and 19 hours after the treatment (ZT1, ZT7, ZT13 and ZT19) with the aim of analyzing the circadian rhythm of

hypothalamus clock genes, major biochemical parameters, and serum hormones.

MATERIALS AND METHODS

Proanthocyanidins extract

The grape seed proanthocyanidin-rich extract (GSPE) used in this study was obtained from white grape seed and is mainly composed of catechin, epicatechin, gallic acid, epicatechin gallate and dimers, trimers and tetramers of proanthocyanidins (44). This extract was provided by Les Dérives Résiniques et Terpéniques (Dax, France).

Animal Procedures

Sixty-four 12-week-old male photosensible Fischer 344 rats from Charles River Labratories (Barcelona, Spain) were housed in pairs under standard (STD) laboratory conditions at 22°C and 12 hours light/dark cycle. After one week of acclimatization period, rats were fed CAF diet for 5 weeks. After this time, animals were weighted and randomly divided into four groups (n=16), and administered VH, which was condensed milk 1/5 diluted, or GSPE (25mg/Kg) dissolved in VH at two different time, ZT0 and ZT12, for 4 weeks. In order to obtain four different time points in a 24-hours period to analyze circadian rhythms, animals were sacrificed at ZT1, ZT7, ZT13 and ZT19 (Figure 1). The CAF diet was prepared freshly every day and contained bacon, cookie with paté, cookie with cheese, carrots, ensaïmada (pastry), STD chow and sweetened milk (22% sucrose w/v), and its caloric distribution was 56.43% carbohydrate, 45.72% lipid and 9.5% protein (45). GSPE was dissolved in condensed milk 1/5 diluted as VH to eliminate the astringent taste of the extract and the rats consumed it voluntarily. Moreover, in order to avoid the effect of our intervention at different times to give the dose, all rats were in the same room, and we made sure that all rats approached the end of the box at two administration times. Body weight (BW) and food intake (FI) were recorded weekly during the whole experimental procedure. After 9 weeks, the animals

were deprived of food for 3 hours and sacrificed by decapitation. Blood was collected in non-heparinized tubes, incubated for 1 h at room temperature and immediately centrifuged at 1200×g for 15 min at 4 °C to collect the serum. The hypothalamic samples were rapidly removed after death, frozen in liquid nitrogen and stored at -80°C until further analyses.

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

Indirect Calorimetry

Indirect calorimetry was performed on all groups for 24 h after receiving GSPE treatment or VH one week before sacrifice. For this experimental procedure were used an Oxylet Pro System (Panlab, Barcelona, Spain) and the Metabolism 2.1.02 (Panlab, Cornellà, Spain) software program. Fat and glucose oxidation were calculated with the oxygen consumption (VO2) and carbon dioxide production (VCO2) values given by the Oxylet LE 405-gas analyzer (PANLAB). At each time point, the program Metabolism 2.1.02 (PANLAB, Barcelona, Spain) calculated the respiratory quotient (RQ) as the VCO2/VO2 ratio. Using the stoichiometric equations of Frayn and assuming a nitrogen excretion rate (n) of 135 µg /kg x min, we used the formula (g/min) = 4.55 × VCO2 − 3.21 × VO2 − 2.87 n for the oxidation of carbohydrates and the formula (g/min) = 1.67 × VO2 − 1.67 × VCO2 − 1.92 n for the oxidation of fat. In order to obtain the Energy Expenditure (EE) from fat and carbohydrate in kJ/min, the fat and carbohydrate rates were multiplied by 37 and 16, respectively, using the Atwater general conversion factor. Due to the differences in metabolic activity in rodents during day and night, the results were also separated in both light and darkness phases to better appreciate differences between groups.

Serum Analysis

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

Serum hormones levels were measured by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ). Serum samples were thawed at 4 °C. 50 µL of serum were mixed with 250 µL of methanol containing the internal STD (2 ng/mL). Then, the mixture was vortexed and centrifuged for 5 minutes at 4 ºC and 252 g. The supernatant was transferred to a new tube and mixed with 700 µL of 0,1 % formic acid in water. The sample was loaded to an SPE system previously conditioned with methanol and 0,1 % formic acid in water. The cartridge was washed with 0,1 % formic acid in water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45 °C and reconstituted with 50 µL of water:methanol (60:40, v/v) and transferred to a glass vial for analysis. The hormones detected were Melatonin, Corticosterone, Triiodothyronine (T3), Thyroxine (T4) and Testosterone, analytical column was Zorbax Eclipse C18 (150 x 2.1 mm) from Agilent Technologies.

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 1 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as a percentage of the STD-VH group using the the 2-ΔΔCt method with Ppia gene as an endogens control, as reported by Schmittgen and Livak (33).

Statistical Analysis

BW gain and indirect calorimetry data were represented as means ± STD error of mean (S.E.M) 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-mesures 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 in order to analyze this data, as indicated in the respective figure legend were applied to explore the origin of outcomes. Theses statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

In order to analyze the circadian rhythms of the different parameters we used Cosinor-based rhythmometry method. We considered the presence of circadian rhythm when the model of the expressions of each gene fitted the cosine curves ($P < 0.05$). For this, a script was developed by J.R. S-R using PyCharm software (v.2018.2.4, JetBrains s.r.o., Prague, Czech Republic) with Python version 3.7.4 and the estimated circadian rhythm was plotted using the CosinorPy package (v.1.1) (47).

RESULTS

Proanthocyanidins administration showed a greater decrease in body weight gain and increase in energy expenditure when was given at ZT12

GSPE administration resulted in a decrease in BW gain unequally between the two groups (Figure 2A and 2B). Thus, while GSPE admisnitration at ZT0 showed a tendency to decrease BW gain compered to VH, treatment at ZT12 resulted in a substantial decrease in BW gain during the 4 weeks of treatment period. Furthermore, analysis of the area under the curve (AUC) mirrored these results (Figure 2C), showing a tendency to decrease in BW gain of rats GSPE administered at ZT12 compared to those administered at ZT0 ($p=0.06$). Interestingly, EE was stimulated by GSPE when was administered at ZT12 (Figure 2D). This group showed 7.51% higher energy expenditure during the whole day compared to VH rats ($p=0.001$). Moreover, this difference tended to be greater than the found in ZT0 rats ($p=0.062$). Analysis of EE separating light (Figure 2E) and dark (Figure 2F) phases revealed that, on the one hand, GSPE treatment at ZT0 tended to increase EE in the light phase ($p=0.076$) while no differences were found in the dark phase compared to their control rats (ZT0-VH). On the other hand, the increase in EE was significant in both phases when GSPE was administered at ZT12 compared to their VH groups ($p=0.015$ and $p=0.005$, respectively). Regarding the source of energy used by the animals (Table S1), GSPE treatment stimulated increased fat oxidation at both dosing times ($p<0.001$ and $p=0.049$, respectively for ZT0 and ZT12). In addition, GSPE administration at ZT0 reduced energy from carbohydrate oxidation, both in the whole day ($p<0.001$) and in the two phases ($p=0.023$ and $p<0.001$, respectively for light and dark phases). These differences were not found for the GSPE-administered groups at ZT12. Additionally, no differences were found in food and energy intake (Table S1).

Circadian rhythmicity of serum biochemical parameters and insulin levels were restored by proanthocyanidins

In order to analyze the impact of treatments on circadian rhythms, the Cosinor method was used to estimate the rhythmicity of the different parameters. Most of the biochemical parameters did not show a clear circadian rhythm in the VH groups (Figure 3 and Table S2). Regarding serum glucose (Figure 3A), the VH groups showed a tendency to have circadian rhythm (p=0.052 and p=0.094, respectively for ZT0-VH and ZT12-VH), however only for the GSPE treatment at ZT12 a clear circadian rhythm was detected (p=0.008). Interestingly, the acrophases were totally different between the GSPE and VH groups at ZT12 (p<0.001) (table S2 and S3). Additionally, GSPE treatment at ZT12 resulted in increased glucose levels in ZT1 (p=0.029) and decreased glucose levels in ZT19 (p=0.021) compared to the ZT12-VH group by Mann-Whitney test (Figure S1). For serum insulin (Figure 3B), no circadian rhythm was detected for the groups receiving VH, nor did GSPE treatment at ZT12 display circadian rhythm. However, the group that received treatment at ZT0 showed a tendency to have circadian rhythm (p=0.055) with an acrophase around ZT7 (Table S2). No differences were found by Mann-Whitney test between GSPE treatments and VH at each time point. Other biochemical parameters such as cholesterol, triglycerides and NEFAs (Figure 3C-3E) were also studied. No circadian rhythms were detected for either group receiving VH at ZT0 or ZT12 in serum cholesterol and NEFAs nor for the ZT12-VH group in serum triglycerides. Interestingly, for both GSPE-treated groups at ZT0 and ZT12 circadian rhythms were detected for these parameters (Table S2)..

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Proanthocyanidin treatment showed the capacity to restore the circadian rhythm of melatonin when administered at ZT12

The circadian rhythm for serum corticosterone was detected for all 4 experimental groups (Figure 4A) showing acrophase at the onset of the dark phase. Regarding the glucocorticoid receptor genes Nr3c1 and Nr3c2 (Figure 4B and 4C), no circadian rhythms were detected for the GSPE-treated groups at ZT0 and ZT12 for the Nr3c1 gene. However, circadian rhythm was observed for the Nr3c2 gene when animals were treated with GSPE at ZT12 (p=0.049) (table S4). The circadian rhythm of melatonin was not detected for the groups receiving VH at both ZT0 and ZT12 (Figure 4D). Interestingly, only the GSPE-treated group at ZT12 showed a clear circadian rhythm for serum melatonin levels (p=0.023) with an acrophase around ZT21 (Table S2), whereas no rhythmicity was detected for the treated group at ZT0. Furthermore, for the groups receiving VH, no rhythmicity was detected for serum testosterone

(Figure 4E). Nevertheless, both GSPE-treated groups at both ZT0 (p=0.042) and ZT12 (p=0.001) showed a clear circadian rhythm with acrophase in the dark phase (Table S2). The two main thyroid hormones were also studied (Figure S2). No circadian rhythm was detected for either group in serum T3, T4 and T3-to-T4 ratio, nor differences between groups at each time point by Mann-Whitney test.

Proanthocyanidin treatment at ZT12 restored the circadian rhythm of leptin and modulated appetite signaling gene expression

Circadian rhythmicity of serum leptin was not detected in CAF-fed rats that received VH (Figure 5A). Moreover, GSPE treatment at ZT0 showed no ability to restore the rhythmicity of this parameter. Interestingly, circadian rhythm was detected for the group treated with GSPE at ZT12 (p=0.018), showing acrophase in the light phase (Table S2). Additionally, *Cart* gene expression tended to show circadian rhythm in all 4 experimental groups (Figure 5B). However, the acrophase of the groups treated at ZT0 was detected in the light phase, whereas the acrophase of the groups treated at ZT12 showed acrophase in the dark phase (p=0.003) (Tables S4 and S5). *Pomc* gene expression showed a circadian rhythm for both groups receiving VH (p=0.008) and GSPE (p=0.016) at ZT0 with acrophase in the dark phase (around ZT16) (Figure 5C and Table S4). Additionally, although no rhythmicity was detected in the groups that received the dose at ZT12, there was a tendency to increase *Pomc* expression with GSPE treatment at ZT1 (p=0.083) and ZT13 (p=0.083) compared to the VH group by Mann-Whitney test (Figure S3). In the case of the *Argp* and *Npy* genes (Figure 5D and Figure 5E), no circadian rhythm was detected in any group that received the dose at ZT0, nor for the *Npy* gene in the groups that were treated at ZT12. Interestingly, *Argp* gene expression did show circadian rhythm in the group treated with GSPE at ZT12 (Table S4). Furthermore, GSPE treatment at this time resulted in a tendency to increase *Argp* expression at both ZT13 (p=0.083) and ZT19 by Mann-Whitney test (p=0.083) (Figure S3).

Proanthocyanidins modulated the expression of clock genes in the hypothalamus in a time-of-day-dependent manner

In order to determine the state of the central clock in a context of obesity, the rhythmicity of the expression of hypothalamic clock genes was analyzed with the Cosinor method. Circadian rhythmicity of *Bmal1* gene expression was not detected when VH was administered at ZT0 (Figure 6A). GSPE treatment in the morning showed circadian rhythmicity ($p=0.014$) with acrophase around ZT1 (Table S4). Interestingly, the rhythmicity of the group receiving VH at ZT12 was detected ($p=0.032$) as well as the GSPE-treated group at ZT12 ($p=0.001$). However, a significant difference ($p<0.001$) in the acrophases of these groups was observed (Table S5). While the acrophase of the VH group is around ZT2, the GSPE-treated animals showed their acrophase around ZT23 (Table S4). No circadian rhythmicity of *Rev-erbα* gene expression was detected for the VH groups at both ZT0 and ZT12 (Figure 6B). Treatment with GSPE resulted in rhythm recovery whether the dose was administered in the morning ($p<0.001$) or in the evening ($p=0.006$). In both cases, acrophase in these groups was detected in the light phase, around ZT9 and ZT6 respectively for ZT0-GSPE and ZT12-GSPE (Table S4). Moreover, GSPE treatment at ZT0 resulted in increased Rev-erbα expression at time point ZT13 compared to the VH group ($p=0.043$) by Mann-Whitney test (Figure S4). In the case of *Rorα* gene expression (Figure 6C), no circadian rhythm was detected for the VH group when administered at ZT0, nor was it possible to detect the circadian rhythm for the groups that received the VH or GSPE dose at ZT12. Interestingly, the group treated with GSPE at ZT0 showed circadian rhythm gene expression ($p=0.02$) with acrophase around ZT9 (Table S4). Additionally, an increase in expression with GSPE treatment at ZT0 was observed at time point ZT1 compared to the VH group ($p=0.043$) by Mann-Whitney test (Figure S4). Finally, for the Nampt gene (Figure 6D), no circadian experiment was detected for any group, only GSPE treatment at ZT12 tended ($p=0.092$) to show circadian rhythm for Nampt expression. Furthermore, GSPE treatment at ZT0 resulted in increased Nampt expression at

time point ZT13 compared to the VH group (p=0.021) by Mann-Whitney test (Figure S4).

DISCUSSION

In recent years, it has been reported that diet compositions have an important impact on the circadian rhythm of the organism, altering its normal rhythmicity and causing changes in behavior, metabolism, and some physiological processes (23,24,26). These alterations could lead to the worsening of conditions associated with diabetes, cardiovascular diseases, dyslipidemia, among others, increasing the risk of developing metabolic syndrome (3–7,25). PACs have demonstrated wide beneficial effects on metabolic disorders related to metabolic syndrome (31–37). Studies about the molecular mechanisms by which PACs exert these activities have showed some of the metabolic pathways by which their effects are mediated, which involved to nuclear receptors and epigenetic modifications (38). In addition, our group has demonstrated PACs effects on central and peripheral clocks (39–42). The administration of other phenolic compounds has been also demonstrated to regulate the circadian rhythms (39,43,48,49). Indeed, the interaction of phenolic compounds with the clock system has been suggested by our group as other potential mechanism involved in phenolic beneficial effects (43). Therefore, the aim of the present study was to evaluate, in an obesogenic context, the GSPE modulator effect on circadian rhythmicity of central clock genes and serum hormones and cardiometabolic risk factors. In addition, since the effects of these compounds can also be affected by the time of day in which they are consumed (50,51), this study also investigated the PACs effects on circadian system in a time-of-day dependent manner. To achieve these purposes, four groups were fed CAF diet for 5 weeks and after this time the groups were administered VH or GSPE at ZT0 or ZT12 for 4 weeks more and sacrificed at four different times to study their circadian system.

Due to its high fat and carbohydrate content, CAF diet can induce a clinical picture of obesity and other comorbidities characteristic of the metabolic syndrome and it is considered a good experimental model for the study of this disease (30,52). As was expected CAF-fed rats showed a progressive increase in BW gain along the study. Interestingly PACs administration resulted in a reduction of BW gain, although different effects were observed depending on PACs time of administration, since the PACs effects on BW gain were higher in the animals administered at ZT12 than at ZT0. In this regard, other studies have reported lower BW gain after PACs administration in CAF-fed rats respect to the animals administered vehicles; however, the PACs were administered in the morning and GSPE doses used either were higher than the 25 mg/kg used in this study (53,54) or was the same dose but PACs were administered combined with other bioactive compounds. Additionally, GSPE administration stimulated EE during the whole day in rats that were treated at ZT12. Treatment at ZT0 only showed this effect during the light phase. In this context, although other studies reported an increase in EE after GSPE administration when treatment was administered in the morning, the doses were also higher than the used in this study (55,56). The higher decrease in BW gain in the animals administered at night could be due to the stimulation of EE by GSPE treatment. Moreover, the difference between the GSPE-administered groups in decreasing BW gain and stimulating EE could be related to the recently reported change in GSPE bioavailability depending on the time of day at which it is consumed (57).

It has been reported that CAF diet-induced obesity results in altered lipid and glucose profiles (52). In addition, both glucose and insulin are controlled by circadian regulation. In addition, it has been described that high-fat diets are able to alter the rhythmicity of insulin and glucose metabolism and the CAF diet is able to alter the circadian rhythm of liver metabolism (29,58,59). The results obtained in this study showed that most of the biochemical parameters studied have a lack of circadian rhythm in serum. On the one hand, GSPE treatment at

ZT0 tended to recover the circadian rhythm of insulin but not serum glucose. On the other hand, treatment at ZT12 restored the circadian rhythm of serum glucose but not insulin. Regarding triglycerides, cholesterol and NEFAs, GSPE treatment restored the rhythmicity of these parameters at both dosing times. In this sense, GSPE has shown its ability to improve the lipid or glucose profile in an obesogenic context (31–33,36). In addition, GSPE showed a modulation of the circadian rhythm of the peripheral clock components in the liver, such as *Bmal1, Cry1, Per2* and *Rorα*, (40), genes that are closely related to an improvement of lipid and glucose metabolism (60–63).

Melatonin, a key circadian marker, increases its concentration during the dark phase, being an important signal for the organism to recognize the light-dark cycle and synchronize peripheral clocks (64). Result of this study showed that melatonin was significantly affected by the CAF diet as it did not exhibit circadian rhythm in any VH group. Interestingly, GSPE treatment at ZT12 resulted in a recovery of rhythmicity in serum melatonin levels whereas this was not observed when treatment was at ZT0. In this regard, the ability of GSPE to increase melatonin levels in healthy rats when administered at ZT0, elongating melatonin circulation beyond dawn, whereas this effect was not seen when treatment was at ZT12, has been reported (42). Thus, the discrepancies in the results could be due to the fact that healthy rats had no diet-induced circadian rhythm alterations, in the present study we observed how in an obesogenic context, GSPE administered at ZT12 restored the melatonin rhythmicity. Another important hormone in the circadian control of metabolism is corticosterone. This hormone plays a key role in the hypothalamic-pituitary-adrenal (HPA) axis and exhibits a marked circadian rhythm (65,66) and its circadian disruption could lead to diseases of glucocorticoid sensitivity or resistance (67). Furthermore, the circadian maintenance of corticosterone release appears to be regulated through the action of the clock system on the expression of glucocorticoid receptors such as Nr3c1 (65,68–70). Specifically, it is known that there is a cross-regulation between Nr3c1 and Rev-erbα that

affects the expression of both receptors (71). In contrast to melatonin, in this study the circadian rhythm of corticosterone was not affected in either group. Although glucocorticoid receptors did not show clear rhythmicity in these CAF diet-fed rats, they were not sufficient to alter the rhythmicity of corticosterone levels. Testosterone and thyroid hormones act as modulators of the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) axes, respectively. These axes are important in processes such as lipid and carbohydrate metabolism and are closely related to biological rhythms (72,73). In this context, the rhythm of these hormones was not detected in VH groups, which could lead to metabolic alterations and, particularly for thyroid hormones, alterations in feeding patterns, since these hormones have an established diet-dependent circadian rhythm (74,75). Although GSPE administration restored the rhythmicity of testosterone levels at both dosing times, thyroid hormones did not recover the rhythmicity.

The most important controller of circadian rhythm is the central clock; this pacemaker is located in the hypothalamus and is able to maintain the synchronization of peripheral clocks with the environment and its oscillators through hormonal signals and neural connections (9,22). In order to study the SCN state in these groups, we analyzed the gene expression of *Bmal1*, Rev-erbα, *Nampt* and *Rorα*. The data showed that all 4 genes lost rhythmicity of gene expression of these genes in the CAF diet-induced obese rats. Regarding GSPE treatment, modulation of circadian rhythm of *Bmal1* and Nampt by GSPE when treatment was at ZT0 while modulation of *Rev-erbα* when treatment was at ZT12 was reported in healthy rats (42). In the present study, although Nampt rhythm was not recovered, gene expression of *Bmal1, Rev-erbα* and *Rorα*, was restored with treatment at ZT0, whereas when treatment was at ZT12, *Bmal1* and Rev-erbα showed circadian rhythmicity. On the one hand, previous studies demonstrated the importance of *Bmal1* in glucose metabolism where they observed that deletion of this gene leads to decreased insulin secretion (76,77). This would be in agreement with the lack of circadian rhythm of glucose levels

in VH rats that was observed in this study. On the other hand, the loss of rhythmicity of *Bmal1* gene expression could explain the modification of the circadian pattern of *Rev-erbα, Rorα*, and *Nampt*, in agreement with the defined role of *Bmal1* as a master regulator of the molecular clock system (78). The expression of Rev-erbα has been related to the maintenance of the central clock and, the regulation of energy metabolism (19). It has been observed that the absence of the expression of this gene is related to the increase in BW in male mice (20). 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 arcuate nucleus of rats promoted increased food intake and decreased *Cart* expression (79). The regulation of *Argp* or *Pomc* genes, and genes related to mitochondrial function have also been related to *Nampt* expression (11-13). In this sense, appetite signaling genes did not show rhythmicity for VH groups, which would be in agreement with the results obtained for clock genes, which lose rhythmicity. Interestingly, together with the recovery of the circadian rhythm of some clock genes, rhythmicity was observed in the expression of genes such as *Cart* or *Pomc* for those treated at ZT0, while *Cart* and *Argp* for ZT12. Finally, serum leptin only showed rhythmicity with GSPE treatment at ZT12. This hormone, secreted by fat cells, exhibit important functions in the regulation of food intake and metabolism, and leptin resistance may promote the development of obesity. In humans, leptin shows a marked circadian rhythm with a peak two hours before the onset of the activity phase, and an alteration in its secretion promotes the loss of the circadian rhythm of metabolism and feeding (80,81).

CONCLUSION

In conclusion, treatment with PACs restored the circadian rhythm of the central clock and various parameters of cardiovascular risk in a time-of-day-dependent manner, altered by the composition of the diet. Moreover, EE and decreased BW gain was stimulated more effectively when the treatment was at night.

Thus, obese rats showed a lack of rhythmicity of several biochemical parameters and key hormones regulating metabolism such as thyroid hormones, testosterone, or melatonin. In addition, the expression of clock and appetite signaling genes in the hypothalamus did not show rhythmicity in these rats. Interestingly, GSPE treatment resulted in circadian modulation of clock genes in the hypothalamus, serum biochemical parameters such as glucose, insulin, leptin, cholesterol, or triglycerides as well as appetite signaling genes and hormones in a time-of-day dependent manner. Especially remarkable was melatonin, a key marker of the light-dark cycle, which was restored with the dose at ZT12.

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FIGURES

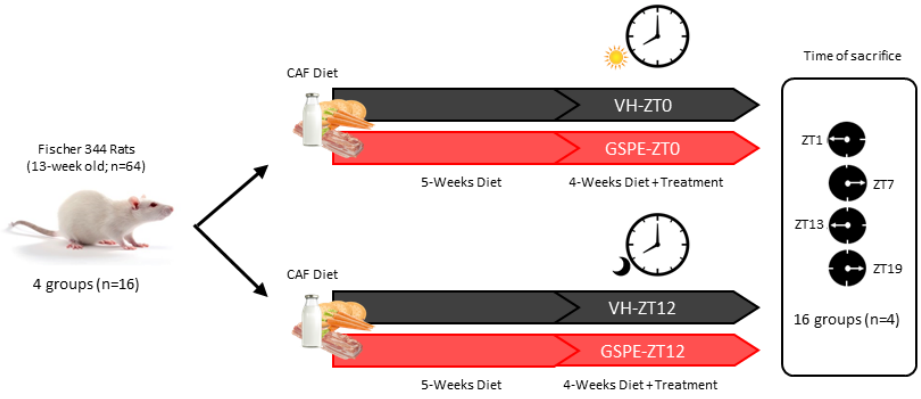

Figure 1. *Experimental design to evaluate the modulator effect of GSPE on circadian rhythm in an obesogenic context. Rats were fed with CAF diet for 5 weeks, after this time VH or GSPE were administered to the animals for 4 weeks. Animals were scarified in 4 time point to study the oscillation of parameters. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract.*

Table 1. Nucleotide sequences of primers used for PCR amplification in hypothalamus.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC
Rora	CCCGATGTCTTCAAATCCTTAGG	TCAGTCAGATGCATAGAACACAAACTC
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG
Rev-erba	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT
Pomc	CCTCAGAGAGCTGCCTTTCC	CCTGAGCGACTGTAGCAGAA
Cart	TTAACAACAATAAAGTTTGCGTTCC	CACACATACCAACACCATTCAAG
Npy	TGGCCAGATACTACTCCGCT	CTCAGGGCTGGATCTCTTGC
Argp	GCAGACCGAGCAGAAGATGT	GCGGTTCTGTGGATCTAGCA
Nr3c1	AGCATTACCACAGCTCACCC	CTGCATACAACACCTCGGGT
Nr3c2	TTCAGTATGCAGCCCTGTGG	CACTGGGAAACTGCCAAAGC
Ppia	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG

Bmal1, hydrocarbon receptor nuclear translocator-like 1; Nampt, Nicotinamide Phosphoribosyltransferase; Npy: Neuropeptide Y; Argp: agouti-related protein; Pomc: pro-opiomelanocortin; Cart: cocaine-amphetamine-regulated transcript ; Ppia, Peptidylprolyl Isomerase A.

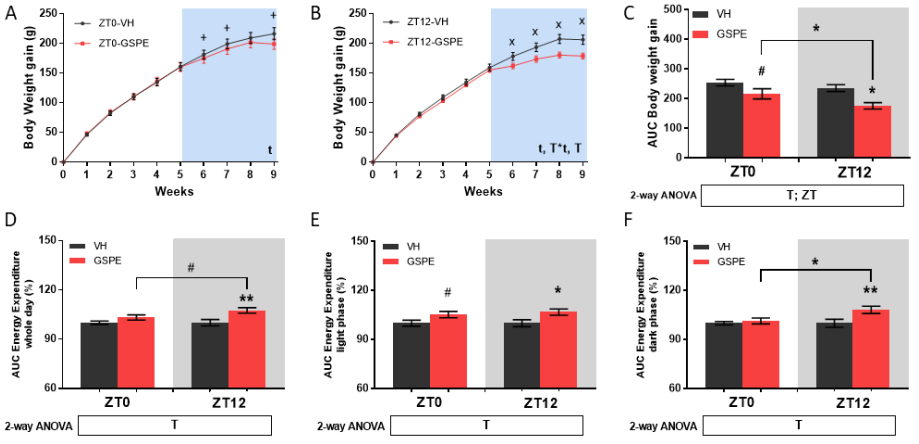

Figure 2. Body Weight Gain and Energy Expenditure. (A) and (B) Body Weight Gain along the experiment. Values are expressed as the mean ± S.E.M. (n=16). t, time effect; T, treatment effect, Txt, interaction between time and treatment effect. x Indicates significant differences using repeated measured-ANOVA followed by Student´s t test between VH vs GSPE ($p \leq 0.05$). + Indicates tendency using repeated measured-ANOVA followed by Student´s t test between VH vs GSPE (p=0.1-0.051). (C) Area under curve of Body Weight Gain during treatment period (las 4 weeks). (D) Area under curve of Energy expenditure for whole day. (E) Area under curve of Energy expenditure for light phase. (F) Area under curve of Energy expenditure for dark phase. Values are expressed as the mean ± S.E.M. (n=14-16). T, treatment effect; ZT, treatment time effect. * Indicates significant differences by treatment effect, $ Indicates significant differences by treatment time effect using 2-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$). # Indicates tendency by treatment, & Indicates tendency by treatment time effect using LSD post-hoc test (p=0.1-0.051). CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

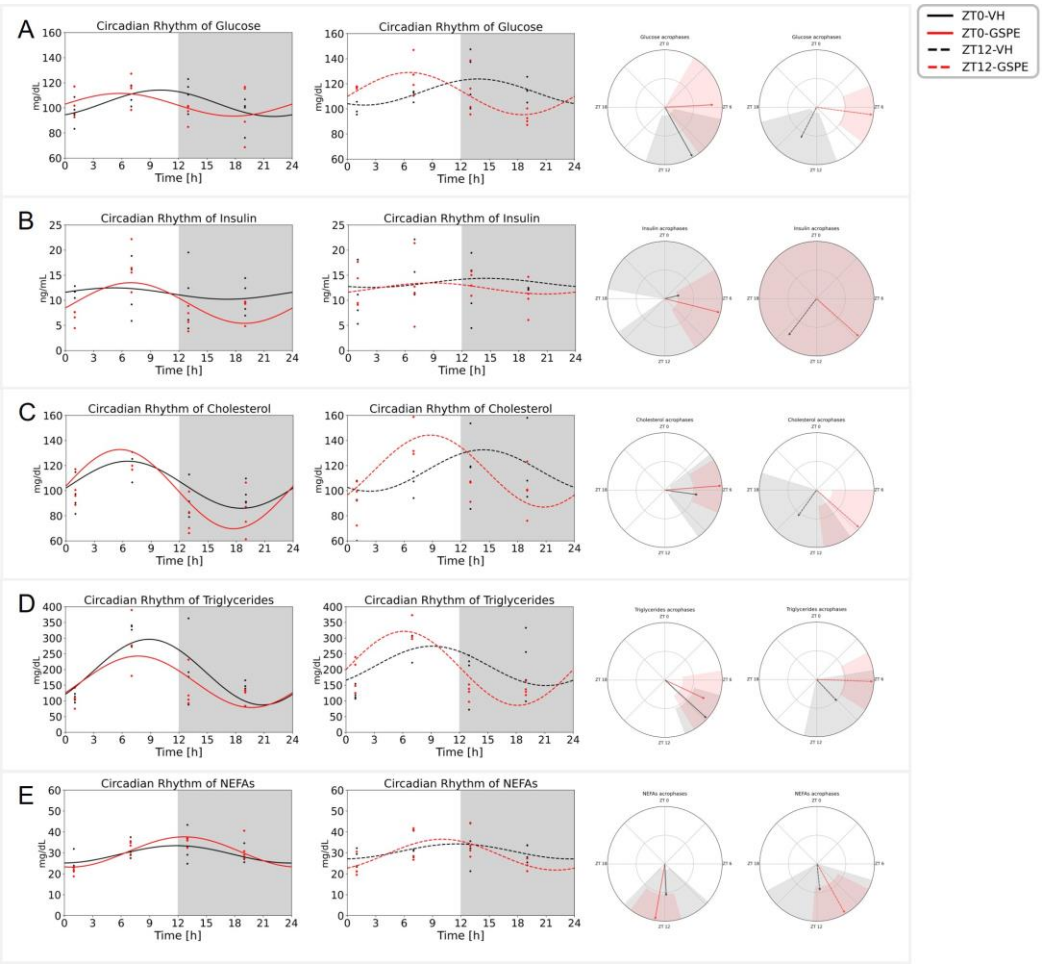

Figure 3. Serum biochemical parameters. (A) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Glucose (mg/dL) for VH and GSPE at ZT0 and ZT12. (B) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum insulin (ng/mL) for VH and GSPE at ZT0 and ZT12. (C) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Cholesterol (mg/dL) for VH and GSPE at ZT0 and ZT12. (D) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Triglycerides (mg/dL) for VH and GSPE at ZT0 and ZT12. (E) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum NEFAs (mg/dL) for VH and GSPE at ZT0 and ZT12. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

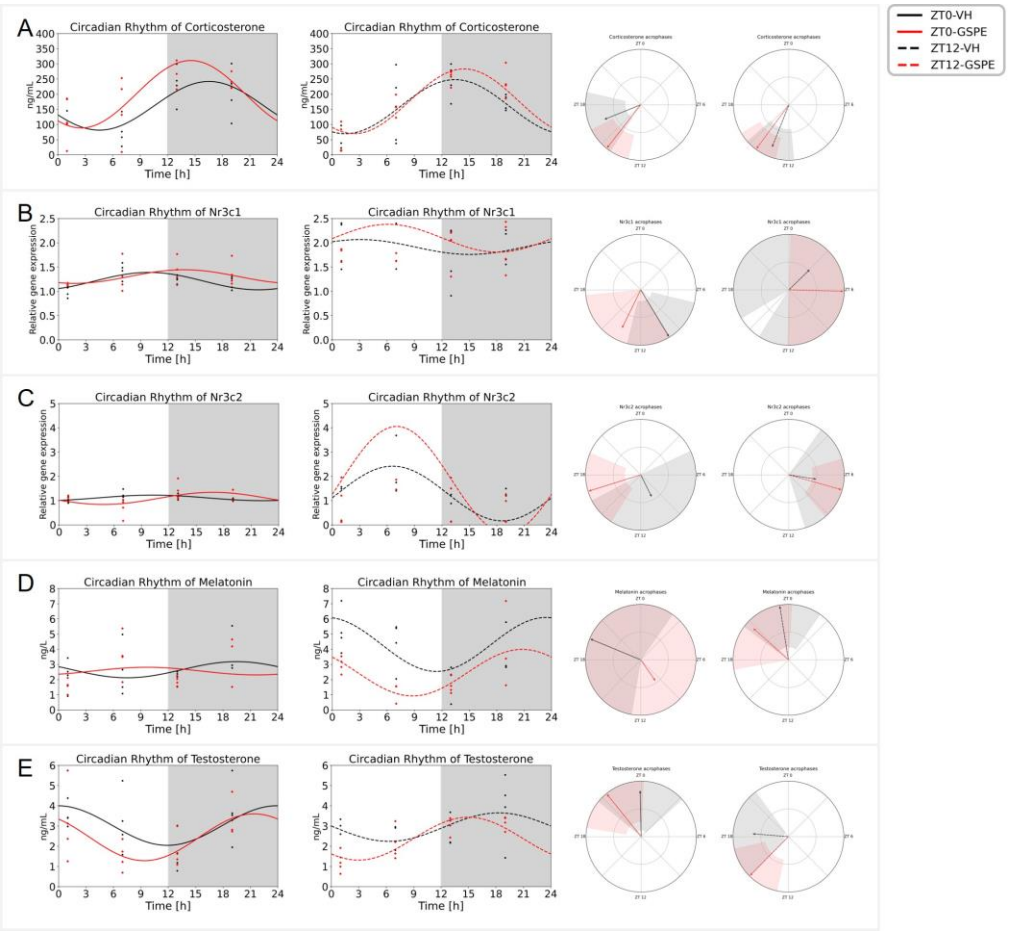

Figure 4. Serum hormones and glucocorticoid receptor genes. (A) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Corticosterone (ng/mL) for VH and GSPE at ZT0 and ZT12. (B) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Nr3c1* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (C) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Nr3c2* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (D) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Melatonin (ng/L) for VH and GSPE at ZT0 and ZT12. (E) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Testosterone (ng/mL) for VH and GSPE at ZT0 and ZT12. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

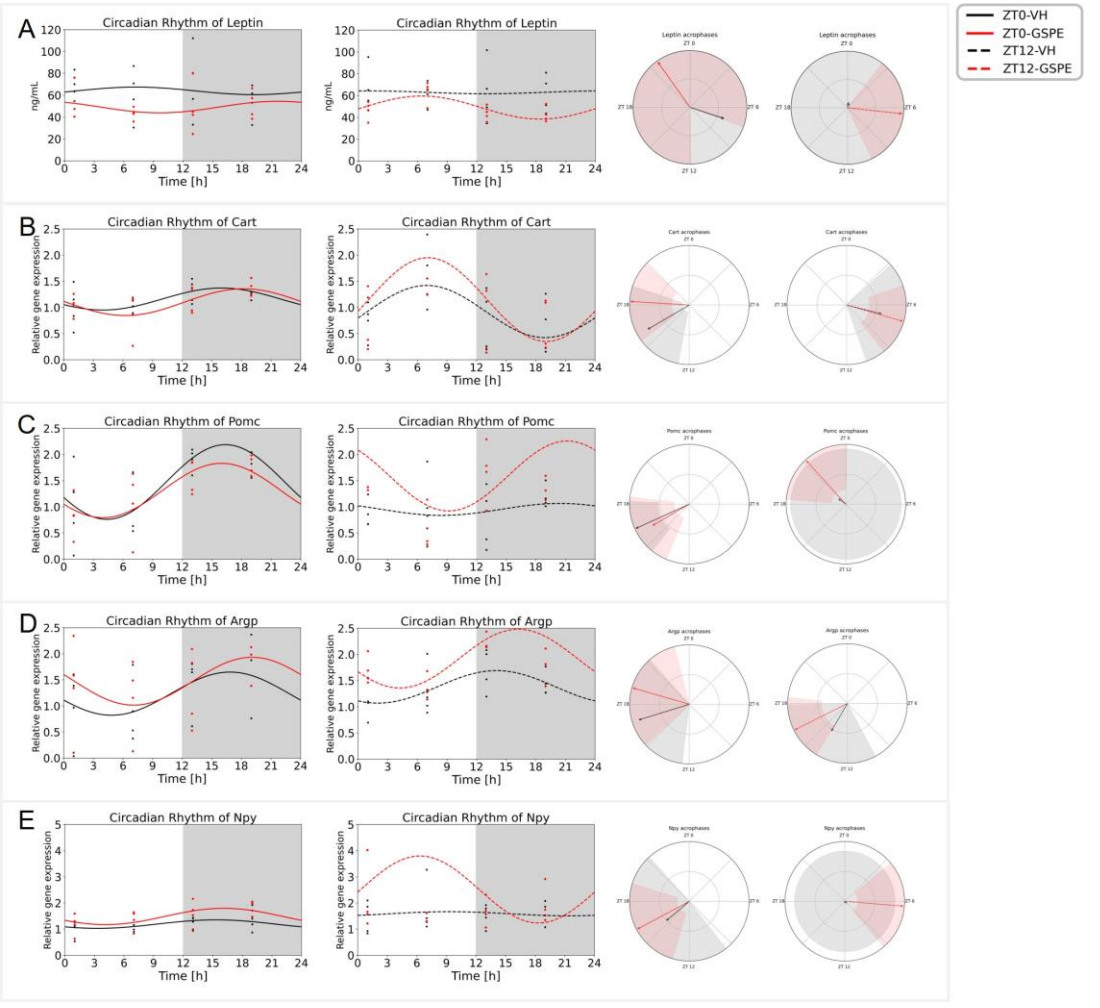

Figure 5. Serum leptin and hypothalamic appetite signaling genes. (A) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum Leptin (ng/mL) for VH and GSPE at ZT0 and ZT12. (B) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Cart* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (C) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Pomc* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (D) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Argp* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (E) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Npy* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

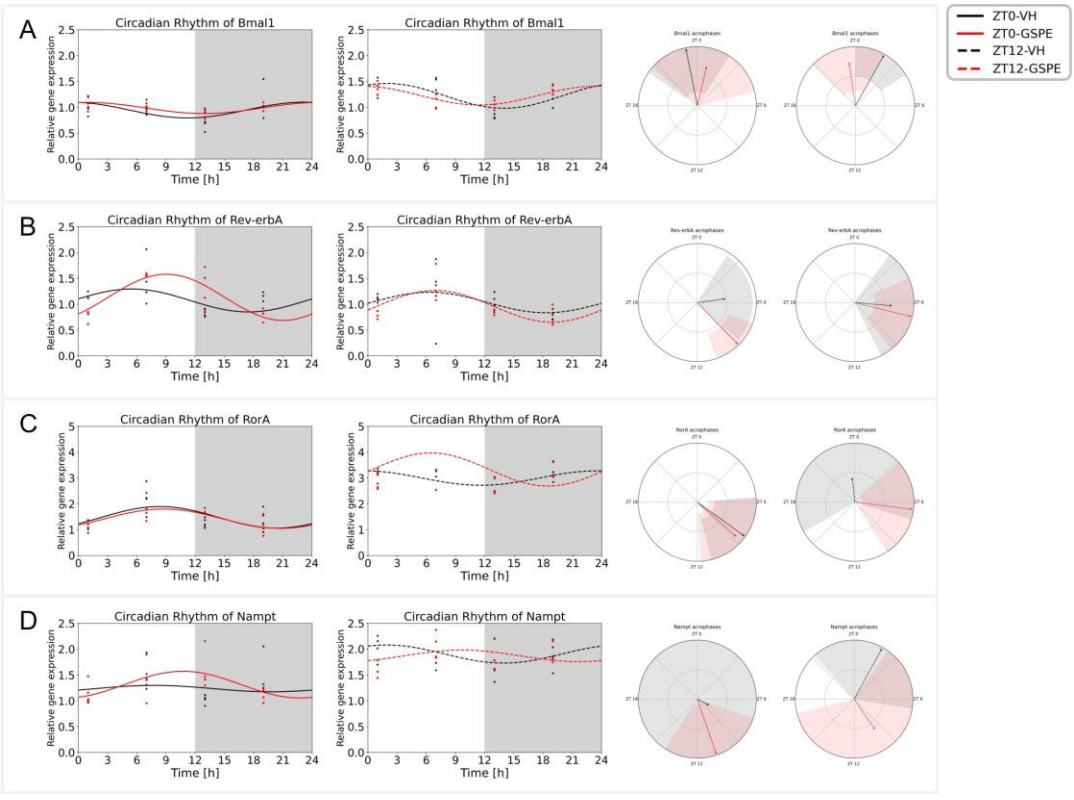

Figure 6. Hypothalamic clock genes. (A) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Bmal1* expression gene (relative gene expression) for VH and GSPE at ZT0 and ZT12. (B) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Rev-erbα* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (C) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Rorα* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. (D) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of *Nampt* gene expression (relative gene expression) for VH and GSPE at ZT0 and ZT12. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

SUPPLEMENTARY

Table S1. GSPE treatment effect on cumulative food energy intake and oxidation energy source along the experiment.

	ZT0-VH	ZT0-GSPE	ZT12-VH	ZT0-GSPE	ANOVA
Total Food Intake (g)	1314.79 (±20.71)	1371.72 (±30.4)	1261.91 (±71.29)	1285.08 (±36.97)	n.s.
Protein Intake (g)	143.05 (±2.2)	137.08 (±3.42)	127.08 (±4.04)	122.08 (±3.5)	n.s.
Fat Intake (g)	225.66 (±4.53)	220.7 (±5.94)	204.23 (±5.28)	189.75 (±7.22)	n.s.
Carbohydrates Intake (g)	913.87 (±25.26)	1000.53 (±33.95)	933.35 (±62.21)	937.33 (±29.56)	n.s.
Sugar Intake (g)	662.26 (±29.99)	770.36 (±30.93)	727.12 (±58.36)	738.5 (±29.18)	n.s.
Total Energy Intake (Kcal)	6191.35 (±129.05)	6512.25 (±190.82)	6038.61 (±278.91)	5933.07 (±162.98)	n.s.
Energy intake from Protein (Kcal)	572.19 (±8.8)	548.31 (±13.68)	508.3 (±16.16)	483.56 (±16.11)	n.s.
Energy intake from Fat (Kcal)	2030.91 (±40.78)	1986.34 (±53.44)	1838.08 (±47.52)	1707.73 (±65)	n.s.
Energy intake from Carbohydrates (Kcal)	3655.5 (±101.05)	4002.11 (±135.81)	3733.42 (±248.84)	3749.33 (±118.24)	n.s.
Energy intake from Sugar (Kcal)	2649.03 (±119.94)	3081.43 (±123.7)	2908.46 (±233.45)	2954 (±116.7)	n.s.

Energy from carbohydrates oxidation (%) whole day	100 (±12.74)	34.81 (±7.70)***	100 (±30.09)	89.22 (±32.15)	T
AUC Energy from carbohydrates oxidation (%) Light phase	100 (±38.79)	4.07 (±4.07)*	100 (±70.08)	163.98 (±110.01)	T
AUC Energy from carbohydrates oxidation (%) Dark phase	100 (±12.62)	37.54 (±8.27)***	100 (±28.32)	81.97 (±28.06)	T
AUC Energy from fat oxidation (%) whole day	100 (±4.46)	124.39 (±3.49)***	100 (±5.78)	118.74 (±6.76)*	T
AUC Energy from fat oxidation (%) Light phase	100 (±5.03)	120.81 (±3.93)**	100 (±4.80)	116.78 (±6.58)*	T
AUC Energy from fat oxidation (%) Dark phase	100 (±4.69)	128.97 (±4.42)***	100 (±8.55)	120.95 (±9.19)#	T

Values are expressed as the mean ± S.E.M. (n=14-16). T, GSPE treatment effect. * Indicates significant differences by treatment effect using 2-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$). # Indicates tendency by treatment effect using LSD post-hoc test (p=0.1-0.051). AUC, Area under curve; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

Table S2. Circadian parameters of biochemical parameters and hormones.

Parameter	Group	Period (h)	p	MESOR	Amplitude	p(amplitude)	Acrophase[h]	p(acrophase)
Glucose	ZT0-VH	24	0.052#	103.64	10.51	0.006**	10.06	<0.001***
	ZT0-GSPE	24	0.25	102.45	9.05	0.079#	5.8	0.008**
	ZT12-VH	24	0.094#	113.38	10.39	0.017*	13.81	<0.001***
	ZT12-GSPE	24	0.008**	112.14	16.8	<0.001***	6.51	<0.001***
Insulin	ZT0-VH	24	0.797	11.33	1.13	0.497	5.13	0.362
	ZT0-GSPE	24	0.055#	9.44	4.04	0.006**	6.94	<0.001***
	ZT12-VH	24	0.921	13.46	0.9	0.684	14.5	0.123
	ZT12-GSPE	24	0.784	12.35	1.1	0.481	8.79	0.105
Cholesterol	ZT0-VH	24	0.08#	104.67	18.74	0.013*	6.53	<0.001***
	ZT0-GSPE	24	0.005**	101.18	31.53	<0.001***	5.71	<0.001***
	ZT12-VH	24	0.39	116.01	16.51	0.154	14.38	<0.001***
	ZT12-GSPE	24	0.029*	115.57	28.68	0.002**	8.76	<0.001***
Triglycerides	ZT0-VH	24	0.004**	191.58	104.54	<0.001***	8.83	<0.001***
	ZT0-GSPE	24	0.018*	160.68	81.93	0.001**	7.67	<0.001***
	ZT12-VH	24	0.242	211.44	62.78	0.075#	9.12	<0.001***
	ZT12-GSPE	24	0.003**	203.64	117.91	<0.001***	6.14	<0.001***
NEFAs	ZT0-VH	24	0.131	29.29	4.15	0.029*	11.83	<0.001***
	ZT0-GSPE	24	0.001**	30.43	7.22	<0.001***	12.67	<0.001***
	ZT12-VH	24	0.295	30.7	3.59	0.101	11.61	<0.001***
	ZT12-GSPE	24	0.018*	29.14	7.37	0.001**	10.04	<0.001***
Melatonin	ZT0-VH	24	0.585	2.65	0.53	0.29	19.49	<0.001***
	ZT0-GSPE	24	0.891	2.56	0.25	0.631	9.66	0.219
	ZT12-VH	24	0.159	4.31	1.78	0.039*	23.39	<0.001***
	ZT12-GSPE	24	0.023*	2.45	1.53	0.001**	20.79	<0.001***

Corticosterone	ZT0-VH	24	0.011*	161.67	80.16	<0.001***	16.55	<0.001***
	ZT0-GSPE	24	0.005**	199.79	111.08	<0.001***	14.55	<0.001***
	ZT12-VH	24	0.013*	158.81	89.14	<0.001***	13.45	<0.001***
	ZT12-GSPE	24	<0.001***	176.55	106.57	<0.001***	14.44	<0.001***
Testosterone	ZT0-VH	24	0.134	3.02	0.98	0.03*	23.91	<0.001***
	ZT0-GSPE	24	0.042*	2.44	1.16	0.004**	21.39	<0.001***
	ZT12-VH	24	0.213	2.94	0.7	0.062#	18.3	<0.001***
	ZT12-GSPE	24	0.001**	2.37	1.06	<0.001***	14.97	<0.001***
T3	ZT0-VH	24	0.243	1.06	0.07	0.075#	12.57	<0.001***
	ZT0-GSPE	24	0.222	1,00	0.11	0.066#	5.88	0.005**
	ZT12-VH	24	0.453	1.07	0.09	0.194	10.37	<0.001***
	ZT12-GSPE	24	0.171	0.96	0.09	0.044*	8.22	<0.001***
T4	ZT0-VH	24	0.373	45.25	2.49	0.145	4.3	0.1
	ZT0-GSPE	24	0.203	45.14	3.48	0.057#	5.92	0.003**
	ZT12-VH	24	0.552	39.78	2.22	0.265	18.1	<0.001***
	ZT12-GSPE	24	0.114	38.55	3.43	0.023*	13.65	<0.001***
T3/T4	ZT0-VH	24	0.401	23.98	1.44	0.161	22.97	<0.001***
	ZT0-GSPE	24	0.713	22.79	1,00	0.404	4.69	0.306
	ZT12-VH	24	0.164	27.52	3.28	0.041*	7.78	<0.001***
	ZT12-GSPE	24	0.107	25.83	1.94	0.021*	5.64	0.001**
Leptin	ZT0-VH	24	0.912	63.96	3.46	0.669	7.22	0.418
	ZT0-GSPE	24	0.625	49.15	5.28	0.323	21.65	<0.001***
	ZT12-VH	24	0.982	62.9	1.33	0.851	0.9	0.964
	ZT12-GSPE	24	0.018*	48.92	10.58	<0.001***	6.42	<0.001***

Acrophase is the time at which the peak of a rhythm occurs; Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The values are the estimation of circadian parameters obtained by Cosinor method. * Circadian rhythm and parameters significantly detected (Cosinor analysis. $p<0.05$). # Indicates tendency (Cosinor analysis. $p<0.1$). CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

Table S3. Comparison of circadian parameters of biochemical parameters and hormones between groups.

Parameter	Groups	p	p(d_amplitude)	p(d_acrophase)
Glucose	ZT0-VH vs ZT0-GSPE	0.133	0.821	0.093#
	ZT12-VH vs ZT12-GSPE	0.007**	0.302	<0.001***
Insulin	ZT0-VH vs ZT0-GSPE	0.173	0.193	0.743
	ZT12-VH vs ZT12-GSPE	0.969	0.942	0.587
Cholesterol	ZT0-VH vs ZT0-GSPE	0.004**	0.241	0.651
	ZT12-VH vs ZT12-GSPE	0.115	0.413	0.046*
Triglycerides	ZT0-VH vs ZT0-GSPE	0.001**	0.521	0.432
	ZT12-VH vs ZT12-GSPE	0.012*	0.212	0.166
NEFAs	ZT0-VH vs ZT0-GSPE	0.003**	0.202	0.645
	ZT12-VH vs ZT12-GSPE	0.038*	0.223	0.542
Melatonin	ZT0-VH vs ZT0-GSPE	0.931	0.701	0.239
	ZT12-VH vs ZT12-GSPE	0.012*	0.809	0.259
Corticosterone	ZT0-VH vs ZT0-GSPE	<0.001***	0.376	0.168
	ZT12-VH vs ZT12-GSPE	<0.001***	0.562	0.405
Testosterone	ZT0-VH vs ZT0-GSPE	0.034*	0.767	0.25
	ZT12-VH vs ZT12-GSPE	0.007**	0.412	0.097#
T3	ZT0-VH vs ZT0-GSPE	0.186	0.561	0.175
	ZT12-VH vs ZT12-GSPE	0.184	0.939	0.563
T4	ZT0-VH vs ZT0-GSPE	0.35	0.691	0.629
	ZT12-VH vs ZT12-GSPE	0.352	0.628	0.219

T3/T4	ZT0-VH vs ZT0-GSPE	0.614	0.784	<0.001***
	ZT12-VH vs ZT12-GSPE	0.095#	0.46	0.467
Leptina	ZT0-VH vs ZT0-GSPE	0.382	0.851	0.111
	ZT12-VH vs ZT12-GSPE	0.102	0.231	0.728

Acrophase is the time at which the peak of a rhythm occurs; Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The first column (p) indicates if there are significant differences between two circadian rhythms. The circadian parameter values are the differences between two groups of each circadian parameter. * The effect of diet within ZT0 vs ZT12 (comparison Cosinor analysis. $p<0.05$). CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

Table S4. Circadian parameters of hypothalamic genes.

Genes	Group	Period (h)	p	MESOR	Amplitude	p(amplitude)	Acrophase[h]	p(acrophase)
Bmal1	ZT0-VH	24	0.192	0.95	0.15	0.053#	23,24	<0.000***
	ZT0-GSPE	24	0.014*	0.99	0.11	0.001**	0.88	0.422
	ZT12-VH	24	0.032*	1,22	0.24	0.003**	1,97	0.120
	ZT12-GSPE	24	0.001**	1,23	0.18	<0.000***	23,44	<0.000***
Rev-erbα	ZT0-VH	24	0.194	1,07	0.22	0.053#	5,49	0.006**
	ZT0-GSPE	24	<0.000***	1,13	0.45	<0.000***	9,06	<0.000***
	ZT12-VH	24	0.312	1,03	0.20	0.110	6,32	0.008**
	ZT12-GSPE	24	0.006**	0.96	0.31	<0.000***	6,93	<0.000***
Nampt	ZT0-VH	24	0.890	1,24	0.06	0.628	7,80	0.322
	ZT0-GSPE	24	0.092#	1,31	0.26	0.016*	10.73	<0.000***
	ZT12-VH	24	0.410	1,90	0.17	0.167	1,92	0.487
	ZT12-GSPE	24	0.480	1,87	0.11	0.212	9,69	0.002**
Rorα	ZT0-VH	24	0.102	1,48	0.42	0.019*	8,38	<0.000***
	ZT0-GSPE	24	0.020*	1,43	0.37	0.001**	8,77	<0.000***
	ZT12-VH	24	0.088#	3,00	0.28	0.015*	23,57	<0.000***
	ZT12-GSPE	24	0.280	3,33	0.64	0.094#	6,46	0.005**
Cart	ZT0-VH	24	0.096#	1,16	0.21	0.018*	15,97	<0.000***
	ZT0-GSPE	24	0.054#	1,10	0.26	0.007**	18,23	<0.000***
	ZT12-VH	24	0.071#	0.92	0.50	0.011*	6,94	<0.000***
	ZT12-GSPE	24	0.055#	1,15	0.80	0.007**	7,08	<0.000***
Pomc	ZT0-VH	24	0.008**	1,47	0.71	<0.000***	16,37	<0.000***
	ZT0-GSPE	24	0.016*	1,31	0.52	0.001**	15,98	<0.000***
	ZT12-VH	24	0.805	0.95	0.11	0.506	20.43	<0.000***
	ZT12-GSPE	24	0.152	1,59	0.67	0.037*	21,19	<0.000***

Argp	ZT0-VH	24	0.207	1,23	0.41	0.059#	16,85	<0.000***
	ZT0-GSPE	24	0.257	1,47	0.46	0.082#	19,08	<0.000***
	ZT12-VH	24	0.101	1,38	0.31	0.019*	14,02	<0.000***
	ZT12-GSPE	24	0.032*	1,92	0.56	0.003**	16,23	<0.000***
Npy	ZT0-VH	24	0.401	1,19	0.16	0.161	15,34	<0.000***
	ZT0-GSPE	24	0.113	1,48	0.31	0.023*	16,12	<0.000***
	ZT12-VH	24	0.944	1,59	0.08	0.735	9,34	0.407
	ZT12-GSPE	24	0.210	2,51	1,28	0.060#	6,31	0.002**
Nr3c1	ZT0-VH	24	0.021*	1,21	0.18	0.001**	9,92	<0.000***
	ZT0-GSPE	24	0.300	1,30	0.14	0.104	13,74	<0.000***
	ZT12-VH	24	0.725	1,91	0.15	0.416	3,06	0.516
	ZT12-GSPE	24	0.609	2,09	0.29	0.309	6,10	0.083#
Nr3c2	ZT0-VH	24	0.072#	1,11	0.11	0.011*	10.23	<0.000***
	ZT0-GSPE	24	0.150	1,09	0.25	0.036*	16,85	<0.000***
	ZT12-VH	24	0.058#	1,29	1,13	0.008**	6,60	<0.000***
	ZT12-GSPE	24	0.049*	1,86	2,20	0.006**	7,05	<0.000***

Acrophase is the time at which the peak of a rhythm occurs; Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The values are the estimation of circadian parameters obtained by Cosinor method. * Circadian rhythm and parameters significantly detected (Cosinor analysis. $p<0.05$). # Indicates tendency (Cosinor analysis. $p<0.1$). CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

Table S5. Comparison of circadian parameters of hypothalamic genes between groups.

Genes	Groups	p	p(d_amplitude)	p(d_acrophase)
Bmal1	ZT0-VH vs ZT0-GSPE	0.106	0.594	<0.000***
	ZT12-VH vs ZT12-GSPE	0.003**	0.505	<0.000***
Rev-erbα	ZT0-VH vs ZT0-GSPE	0.002**	0.108	0.059#
	ZT12-VH vs ZT12-GSPE	0.050*	0.463	0.798
Nampt	ZT0-VH vs ZT0-GSPE	0.388	0.241	0.695
	ZT12-VH vs ZT12-GSPE	0.602	0.684	0.080#
Rorα	ZT0-VH vs ZT0-GSPE	0.037*	0.834	0.848
	ZT12-VH vs ZT12-GSPE	0.223	0.367	<0.000***
Cart	ZT0-VH vs ZT0-GSPE	0.043*	0.726	0.294
	ZT12-VH vs ZT12-GSPE	0.029*	0.395	0.951
Pomc	ZT0-VH vs ZT0-GSPE	0.001**	0.424	0.804
	ZT12-VH vs ZT12-GSPE	0.047*	0.127	0.931
Argp	ZT0-VH vs ZT0-GSPE	0.227	0.895	0.460
	ZT12-VH vs ZT12-GSPE	0.002**	0.272	0.329
Npy	ZT0-VH vs ZT0-GSPE	0.049*	0.402	0.816
	ZT12-VH vs ZT12-GSPE	0.123	0.095#	0.902

Nr3c1	ZT0-VH vs ZT0-GSPE	0.072#	0.701	0.127
	ZT12-VH vs ZT12-GSPE	0.798	0.690	0.636
Nr3c2	ZT0-VH vs ZT0-GSPE	0.133	0.284	0.046*
	ZT12-VH vs ZT12-GSPE	0.023*	0.235	0.853

Acrophase is the time at which the peak of a rhythm occurs; Amplitude is the difference between the peak and the mean value of a wave; MESOR is a circadian rhythm-adjusted mean. The first column (p) indicates if there are significant differences between two circadian rhythms. The circadian parameter values are the differences between two groups of each circadian parameter. * The effect of diet within ZT0 vs ZT12 (comparison Cosinor analysis. $p<0.05$). CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

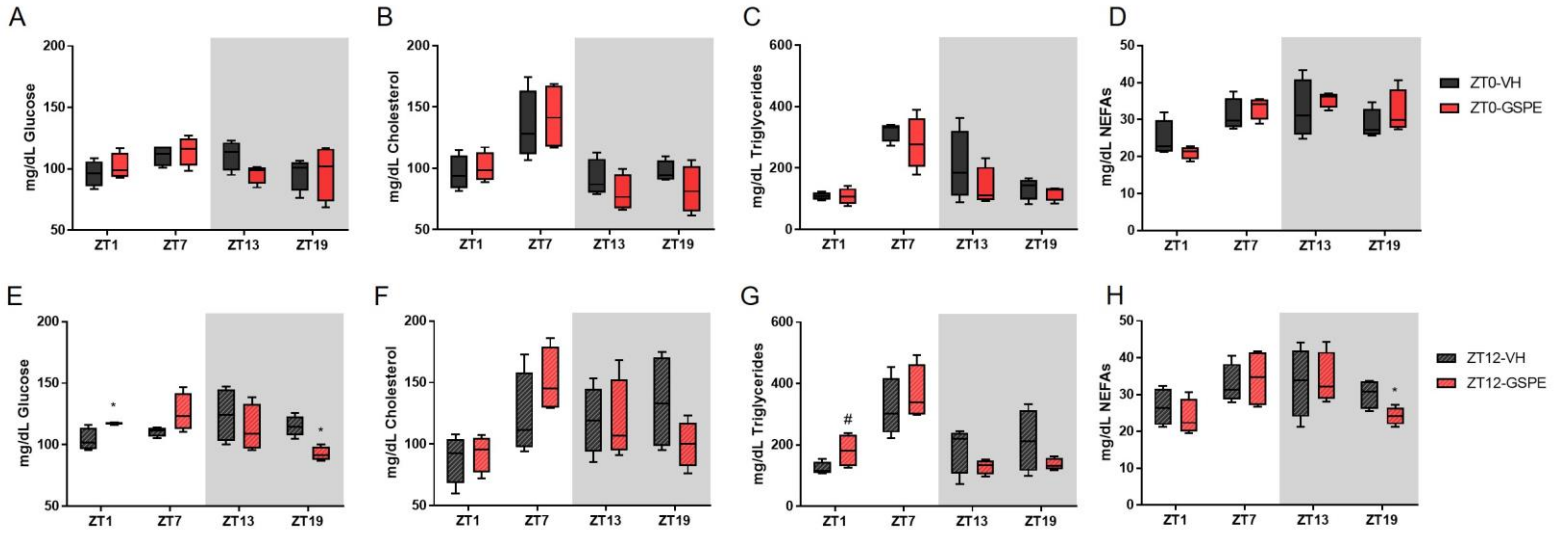

Figure S1. Serum biochemical parameters. (A) and (E) Data shown as Median and Interquartile Range of Serum Glucose (mg/dL) for VH and GSPE at ZT0 and ZT12. (B) and (F) Data shown as Median and Interquartile Range of Serum Cholesterol (mg/dL) for VH and GSPE at ZT0 and ZT12. (C) and (G) Data shown as Median and Interquartile Range of Serum Triglycerides (mg/dL) for VH and GSPE at ZT0 and ZT12. (D) and (H) Data shown as Median and Interquartile Range of Serum NEFAs (mg/dL) for VH and GSPE at ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney $p < 0.5$). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney $p < 0.1$). CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

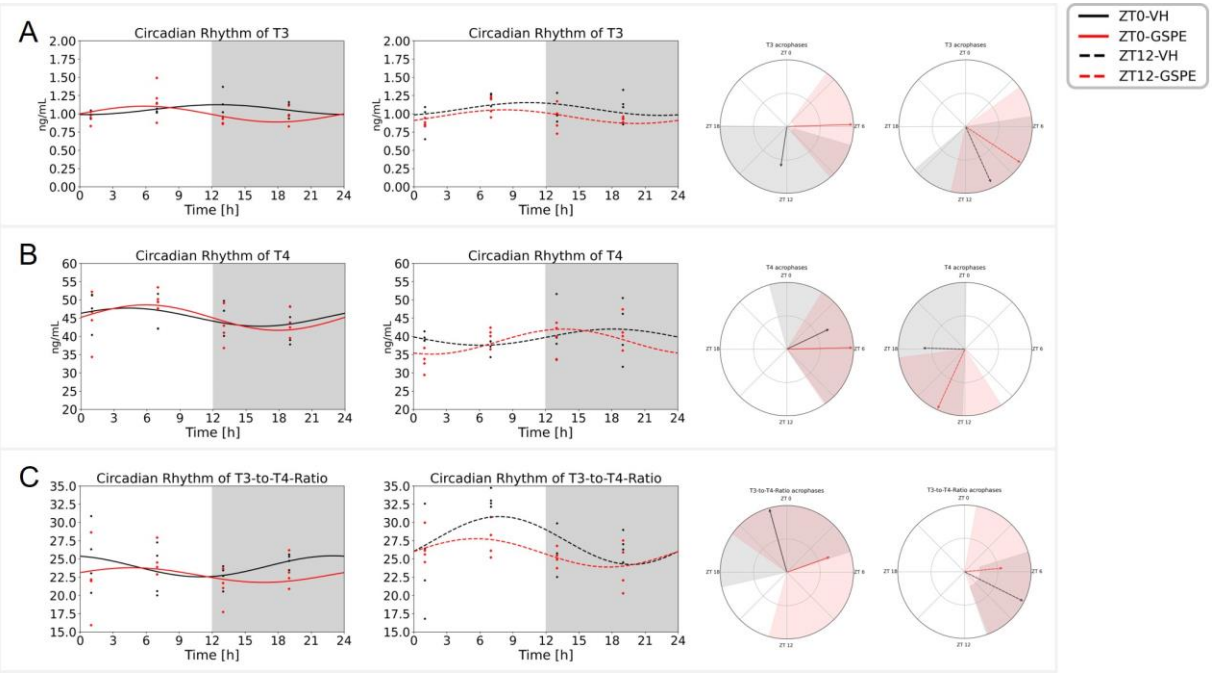

Figure S2. Serum thyroid hormones. (A) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum T3 (ng/mL) for VH and GSPE at ZT0 and ZT12. (B) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum T4 (ng/mL) for VH and GSPE at ZT0 and ZT12. (C) Circadian rhythms estimated and Acrophases with their amplitude represented by cosinor method of Serum T3-to-T4 ratio for VH and GSPE at ZT0 and ZT12. CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

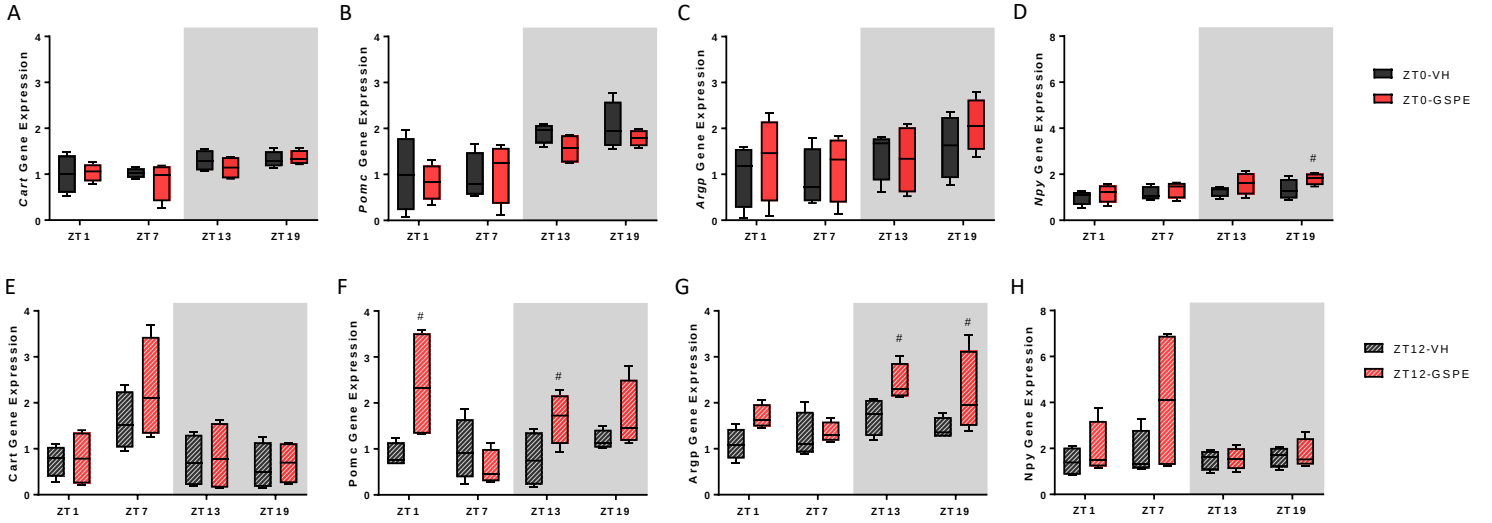

Figure S3. Appetite signaling genes. (A) and (E) Data shown as Median and Interquartile Range of *Cart* gene expression for VH and GSPE at ZT0 and ZT12. (B) and (F) Data shown as Median and Interquartile Range of *Pomc* gene expression for VH and GSPE at ZT0 and ZT12. (C) and (G) Data shown as Median and Interquartile Range of *Argp* gene expression for VH and GSPE at ZT0 and ZT12. (D) and (H) Data shown as Median and Interquartile Range of *Npy* gene expression for VH and GSPE at ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney $p < 0.5$). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney $p < 0.1$).CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

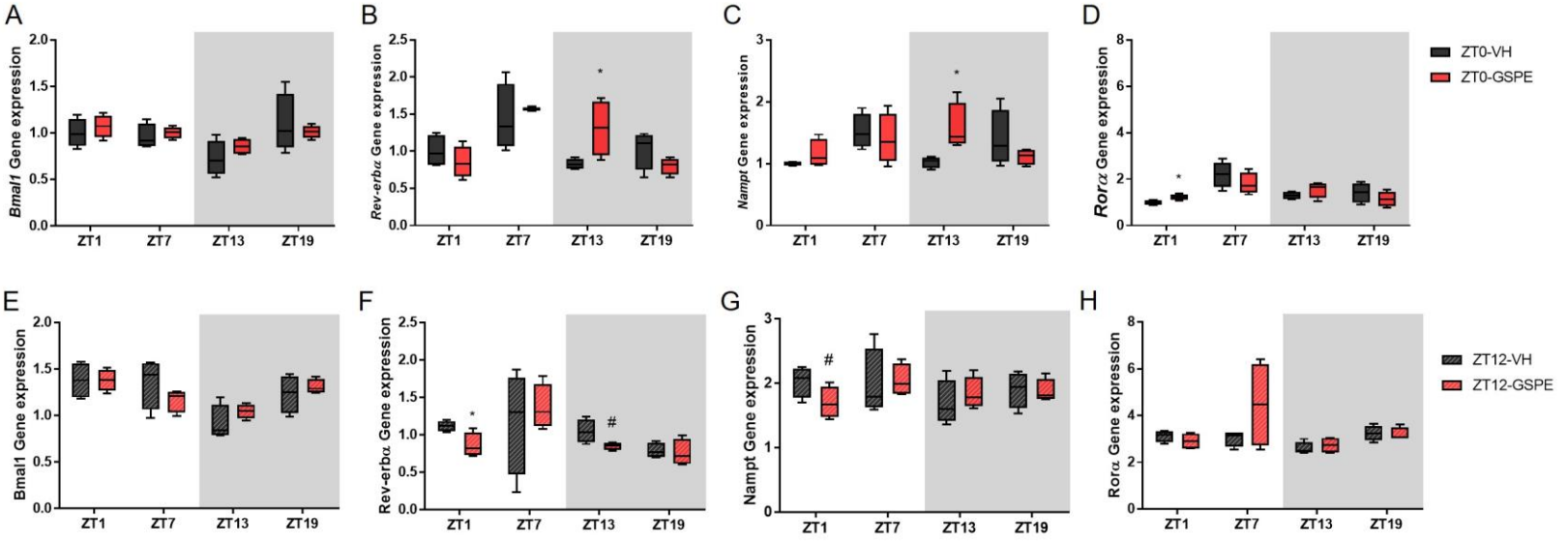

Figure S4. Hypothalamic clock genes. (A) and (E) Data shown as Median and Interquartile Range of *Bmal1* gene expression for VH and GSPE at ZT0 and ZT12. (B) and (F) Data shown as Median and Interquartile Range of *Rev-erbα* gene expression for VH and GSPE at ZT0 and ZT12. (C) and (G) Data shown as Median and Interquartile Range of *Nampt* gene expression for VH and GSPE at ZT0 and ZT12. (D) and (H) Data shown as Median and Interquartile Range of *Rorα* gene expression for VH and GSPE at ZT0 and ZT12. * Indicates significant differences between ZT0 vs ZT12 at each time point by Mann-Whitney $p < 0.5$). # Indicates tendency between ZT0 vs ZT12 at each time point by Mann-Whitney $p < 0.1$).CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; ZT0. Treatment time 8 a.m.; ZT12, Treatment time 8 p.m.

Chapter 2

To investigate the effect of PACs on circadian rhythms in a jetlag situation in healthy and CAF-induce obese Fischer 344 rats

Manuscript 4

Objective: to investigate the effects of PACs on physiological and circadian modulators in healthy and CAF-induce obese Fischer 344 rats subjected to a sudden change of light/dark cycle

Grape seed Proanthocyanidins Mitigate the Disturbances Caused by an Abrupt Photoperiod Change in Healthy and Obese Rats

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Keywords: Cafeteria diet; Chrononutrition; Circadian Rhythms; Phenolic compounds; Seasonal Rhythms; Zeitgebers.

ABSTRACT

Variations in light/dark cycle and obesogenic diets trigger physiological and behavioral disorders. Proanthocyanidins, in addition to their healthy properties, have recently demonstrated a modulating effect on biological rhythms. Therefore, the aim of this study was to evaluate the administration of a grape seed proanthocyanidin-rich extract (GSPE) to mitigate the disruption caused by a sudden photoperiod change in healthy and cafeteria (CAF)-diet obese rats. For this, 48 photoperiod-sensitive Fischer 344 rats were fed standard or CAF diets for 6 weeks under standard (12 h light/day, L12) conditions. Then, rats were switched to long (18 h light/day, L18) or short (6 h light/day, L6) photoperiod and administered vehicle or GSPE (25 mg/kg) for 1 week. Body weight (BW) and food intake (FI) were recorded weekly. Animal activity and serum hormone concentrations were studied before and after photoperiod change. Hormone levels were measured both at 3h (ZT3) and 15h (ZT15) after the onset of light. Results showed the impact of CAF diet and photoperiod on BW, FI, activity and hormonal status of the animals. GSPE administration resulted in an attenuation of the changes produced by the photoperiod disruption. Specifically, GSPE in L6 CAF-fed rats reduced serum corticosterone concentration, restoring its circadian rhythm, increased T3-to-T4 ratio and increased light phase activity, while under L18 decreased BW and testosterone concentration and increased the animal activity. These results suggest that GSPE may contribute to the adaptation to the new photoperiods. However, further studies are needed to elucidate the metabolic pathways and processes involved in these events

1. Introduction

Biological rhythms play an important role in the physiological and metabolic adaptation of the organism to external variations such as the rotation of the Earth around its axis or its translation around the Sun. This adaptation to the time of day (circadian rhythm) or year (seasonal rhythm) allows the optimization of the metabolism and energy expenditure (EE) [1,2]. Processes such as cardiovascular activity, endocrine system, blood pressure, body temperature, sleep-wake cycle, kidney activity, gastrointestinal tract and liver metabolism are regulated to a greater or lesser extent by the circadian rhythm, whose period is usually around 24 hours [3,4]. The daylight hours during these 24 hours also regulates these processes and allows the organisms to have a reference of the time of the year in which is found itself. The control center of circadian rhythm is located in the hypothalamic suprachiasmatic nucleus (SCN), and it can maintain the synchronization of the rest of the organism with the external environment via the retina, through the retino-hypothalamic tract (RHT) [2,5]. The central master clock can function autonomously, but it can be also reset by external signals or modulators called zeitgebers such as light, which plays a crucial role in reprograming or altering the circadian rhythm [2,6]. In this regard, circadian alteration via changes in day length (photoperiod) have been related to disorders in lipid metabolism and in colon functionality [7,8]. Changes on behavioral, feeding patterns and body weight (BW) gain produced by light/dark shifts have been also reported in the photoperiod-sensitive Fischer 344 (F344) rats [9,10].

The central clock sends signals, via the autonomic nervous system or circulating humoral factors including melatonin and cortisol, to the peripheral clocks to maintain rhythmicity and to ensure temporally coordinated physiology [11]. These oscillators, which are present in almost all mammalian tissues, maintain circadian rhythms and regulate tissue-specific gene expression and functionality. Peripheral clocks are also regulated by behavioral signals such as physical activity and, most notably, fasting/feeding states [12]. Diet

composition is another important zeitgeber for these oscillators and for instance circadian disruptions in hepatic metabolites have been reported in rats fed cafeteria (CAF) diet [13]. CAF diet consists in highly palatable; energy dense and unhealthy human food and rats fed-CAF diet are considered a robust model of human metabolic syndrome [14]. Alterations of the master clock in the hypothalamus has been also reported in mice fed high-fat diet, indicating that diet composition can also modify circadian synchronization to light [6,15]. In fact, in an obesogenic context, alterations of clock gene expressions have been reported in liver, adipose tissue, and hypothalamus, as well as the loss of rhythmicity of hormones and nuclear hormone receptors involved in metabolism and energy utilization, such as corticosterone and thyroid stimulating hormone (TSH), and testosterone both in rodents and humans [6,16,17].

Nevertheless, the interaction between metabolic diseases and circadian rhythms is bidirectional so that also circadian misalignment has been identified as a risk factor for developing metabolic disorders [18]. In this regard, the disruption of hepatic circadian rhythms has been related to non-alcoholic fatty liver disease (NAFLD) [19]. In addition, the circadian rhythm disturbance due to modern lifestyles (shift work, artificial light, fast food, eating time, etc.) has been related to the development of metabolic disorders that in the long term could lead to type 2 diabetes, cardiovascular diseases, overweight and obesity [20,21]. Additionality, light/dark cycle shifts, and high fat diet alter the cognitive response in F344 rats [22]. In this regard, it has been pointed out that metabolic disorders typical of the metabolic syndrome, together with other comorbidities such as depression, sleep disturbances, cognitive dysfunction and steatohepatitis could form part of what would be known as the "Circadian Syndrome" [23].

Proanthocyanidins (PACs) are a class of polyphenols constituted by polymers of flavanols and its gallate derivatives, whose healthy activities, including beneficial effects on different key aspects of metabolic syndrome, have been

extensively investigated [24]. Several molecular mechanisms have been demonstrated to be involved in their effectivity, including epigenetic modifications which have recently emerged as important mediators of their properties [25]. In addition, different studies have demonstrated that PACs can modulate both central and peripheral biological rhythms in healthy animals under jet-lag conditions, but also in CAF fed obese rats [26–29]. In this regard, the interaction of these phenolic compounds with the clock system has been recently pointed out by our group as another mechanism involved in their beneficial effects [30].

Therefore, the aim of the present study was to investigate if PACs can contribute to restore circadian disruption caused by light/dark cycle shifts in standard (STD) conditions and diet-induced obesity. For this purpose, STD and CAF-fed rats were transferred abruptly from STD (12 h light/day, L12) to long (18 h light/day, L18) or short (6 h light/day, L6) photoperiods. BW gain and food intake were recorded weekly and animal activity and the serum hormones levels of T3 and T4, corticosterone, testosterone (as representative hormones of hypothalamic-pituitary-thyroidal, -adrenal and-gonadal axis modulating hormones, respectively) and melatonin were analyzed before and after photoperiod disruption

2. Materials and Methods

2.1. Grape seed proanthocyanidin-rich extract

The grape seed proanthocyanidin-rich extract (GSPE) used in this study was provided by Les Dérives Résiniques et Terpéniques (Dax, France). This was obtained from white grape seed. The phenolic profile of this extract is mainly composed of catechin, epicate-chin, gallic acid, epicatechin gallate and dimers, trimers and tetramers of proanthocyanidins [31].

2.2. Animal experiment procedure

The animals used were 13-week-old male F344 rats from Charles Rives Laboratories (Barcelona, Spain) housed under STD laboratory conditions at 22

°C and 12 hours light/dark cycle with ad libitum access to food and drinking water. After two weeks of ac-climatization period, rats were weighted and randomly divided into two dietary groups (Figure 1). One group (L12-STD) was fed STD chow diet (2.90 kcal/g; A04, Panlab, Barcelona, Spain), and the other group (L12-CAF) CAF diet for 6 weeks (n=24/group). After this time, animals were transferred from L12 to a L18 or L6 photoperiods and administered vehicle (VH), which was condensed milk 1/5 diluted, or GSPE (25 mg/kg) dissolved in VH for 1 week more. Thus, the animals were grouped into 8 different groups (n=6). CAF diet was prepared every day and contained bacon, cookie with paté, cookie with cheese, carrots, ensaïmada (pastry), STD chow and sweetened milk (22% sucrose w/v), and its caloric distribution was 56.43% carbohydrate, 45.72% lipid and 9.5% protein [32]. The onset of light was at 8:00 a.m. and defined as zeitgeber time 0 (ZT0). Both VH and GSPE treatments were administered to the rats at ZT0. BW and food intake (FI) were recorded weekly during the whole experimental procedure. Blood was collected from saphenous vein in non-heparinized tubes, incubated for 1 h at room temperature and immediately centrifuged at 1200×g for 15 min at 4 °C to collect the serum. To study the light/dark cycle, blood was extracted at two different times, ZT3 and ZT15 (11:00 a.m. and 11:00 p.m.) in the sixth and seventh weeks.

Animal procedures were approved by The Animal Ethics Committee of the Univer-sity Rovira i Virgili (Tarragona, Spain) and the Generalitat de Catalunya (reference number 9495, 18/09/19) and were carried out in accordance with Directive 86/609EEC of the Council of the European Union and the procedure established by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

2.3. Indirect Calorimetry

Indirect calorimetry was performed on all animals, after the administration of VH or GSPE, for 12 h (from ZT6 to ZT18), one week before photoperiod change and two days be-fore sacrifices, using an Oxylet Pro System (Panlab, Barcelona,

Spain) and the Metabolism 2.1.02 (Panlab, Cornellà, Spain) software program. Lipid and glucose oxidation were calculated with the oxygen consumption (VO2) and carbon dioxide production (VCO2) values given by the Oxylet LE 405-gas analyzer (PANLAB). At each time point, the program Metabolism 2.1.02 (PANLAB, Barcelona, Spain) calculated the respiratory quotient (RQ) as the VCO2/VO2 ratio. Using the stoichiometric equations of Frayn [33] and assuming a nitrogen excretion rate (n) of 135 µg/kg x min [34], we used the formula (g/min) = 4.55 × VCO2 − 3.21 × VO2 − 2.87 n for calculating the oxidation of carbohydrates and the formula (g/min) = 1.67 × VO2 − 1.67 × VCO2 − 1.92 n for calculating the oxidation of lipids. To obtain the EE from lipid and carbohydrate, the lipid and carbohydrate rates were multiplied by 37 and 16, respectively, using the Atwater general conversion factor [35]. In addition, indirect calorimetry allows us to measure the vertical activity of the rat with sensors that detect when rats stand up on their two hind legs. In order to study photoperiod, change effects and the adaptation to the new light/dark cycle of the animals, we separated the results of light and dark phases.

2.4. Hormone Analysis

Serum hormones concentrations were measured by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ). Serum samples were thawed at 4 °C and 50 µL of serum were mixed with 250 µL of methanol containing the internal STD (2 ng/mL). Then, the mixture was vortexed and centrifuged for 5 minutes at 4 ºC and 252 g. The supernatant was transferred to a new tube and mixed with 700 µL of 0.1 % formic acid in water. The sample was loaded to a SPE system previously conditioned with methanol and 0.1 % formic acid in water. The cartridge was washed with 0.1 % formic acid in water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45 ºC and reconstituted with 50 µL of water:methanol (60:40, v/v) and transferred to a glass vial for analysis. The hormones detect-ed were melatonin, corticosterone,

triiodothyronine (T3), thyroxine (T4) and testosterone, analytical column was Zorbax Eclipse C18 (150 x 2.1 mm) from Agilent Technologies.

2.5. Statistical Analysis

Data were represented as means ± STD error of mean (S.E.M.) of each group and for this data normality and homogeneity of variance were tested by Shapiro-Wilk test and Levene test, respectively. Differences between groups were assessed by repeated-measures ANOVA followed by LSD post hoc test for BW gain and accumulative food intake, and for the rest of parameters three-way and two-way ANOVA was used followed by LSD post-hoc test. In order to study two time points to analyze diurnal/nocturnal cycle paired Student's t-test was carried out. Theses statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Abrupt photoperiod transfer caused changes in body weight gain and food intake pattern in CAF-fed rats: effects of grape seed proanthocyanidins

Rats fed CAF diet showed a significantly increased BW gain compared to rats fed STD diet (Figure 2A). The increase in BW gain was observed from the first week of CAF administration and during the 6 weeks that the rats were in L12 conditions. According to this, increases in food and energy intakes were observed in the CAF-fed group during the 6-week period of the experiment compared to STD-rats (Table 1 and Figure 2B).

After 6 weeks in the L12 photoperiod, the rats were abruptly transferred to the L18 or L6 photoperiods. This change in light/dark cycles caused a loss of BW gain in some of the groups (Figure 2C). Regarding animals transferred to L18, an effect of diet was observed in CAF-fed rats administered VH, since this group tended to lose less BW compared to L18-STD-VH animals. In addition, GSPE administration to CAF-rats resulted in a substantial loss of BW gain compared to CAF-VH rats (p=0.005). Interestingly, a photoperiod effect was observed in

the CAF-VH group since this group did not lose BW when were transferred to L18 photoperiod but tended to lose it in L6 conditions (p=0.021). GSPE administration in L6 conditions did not cause any change in BW loss.

The energy intake after photoperiod change was higher in CAF-fed rats than in STD-rats both in L18 an L6 animals, especially due to the consumption of carbohydrates and fats (Table 1 and Figure 2D). Interestingly, the ingestion of carbohydrates was affected by photoperiod since CAF-fed rats under L6 conditions ingested more energy from carbohydrates than CAF-fed rats under L18 conditions (p=0.033). GSPE administration pro-moted a higher energy intake from fat in CAF-fed rats in both L18 and L6 photoperiods (p<0.001 and p=0.018, respectively). Moreover, this effect was different depending on the photoperiod since the increase of energy intake from fat was higher in L18 compared to L6. It was also observed that GSPE administration tended (p=0.064) to increase the energy in-take from protein intake in STD-fed rats in L6 photoperiod.

3.2. Grape seed proanthocyanidins attenuated the impact of photoperiod change on energetic expenditure

The EE data obtained along the day in all the groups are shown in Figures 3A-E. In-direct calorimetry data showed differences in EE between CAF and STD-fed rats in L12 animals, before of photoperiod change. The expenditure was lower in STD-fed diet rats compared to their CAF-fed counterparts (Figure 3A). The increase in EE in CAF animals under L12 conditions disappeared when photoperiod transfer was performed (Figure 3F and 3G). Only a tendency to increase EE (p=0.053) by CAF animals was found in the L18 photoperiod in the transition phase, period changed from dark in L12 conditions to light in L18 photoperiod (from ZT12 to ZT18). In this context, GSPE administration to CAF-fed rats in the L18 photoperiod reduced EE during the light phase (p=0.041) and tended to reduce it during the transition phase (p=0.057). Despite this effect, the rhythm set by the light/dark cycle was maintained for both groups. This rhythm was only minimally altered in STD-VH rats in L6 photoperiod, where the

difference between light and dark phases in EE was not significant (p=0.055) while it recovered with GSPE administration (p=0.008).

Regarding the energy source used by the animals, the results showed that STD-fed rats preferentially oxidized carbohydrates (Table 1), both in the light and dark phases, although the two groups showed a clear rhythm marked by the light/dark cycle with higher carbohydrate oxidation during the night (Figure 4A). However, CAF animals used mainly fat throughout the day, both in the light and dark periods. Moreover, only the CAF-group showed a clear rhythm marked by the light/dark cycle with higher fat oxidation during the light phase (Figure 4B). The energy from carbohydrate oxidation (Figure 3C and Table 1) tended to decrease with GSPE administration in CAF-fed rats in the L18 photoperiod in the light phase (p=0.070) and significantly decreased in the dark phase (p=0.044). In addition, photoperiod change promoted a loss of the rhythm marked by light/dark cycle in CAF-fed rats, which was maintained in STD-fed rats in both photoperiods. Regarding energy from fat oxidation (Figure 4D and Table 1), the higher fat oxidation by CAF rats was generally maintained. The main change was the alteration of rhythm by CAF-fed rats when they are transferred to L6 and L18. Furthermore, in L6 photoperiod no differences were found between STD and CAF-fed rats during the light phase.

3.3. Cafeteria diet decreases the activity of animals in standard conditions and grape seed proanthocyanidins attenuated photoperiod changes in the activity of rats.

A change in the behavioral pattern of the rats could be observed depending on the di-et administered since the CAF-fed rats displayed less activity than their STD-fed counterparts (Figure 5A). STD and CAF-groups displayed less activity in their resting period, light phase, but both in light and dark phases the activity was decreased in the CAF-fed animals (Figure 5B). The change in photoperiod also had a major impact on the activity pattern of the rats (Figure 5C-5F). A reducing effect on activity because of CAF diet was observed in both photoperiods and in both phases, light and dark. The activity of the rats was

increased when they were transferred to L6 photoperiod compared to rats that were transferred to L18 photoperiod, both STD-fed (p=0.002) and CAF-fed rats (p=0.032), during light phase. Interestingly, GSPE administration reduced activity in this phase only in STD-fed rats in L6 photoperiod (p=0.024). During the dark phase GSPE administration tended to increase activity in both L6 (p=0.060) and L18 (p=0.051). GSPE- treated and CAF-fed rats increase the activity when it was analyzed in whole day (Figure 5D). Despite these changes, the two CAF-fed groups maintained the rhythm set by the light/dark cycle when the activity was analyzed separated (Figure 5G). Additionally, the STD-fed rats in L6 photoperiod lost the rhythm set by the light/dark cycle, which recovered with GSPE administration (p=0.014).

3.4. GSPE modulates rhythm alterations caused by abrupt photoperiod change in serum hormones

A rhythm marked by the light/dark cycle was also detected by serum hormones. No differences by diet were found in the serum melatonin and corticosterone concentration (Figure 6A and 6B). Additionally, in both groups a marked rhythm was observed de-pending on light/dark phase with higher concentration in ZT15 compared to ZT3. The CAF-fed rats showed an increase in serum testosterone concentration and T3-to-T4 ratio at both ZT3 and ZT15 (Figure 6C and 6D). Furthermore, differences were observed between these two measurement points in both groups for both parameters, showing a rhythm marked by the light/dark cycle with higher concentration in ZT3 respect to ZT15 and in CAF rats compared to STD animals.

The change of photoperiod had a significant light-hour-dependent effect on serum melatonin concentration (Figure 6E). Rats under L18 conditions showed no difference in melatonin between ZT3 and ZT15. However, when rats were transferred to L6 photoperiod the rhythm set by the light/dark cycle was remarkably maintained. Regarding corticosterone (Figure 6F), a photoperiod effect was found in CAF-fed rats that showed higher concentrations in L6 conditions at ZT3 time point compared to L18 conditions (p=0.031). This

increase was mitigated by GSPE administration (p=0.035). Interestingly, STD-fed rats also showed a lower concentration at ZT3 with GSPE administration under L18 conditions (p=0.020). The rhythm set by the light/dark cycle was altered because of the increased concentration in CAF-VH rats in ZT3 under L6, while GSPE administration tended to restore this rhythm (p=0.060). Serum testosterone concentration was also altered in a photoperiod-dependent manner (Figure 6G). Thus, the light/dark rhythm of the rats was lost under L18 photoperiod. Additionally, CAF-fed rats in this photoperiod showed higher concentrations than STD-fed rats at both ZT3 and ZT15. At ZT15 time point, GSPE administration reduced the hormone concentration (p=0.010). When rats were under L6 conditions, they showed lower testosterone concentration at ZT15 compared to L18 photoperiod rats. Finally, the T3-to-T4 ratio showed no rhythm in CAF-fed rats at any photoperiod (Figure 6H). In addition, GSPE administration restored this rhythm in STD-fed rats at L6 photoperiod.

4. Discussion

Circadian rhythm disruption has been related to behavior, metabolic and physiological disorders [23]. This disturbance could be caused by changes in daylight or alterations in the normal light/dark cycle and may contribute to the development, jointly with a hypercaloric diet, of obesity and other metabolic syndrome comorbidities [23]. In addition, phenolic compounds have shown beneficial effects on several metabolic disorders, and recently some of these compounds including PACs have been related to the restoration of circadian clock [30,36–38]. Therefore, the aim of our study was to evaluate the impact of abrupt light/dark-cycle change on healthy and CAF-induced obese rats and if PACs were able to attenuate this photoperiod change effects.

As expected, CAF diet produced a significant increase in BW gain compared to STD-fed rats. Because of the high fat and carbohydrate content, the CAF diet can induce a clinical picture of obesity and other comorbidities characteristic of the metabolic syn-drome [14,39]. Moreover, it was observed that CAF-diet-fed

rats present hyperphagia since food and energy intakes reflected large difference between CAF and STD-fed rats. Alterations of photoperiod has also been related to an increase in BW [8,9,40]. Nevertheless, in this study, the sudden light/dark shift was not accompanied by body weight gain neither in STD nor CAF rats. These discrepancies could likely be due to the length of the study since we focus on the short-term effects of altered photoperiod to avoid a potential adaptation of the animal to the new light/dark cycle (only one week). Therefore, a BW gain after a longer exposure time to the new photoperiod cannot be ruled out, since an adaptation of at least 5 days in the eating patterns after the reversion of the light-dark cycle has been re-ported [10]. In addition, the stress associate to the sudden change in photoperiod could affect the body weight [41,42]. Interestingly, it was observed that CAF-fed rats switched to L6 photoperiod showed a greater loss of BW than their counterparts in L18 photoperiod, demonstrating a diet-photoperiod interaction effect.

Regarding PACs effects, GSPE administration caused a BW loss in CAF-fed rats un-der L18 conditions, showing an interaction between photoperiod, diet and GSPE since the same effect was not observed in the rest of groups. Several studies have reported lower BW gain after GSPE administration in obese animals, however, the rats were under L12 conditions, and the doses were higher than the 25 mg/kg used in this study [43,44]. Differences in GSPE bioavailability and bioactivity between healthy and diseased rats, including CAF-fed rats, has been previously reported by our group [45,46]. Related to the interaction between polyphenol consumption and photoperiods it could be due to the different bioavailability of these compounds depending on the circadian rhythm [47], and more specifically on exposure to daylight hours [48]. In this context, it has been reported that seasonal fruits rich in phenolic compounds such as cherries, red grape and oranges showed photoperiod-dependent effects [38,49–52]. Regarding food intake, no differences in total energy intake were observed between the GSPE-treated CAF groups and CAF-fed VH groups

after the photoperiod change. However, a change in feeding pattern was observed as GSPE administration increased fat intake compared to the corresponding CAF-fed VH groups. Moreover, this increase was photoperiod dependent since it was significantly higher un-der L18 than under L6 photoperiod. Disruption of the light/dark cycle combined with CAF diet has shown to increase in rats the consumption of fat- and carbohydrate-rich foods with CAF diet [53]. The results of this study show that this effect appears to be stimulated by GSPE administration in a photoperiod-disturbance context.

As previously was reported, CAF-fed rats under L12 photoperiod showed higher EE than STD-fed rats [49]. The disruption of the light/dark cycle eliminated this difference between CAF-fed and STD-fed rats. A lower EE was reported in CAF-fed rats under expo-sure to L18 or L6 photoperiods compared to L12 photoperiod [54], which could explain in part the lack of differences in EE between CAF and STD rats after the photoperiod change. Interestingly, all groups showed differences in EE between light and dark phases, indicating that the rhythm marked by the light/dark cycle would not be altered by the sudden change of photoperiod. In this context, GSPE administration did not show effects in EE in rats under L6 photoperiod whereas a decrease in EE was observed in CAF-fed rats ad-ministered GSPE, both in light and dark phases under L18 conditions. Although other studies reported an increase in EE after GSPE administration, the animals were under L12 conditions and without light/dark shifts [55,56]. The decrease in EE in L18-CAF-GSPE group may be a result of the lower carbohydrate oxidation promoted by GSPE in CAF-fed rats under L18 conditions both light and dark phases. GSPE has been shown to enhance fat oxidation, stimulating it in an obesogenic state [55,56], but no changes in carbohydrate oxidation have been reported with doses similar to 25 mg/kg [56]. Nevertheless, a photo-period-dependent decrease in carbohydrate oxidation has been reported after cherry consumption [50].

Activity was also studied, and it was observed that CAF promoted sedentarism, since animals displayed less activity compared to the STD-fed rats both in light

and dark phases. A decrease in activity has been reported in CAF-diet obese rats under in L12 conditions and in rat jetlag and shift-work models [39,40], but the mechanisms leading to this reduction are unclear. In this study, after the sudden change of photoperiod the activity was in-creased in STD-fed rats under L6 conditions from ZT6 to ZT12 compared to STD-fed rats under L18 conditions. This transition period was in light before the photoperiod change but in dark after the light/dark shift to L6. This increase in activity was mitigated by GSPE administration, thus maintaining the differences between light and dark phases. Interestingly, the GSPE tended to increase activity in obese rats compared to VH rats under both photoperiods but only in the dark phase under L6 photoperiod and from ZT12 to ZT18 under L18 conditions. This period was in dark before the photoperiod change but in light after the light/dark shift.

The main marker of the light/dark cycle, melatonin, was not affected by CAF diet un-der L12 conditions. This hormone increases its concentration during the dark phase, being an important signal for the organism to recognize the photoperiod to which it is exposed [57]. Thus, as expected, melatonin concentration showed a large difference between that measured at ZT3 and ZT15 under L12 photoperiod both in STD and CAF groups. However, after the switch of photoperiod changes in melatonin were observed since this hormone is the first to adapt to the new photoperiod [58–60], particularly under the L18 photoperiod, in which the light was switched off at ZT18. While melatonin signals to the organism to adapt its metabolism under changes in the photoperiod, corticosterone is another hormone with important metabolic implications. This hormone plays a key role in the hypo-thalamic-pituitary-adrenal axis (HPA) and presents a marked circadian rhythm [61,62]. Nevertheless, in this study corticosterone did not adapt at the same rate to melatonin. An increase in corticosterone is related to stress [41,62] and its circadian disruption could lead to diseases of glucocorticoid sensitivity or resistance [63]. In this context, the increase in corticosterone levels observed in STD rats when they were switched to L18 photoperiod, which were reversed by

GSPE administration, could be due to the sudden change of photoperiod and would relate to the increased stress of these animals. Interestingly, the CAF-VH group under L6 photoperiod showed high corticosterone levels in the morning and did not follow their normal rhythm between ZT3 and ZT15. However, this effect was reversed by GSPE administration, recovering its rhythm. Therefore, GSPE could be attenuating the stress induced by the sudden change of photoperiod. Hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) axes are important in processes such as lipid and carbohydrate metabolism and are closely related to biological rhythms. Testosterone and the T3-to-T4 ratio, respectively, act as modulators of these axes [64,65]. The rhythm of both hormones was lost to change the photoperiod, which could lead to metabolic disturbances. Despite of GSPE administration reduced testosterone in L18 at ZT15 in CAF-fed rats, the rhythm was not recovered. Regarding the T3-to-T4 ratio, the rhythm was lost in practically all groups, which could be leading to metabolic disorders produced by changes in feeding patterns since this ratio has an established circadian rhythm dependent on diet [66,67].

CONCLUSIONS

In summary, the results suggested that grape seed PACs might be mitigating, in a photoperiod- and diet-dependent manner, the metabolic disturbances induced by an alteration of the light/dark cycle by suddenly changing the photoperiod. This photoperiod change could be inducing disorders in the HPG and HPT axes as their main modulators such as testosterone and T3-to-T4 ratio, respectively, lost the rhythm set by the light/dark cycle. Particularly, T3-to-T4 ratio could also be indicating an alteration in food intake and activity patterns since their circadian rhythm is closely linked to them. In addition, CAF-fed rats in the L6 photoperiod showed a significant increase of corticosterone in ZT3 causing a loss of rhythm for this hormone, closely linked to stress. GSPE administration was able to reduce serum corticosterone concentration, restoring its circadian rhythm, in-creased T3-to-T4 ratio and increased light

phase activity in CAF-fed rats at L6 photoperiod. In STD-fed rats for this same photoperiod, the administration of PACs improved testosterone circadian rhythm and T3-to-T4 ratio and reduced activity in the period which changed from light to dark phase compared to the VH group. CAF-fed rats administered GSPE showed lower testosterone concentration, increased activity in the period which changed from dark to light phase and lost BW when switched to the L18 photoperiod. Finally, STD-fed rats in L18 showed lower corticosterone concentration in the light phase after GSPE administration. Nevertheless, further studies are needed to elucidate the metabolic pathways and processes involved in these events.

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FIGURES

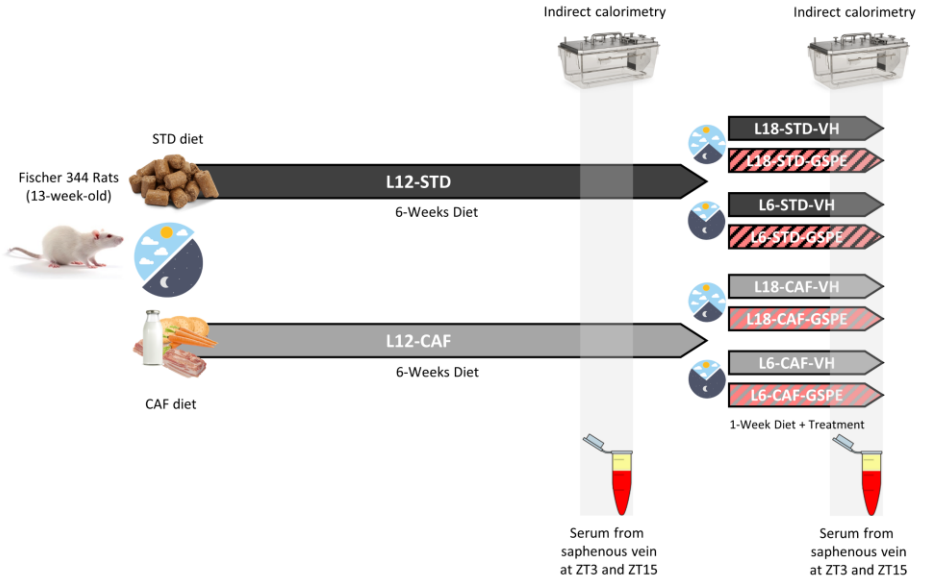

Figure 1. Experimental design to evaluate the effect of GSPE after an abrupt disturbance of photoperiod*. STD and CAF-fed rats were switched to a new light-dark cycle and VH or GSPE were administered to the animals. STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

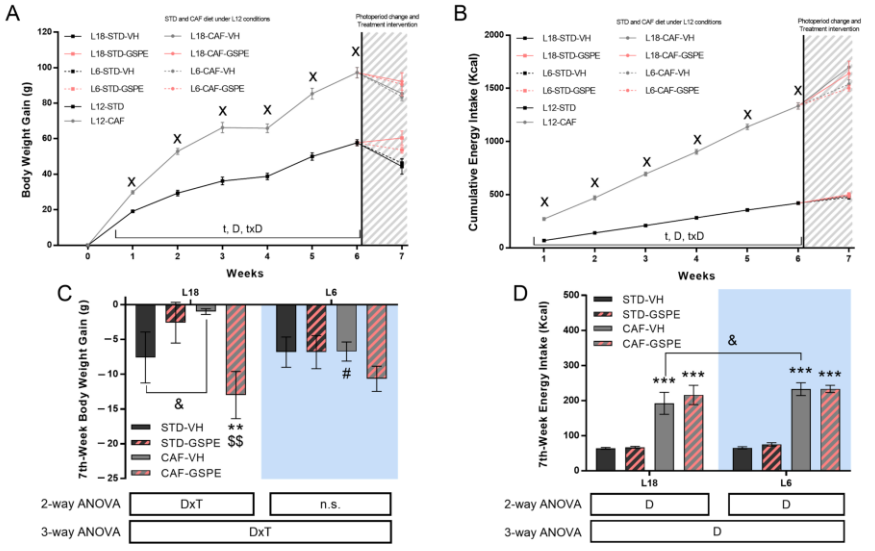

Figure 2. Body Weight Gain and Cumulative Food Intake. *(A) Body Weight Gain and (B) Cumulative Energy Intake along the experiment. (C) Body Weight Gain in the seventh week for L18 and L6 conditions and (D) Cumulative Energy Intake for L18 and L6 conditions (STD-VH, STD-GSPE, CAF-VH and CAF-GSPE) in the seventh week. Values are expressed as the mean ± S.E.M. (n=24 for L12 groups and n=6 for L18 and L6 groups). x Indicates significant differences using repeated measured-ANOVA followed by Student´s t test between L12-STD vs L12-CAF (p ≤ 0.001). D, diet effect; DxT, interaction between treatment and Diet. n.s., no significant differences. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant differences by Photoperiod effect using 2-way and 3-way ANOVA followed by LSD post-hoc test (p ≤ 0.05). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

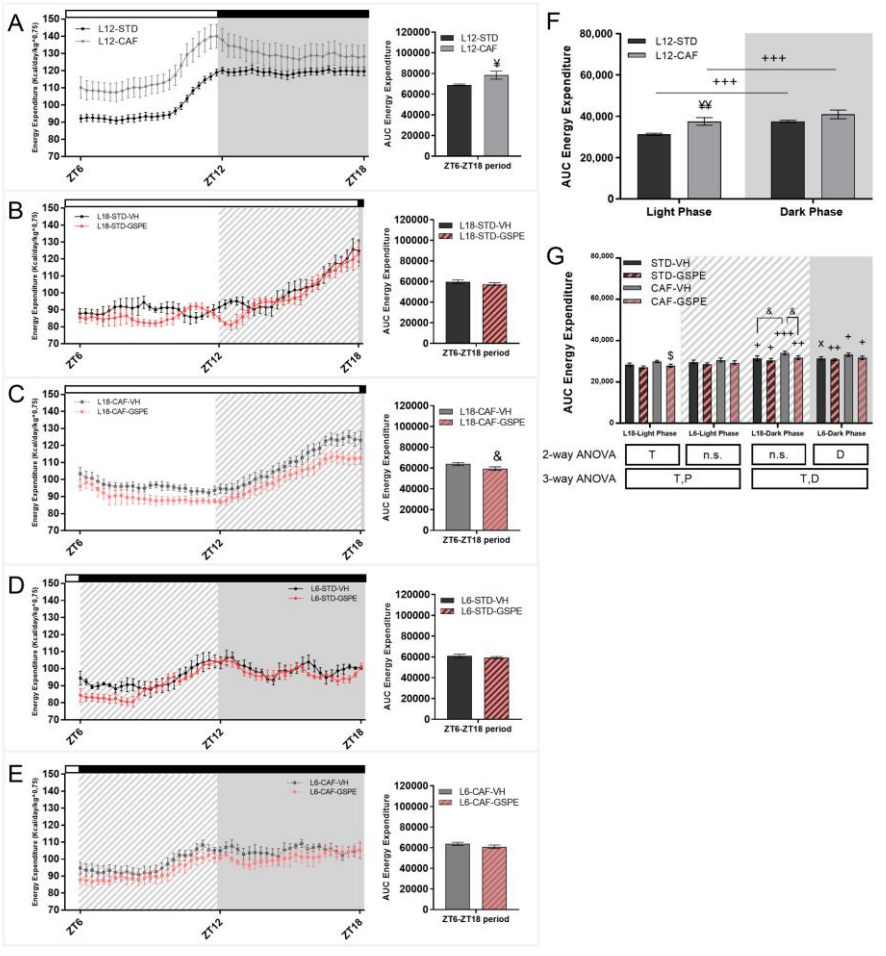

Figure 3. Energy Expenditure of STD and CAF-fed rats in the sixth week of the experiment under L12 conditions and in the seventh week of the experiment under L18 and L6 conditions*. (A) EE for STD- and CAF-fed rats under L12 photoperiod and the corresponding AUC. (B) EE for STD-fed rats and VH or GSPE administration under L18 photoperiod and AUC. (C) EE for CAF-fed rats administered VH or GSPE under L18 photoperiod and the corresponding AUC. (D) EE for STD-fed rats administered VH or GSPE under L6 photoperiod and the corresponding AUC. (E) EE for CAF-fed rats administered VH or GSPE under L6 photoperiod and the corresponding AUC. (F) AUC of EE for L12 conditions groups. (G) AUC of EE for L6 and L18 conditions groups. For L12 conditions values are expressed as the mean ± S.E.M. (n=24). ¥ Indicates significant differences using unpaired Student´s t test between L12-STD vs L12-CAF (p ≤ 0.05). Values are expressed as the mean ± S.E.M. (n=6). D, diet effect; T, GSPE treatment effect; P, photoperiod effect; n.s., no significant differences. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant differences by Photoperiod effect using 2-way and 3-way ANOVA followed by LSD post-hoc test (p ≤ 0.05). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). + Indicates significant differences using paired Student´s t test between Light Phase vs Dark Phase for each group (p ≤ 0.05). x Indicates tendency using paired Student´s t test between Light Phase vs Dark Phase for each group (p=0.1-0.051). EE, Energy Expenditure; AUC, Area under curve; STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich*

extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.

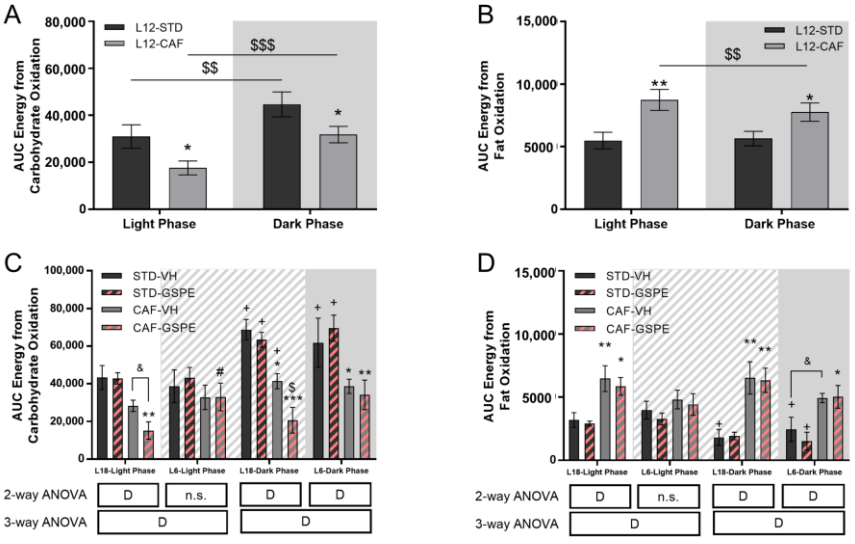

Figure 4. Energy from carbohydrate or fat oxidation of STD and CAF-fed rats in the sixth week of the experiment under L12 con-ditions and in the seventh week of the experiment under L18 and L6 conditions*. (A) AUC of energy from carbohydrate oxidation for STD- and CAF-fed rats under L12 photoperiod. (B) AUC of energy from fat oxidation for STD-fed rats and VH or GSPE administration under L18 photoperiod. (C) AUC of energy from carbohydrate oxidation for L6 and L18 conditions groups. (D) AUC of energy from fat oxidation for L6 and L18 conditions groups. For L12 conditions values are expressed as the mean ± S.E.M. (n=24). ¥ Indicates significant differences using unpaired Student´s t test between L12-STD vs L12-CAF (p ≤ 0.05). Values are expressed as the mean ± S.E.M. (n=6). D, diet effect; n.s., no significant differences. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant differences by Photoperiod effect using 2-way and 3-way ANOVA followed by LSD post-hoc test (p ≤ 0.05). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). + Indicates significant differences using paired Student´s t test between Light Phase vs Dark Phase for each group (p ≤ 0.05). x Indicates tendency using paired Student´s t test between Light Phase vs Dark Phase for each group (p=0.1-0.051). AUC, Area under curve; STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

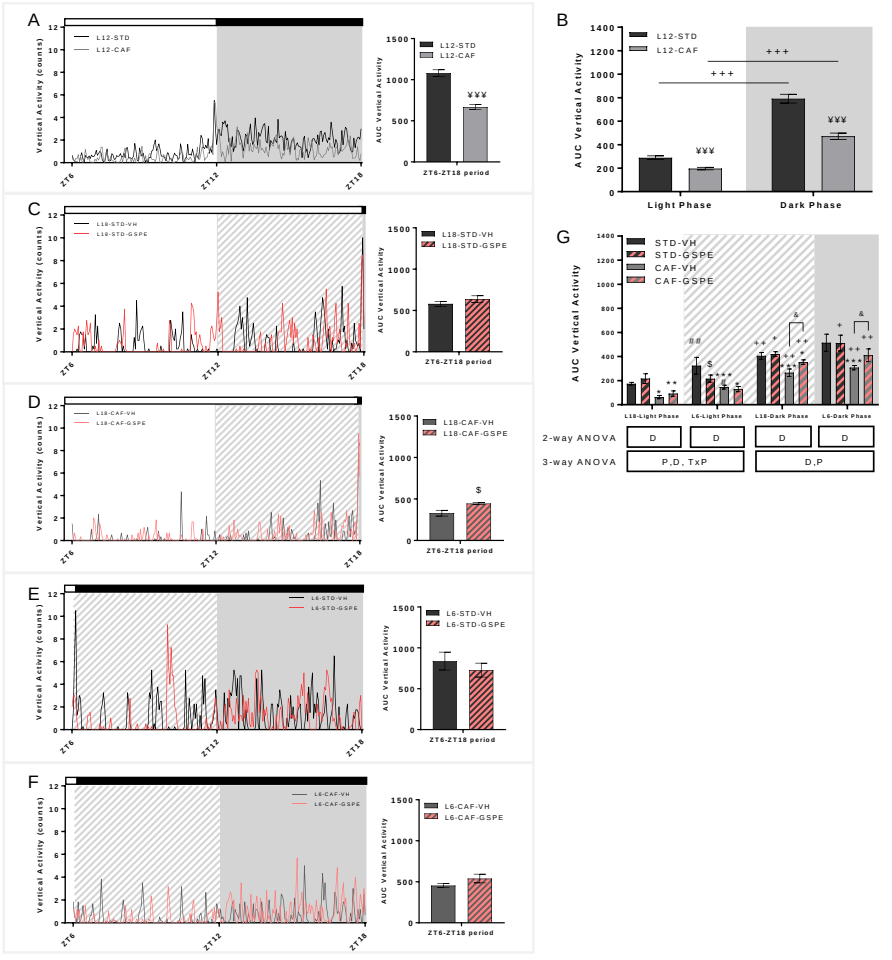

Figure 5. Activity of STD and CAF-fed rats in the sixth week of the experiment under L12 conditions and in the seventh week of the experiment under L18 and L6 conditions. *(A) Activity for STD- and CAF-fed rats under L12 photoperiod and the corresponding AUC. (B) Activity for STD-fed rats administered VH or GSPE under L18 photoperiod and the corresponding AUC. (C) Activity for CAF-fed rats administered VH or GSPE under L18 photoperiod and the corresponding AUC. (D) Activity for STD-fed rats ad-ministered VH or GSPE under L6 photoperiod and the corresponding AUC. (E) Activity for CAF-fed rats administered VH or GSPE under L6 photoperiod and the corresponding AUC. (F) AUC of activity for L12 conditions groups with light phase and dark phase separated. (G) AUC activity for L6 and L18 conditions groups with light phase and dark phase separated. For L12 conditions values are expressed as the mean ± S.E.M. (n=24). ¥ Indicates significant differences using unpaired Student´s t test between L12-STD vs L12-CAF ($p \leq 0.05$. Values are expressed as the mean ± S.E.M. (n=6). D, diet effect; P, photoperiod effect; TxP, interaction between Treatment and Photoperiod. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant differences by Photoperiod effect using 2-way and 3-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). + Indicates significant differences using paired Student´s t test between Light Phase vs Dark Phase for each group ($p \leq 0.05$). x Indicates tendency using paired Student´s t test between Light Phase vs Dark Phase for each group (p=0.1-0.051). AUC, Area under curve; STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats*

administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.

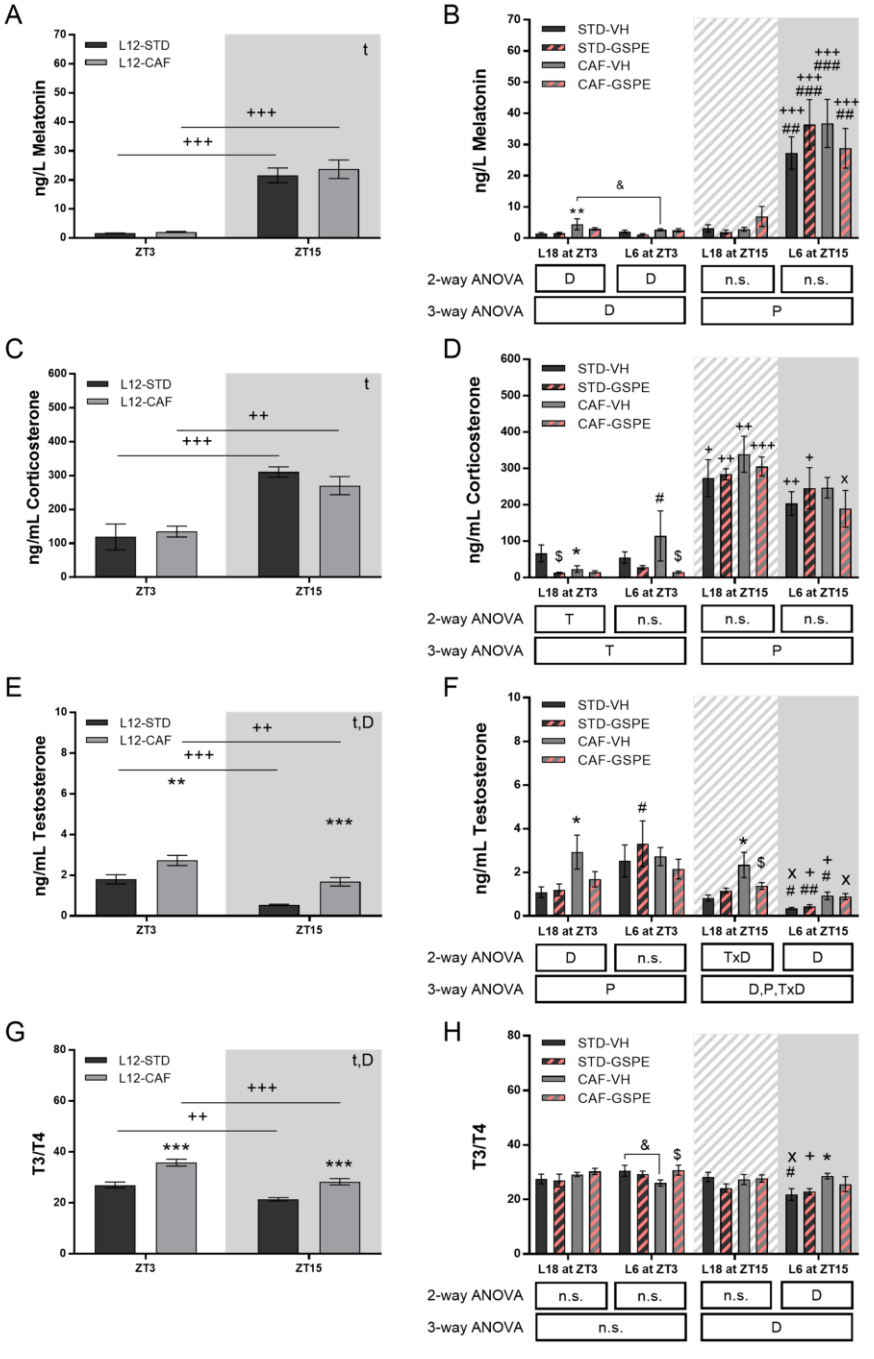

Figure 6. Serum hormones of STD and CAF-fed rats in the sixth week of the experiment under L12 conditions and in the seventh week of the experiment under L18 and L6 conditions. *Serum Melatonin at ZT3 and ZT15 (A) in L12 and (B) L18 and L6 conditions. Serum Corticosterone at ZT3*

*and ZT15 (C) in L12 and (D) L18 and L6 conditions. Serum Testosterone at ZT3 and ZT15 (E) in L12 and (F) L18 and L6 conditions. Serum T3-to-T4 Ratio at ZT3 and ZT15 (G) in L12 and (H) L18 and L6 conditions. Values are expressed as the mean ± S.E.M. (n=6). D, diet effect; T, GSPE treatment effect; P, photoperiod effect; TxD, interaction between treatment and Diet; DxP, interaction between Diet and Photoperiod; n.s., no significant differences. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant differences by Photoperiod effect using 2-way and 3-way ANOVA followed by LSD post-hoc test (p ≤ 0.05). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). + Indicates significant differences using paired Student´s t test between ZT3 vs ZT15 for each group (p ≤ 0.05). x Indicates tendency using paired Student´s t test between ZT3 vs ZT15 for each group (p=0.1-0.051). AUC, Area under curve; GSPE, grape seed proanthocyanidin-rich extract, STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg GSPE; L12, normal photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

Table 1. Effect of abrupt change of photoperiod and grape seed proanthocyanidins administration on cumulative food and energy intake along the six weeks under L12 conditions and the seventh week of experiment under L18 and L6 conditions.

	Diet	L12 Cumulative 6 weeks	t-student	Photoperiod	VH 7th week	GSPE 7th week	ANOVA
Food Intake (g)	STD	120.21 (±3.41)	p<0.001	L6	19.69 (±0.97)	22.61 (±1.44)	D
				L18	19.23 (±0.82)	20.05 (±0.80)	
	CAF	321.81 (±6.98)		L6	57.99 (±3.79)*	57.71 (±2.30)*	
				L18	48.93 (±5.64)*	51.94 (±4.61)*	
Energy Intake (Kcal)	STD	419.70 (±8.25)	p<0.001	L6	65.76 (±3.24)	75.51 (±4.81)	D
				L18	64.22 (±2.74)	66.97 (±2.68)	
	CAF	1335.17 (±28.45)		L6	233.08 (±18.34)*	233.64 (±10.32)*	
				L18	192.59 (±31.13)*	216.21 (±27.48)*	
Energy intake from Protein (Kcal)	STD	79.74 (±1.57)	p<0.001	L6	12.50 (±0.62)	14.35 (±0.91)&	D
				L18	12.20 (±0.52)	12.72 (±0.51)	
	CAF	119.62 (±1.38)		L6	16.06 (±0.76)*	17.27 (±0.43)*	
				L18	16.52 (±0.65)*	17.78 (±1.33)*	
	STD	302.19 (±5.94)	p<0.001	L6	47.35 (±2.33)	54.36 (±3.46)	D. P
				L18	46.24 (±1.97)	48.22 (±1.93)	

Energy intake from Carbohydrates (Kcal)	CAF	911.50 (±28.22)		L6	179.12 (±15.87)*	172.16 (±10.30)*	
				L18	138.59 (±27.29)*#	145.83 (±26.77)*	
Energy intake from Fat (Kcal)	STD	33.58 (±0.66)	p<0.001	L6	5.26 (±0.26)	6.04 (±0.38)	D, T, DxT, DxP
				L18	5.14 (±0.22)	5.36 (±0.21)	
	CAF	416.76 (±7.35)		L6	50.04 (±3.57)*	57.08 (±1.23)*$	
				L18	52.80 (±3.76)*	66.81 (±4.67)*$#	
AUC Energy from carbohydrates oxidation	STD	75639.10 (±9703.77)	p=0.02	L6	100366 (±21217)	112676 (±11496)	D
				L18	112095 (±11294)	106221 (±5877)	
	CAF	49412.4 (±6075.05)		L6	71406 (±9041)*	67067 (±14008)*	
				L18	69592 (±6221)*	35767 (±11233)*$	
AUC Energy from fat oxidation	STD	111461 (±11654)	p=0.008	L6	64388 (±16072)	48017 (±11396)	D
				L18	50167 (±11689)	48442 (±2447)	
	CAF	165171 (±15493)		L6	97490 (±9362)*	94484 (±16432)*	
				L18	130001 (±22824)*	122074 (±16528)*	

Values are expressed as the mean ± S.E.M. (n=24) for L12 conditions. P value was calculated using Student´s t test between L12-STD vs L12-CAF ($p \leq 0.05$). Values are expressed as the mean ± S.E.M. (n=6) for L18 and L6 conditions. D, diet effect; T, GSPE treatment effect; P, photoperiod effect; TxD, interaction between treatment and Diet; DxP, interaction between Diet and Photoperiod. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant differences by Photoperiod effect using 3-way ANOVA followed by LSD post-hoc test ($p \leq 0.05$). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). AUC, Area under curve; STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats

administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.

SUPPLEMENTARY

Table S1. Statistical summary.

Parameter	2-way ANOVA for L18 conditons	2-way ANOVA for L6 conditons	3-way ANOVA
Body weight gain in 7th week	DxT	n.s.	TxD
Energy intake in 7th week	D	D	D
AUC Energy Expenditure light phase	T	n.s.	T, P
AUC Energy Expenditure dark phase	n.s.	D	T, D
AUC Energy from carbohydrate Oxidation light phase	D	n.s.	D

AUC Energy from carbohydrate Oxidation dark phase	D	D	D
AUC Energy from fat Oxidation light phase	D	n.s.	D
AUC Energy from fat Oxidation dark phase	D	D	D
AUC Vertical activity light phase	D	D	P, D, TxP
AUC Vertical activity dark phase	D	D	D, P
Melatonin light phase	D	D	D
Melatonin dark phase	n.s.	n.s.	P
Corticosterone light phase	T	n.s.	T

Corticosterone dark phase	n.s.	n.s.	P
Testosterone light phase	D	n.s.	P
Testosterone dark phase	TxD	D	D, P, TxD
T3/T4 light phase	n.s.	n.s.	n.s.
T3/T4 dark phase	n.s.	D	D

D, diet effect; T, GSPE treatment effect; P, photoperiod effect; TxD, interaction between treatment and Diet; TxP, interaction between GSPE treatment and Photoperiod.

Manuscript 5

Objective: to investigate the effects of PACs on metabolism in healthy and CAF-induce obese Fischer 344 rats subjected to a sudden change of light/dark cycle.

The disruption of metabolism by light/dark shift depends on rat heath status: a role for proanthocyanidins

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The disruption of metabolism by light/dark shift depends on rat heath status: a role for proanthocyanidins

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ABSTRACT

Biological rhythms play an important role in regulating metabolism, controlling changes in behavior, growth, food intake and reproductive status. Changes in light-dark cycles and obesogenic diets are related to the disruption of circadian rhythms and the development of metabolic disorders. Grape seed proanthocyanidins (PACs) have shown beneficial effects on metabolic disease and, recently, it has been suggested that a modulation of circadian system could be mediating their healthy properties. Therefore, the aim of this study was to evaluate the effects of a grape seed PAC-rich extract (GSPE) on metabolic profile and central clock in healthy and obese rats after an abrupt change of light-dark cycle. For this, forty-eight rats were fed standard (STD) or cafeteria (CAF) diet for 6 weeks under standard (12 h light/day, L12) conditions. Then, animals were switched to a long (18 h light/day, L18) or short (6 h light/day, L6) photoperiod and administered vehicle (VH) or GSPE (25mg/kg) for 1 week. The results showed changes in lipid and glucose profile after light-dark shift dependent on photoperiod and diet. CAF diet increased serum cholesterol and triglyceride levels under L18 conditions and insulin was affected under L6 conditions, while PACs administration improved both metabolism disorders. Furthermore, GSPE increased the T3-to-T4 ratio CAF rats under L18 conditions and changed serum metabolomic profiles in a photoperiod-dependent manner. These results suggest that PACs could change the serum metabolomic status and ameliorate the disorders caused by light and dark disturbance in a photoperiod-dependent manner. However, further studies are needed to elucidate the pathways involved in these results.

INTRODUCTION

The rotation of the Earth around its axis and its translation around the sun are factors that generate variations that the biological clocks of the organism are able to capture and promote physiological and metabolic changes to adapt and be more energetically efficient [1,2]. Thus, the light-dark cycle generated by these variations sets the rhythm of processes such as blood pressure, body temperature, sleep-wake cycle, among others [3–6]. The central clock, located in the suprachiasmatic nucleus (SCN), is the pacemaker of the organism's biological rhythm, capable of translating external signals into hormonal signals. In this context, light is an important external signal (zeitgeber) captured by the retina through retinohypothalamic tract (RHT) that keeps the organism synchronized with the environment and with the light-dark cycle [2,7]. In addition to light, food can also be a modulator of the biological rhythm and reprogram or disrupt the circadian rhythm [2,8,9]. Recently, alterations in colon functionality and lipidic metabolism disorders due to an altered light-dark cycle have been reported [10,11]. Moreover, feeding patterns were affected by repeated alterations in the light-day length resulting in an impact on body weight gain [12,13]. Furthermore, disorders in clock genes have been shown in peripheral tissues by diet composition. In this regard, alterations in clock genes in peripheral tissues or the central clock have been demonstrated by diet composition. In this regard, obesogenic diet, such as the cafeteria diet (CAF), promotes the alteration of circadian rhythms of hepatic metabolism resulting in metabolic disorders that increase the risk of metabolic syndrome [14]. Additionality, light/dark cycle shifts, and high fat diet alter the cognitive response in F344 rats [15].

At molecular level, central clock is self-regulated by transcriptional-translational feedback loops with 24-hour cycles [16]. Heterodimers of CLOCK (circadian cycles of locomotor kaput protein production) and BMAL1 (brain and muscle ARNT 1) act as positive regulators to promote transcription of period (PER) and cryptochrome (CRY) proteins. PER and CRY proteins form a complex in the

nucleus that acts as a negative regulator during the day by binding to the E-box regulatory sequence, inhibiting BMAL1 and CLOCK transcription and, at the same time, PER and CRY transcription. Finally, the CLOCK - BMAL1 heterodimer potentiates the transcription of metabolic genes, such as nicotinamide phosphoribosyltransferase (Nampt), which are involved in many processes of metabolism via sirtuina 1 (Sirt1) stimulation and regulation of the nicotinamide adenine dinucleotide (NAD) [17–19]. This evidence of the relationship between the central clock mechanism and metabolism explains the association between circadian rhythm disruption and metabolic disorders. Therefore, several factors, such as artificial light shift work, meal timing, etc, disrupt biological rhythms in modern societies and are associated with the development of metabolic disorders that, in the long term, could lead to type 2 diabetes, cardiovascular disease, overweight and obesity [20–24]. In addition, metabolism is also modulated by chronic axes such as hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) [25,26], which play an important role in energy metabolism, food intake, activity and reproduction and are also closely linked to biological rhythms, showing a marked circadian rhythm. Corticosterone plays a key role in the HPA axis, the T3-to-T4 ratio for the HPT axis and testosterone for the HPG axis and shows a marked circadian rhythm [27,28]. Thus, alterations in the light-dark cycle could result in diseases of glucocorticoid sensitivity or resistance [63]. Increased corticosterone is related to stress [28,29] and its circadian alteration could lead to diseases of glucocorticoid sensitivity or resistance [30].

Proanthocyanidins, a sub-class of flavonoids, has been extensively studied by our group, has demonstrated its important beneficial effects on different key aspects of metabolic syndrome [31,32]. Interestingly, in the last studies carried out, a modulating effect of grape seed proanthocyanidins (PACs) on the circadian rhythm of hepatic peripheral clock has been observed [33]. Thus, PACs was able to modulate the circadian clock by its apparent ability to mimic the

action of melatonin by positively regulating Bmal1 and in turn the molecular clock, in healthy and obese rats [34–37]. Moreover, previous results of this project have demonstrated mitigation effects on alterations by an abrupt photoperiod change in a photoperiod- and diet-dependent manner [Paper 3]. Therefore, PACs may act as zeitgebers for the molecular clock and contribute to restore circadian disruption caused by light/dark shifts or diet-induced obesity.

Considering these previous considerations, the aim of the present study was to evaluate whether grape seed proanthocyanidin extract (GSPE) is able to act on the metabolic disorders produced by the abrupt disruption of the light-dark cycle in both healthy and CAF-induced obese rats. For this purpose, rats fed with standard diet (STD) and CAF were abruptly transferred from a standard (12 h of light/day, L12) to a long (18 h of light/day, L18) or short (6 h of light/day, L6) photoperiod and the gene expression of the central clock and the metabolic profile of these rats were studied.

MATERIALS AND METHODS

Grape seed proanthocyanidin-rich extract

The grape seed proanthocyanidin-rich extract (GSPE) used in this study was provided by Les Dérives Résiniques et Terpéniques (Dax, France). This was obtained from white grape seed. The phenolic profile of this extract is mainly composed of catechin, epicatechin, gallic acid, epicatechin gallate and dimers, trimers and tetramers of proanthocyanidins [41].

Animal Procedures

Forty-eight 13-week-old male Fischer 344 rats from Charles River Laboratories (Barcelona, Spain) were housed under standard laboratory conditions at 22°C and 12 hours light-dark cycle (L12) with ad libitum access to food and drinking water. After two weeks of acclimatization period, rats were weighted and randomly divided into two groups (n=24), one group (L12-STD) was fed a STD diet (2.90 kcal/g; A04, Panlab, Barcelona, Spain), and the other group (L12-CAF) was fed a CAF diet for 6 weeks. After this time, animals were switched from a

L12 to a long (18 h light/day, L18) or short photoperiod (6 h light/day, L6) and administered vehicle (VH), which was condensed milk 1/5 diluted, or GSPE (25mg/Kg) dissolved in VH for 1 week. Thus, the animals were finally grouped into 8 different groups (n=6). The onset of light was at 8:00 a.m. and defined as zeitgeber time 0 (ZT0) and VH and GSPE treatment were administered at ZT0. The CAF diet was prepared every day and contained bacon, cookie with paté, cookie with cheese, carrots, ensaïmada (pastry), standard chow and sweetened milk (22% sucrose w/v), and its caloric distribution was 56.43% carbohydrate, 45.72% lipid and 9.5% protein [42]. After experimental period, the animals were deprived of food for 3 hours before being sacrificed by decapitation from ZT4 to ZT6. The hypothalamic samples were rapidly removed after death, frozen in liquid nitrogen and stored at -80°C until further analyses. Blood was collected in non-heparinized tubes, incubated for 1 h at room temperature and immediately centrifuged at 1200×g for 15 min at 4 °C to collect the serum.

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

Serum Biochemical Parameters and hormones

In order to carry out the analysis of serum biochemical parameters, enzymatic colorimetric assays were used for the analysis of glucose, total cholesterol (TC) and triglycerides (TAG) (QCA, Amposta, Tarragona, Spain) and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany) according to the manufacturer's instructions.

The serum hormones concentrations were measured by liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ). Serum samples were thawed at 4 °C. 50 µL of serum were mixed with 250 µL of methanol

containing the internal standard (2 ng/mL). Then, the mixture was vortexed and centrifuged for 5 minutes at 4 °C and 252g. The supernatant was transferred to a new tube and mixed with 700 µL of 0,1 % formic acid in water. The sample was loaded to an SPE previously conditioned with methanol and 0,1 % formic acid in water. The cartridge was washed with 0,1 % formic acid in water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45 °C and reconstituted with 50 µL of water:methanol (60:40, v/v) and transferred to a glass vial for analysis. The hormones detected were Melatonin, Corticosterone, T3, T4 and Testosterone. For these measures, the Eurecat research center (Reus, Spain) worked with us to carry out the technique.

Metabolomic analysis

Metabolomic analysis in the 96 rat serum samples was performed at Centre for Omic Sciences (COS, Tarragona, Spain) using gas chromatography coupled to Quadrupole Time-of-Flight Mass Spectrometry (GC-qTOF). The extraction was performed by adding 400 µL of methanol:water (8:2) containing internal standard mixture to serum samples (approx. 100 µL). Then, the samples were mixed, incubated at 4ºC for 10 min, centrifuged at 252g 4°C for 10 min and supernatant was evaporated to dryness before compound derivatization (methoximation and silylation). The derivatized compounds were analyzed by GC-qTOF (model 7200 of Agilent, USA). The chromatographic separation was based on Fiehn Method [43] using a J&W Scientific HP5-MS (30 m x 0.25 mm i.d., 0.25 µm film capillary column and helium as carrier gas using an oven program from 60 to 325ºC. Ionization was done by electronic impact (EI), with electron energy of 70eV and operating in full Scan mode. Identification of metabolites was performed using commercial standards and by matching their EI mass spectrum and retention time to metabolomic Fiehn library (from Agilent) which contains more than 1.400 metabolites. After putative identification of metabolites, these were semi-quantified in terms of internal standard response ratio.

Gene Expression Analysis

The total RNA, containing the microRNA, 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 1 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as a percentage of the L18-STD-VH group using the 2-ΔΔCt method with Ppia gene as an endogens control, as reported by Schmittgen and Livak [44].

Statistical Analysis

Data were represented as means ± 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 two-way ANOVA followed by LSD post-hoc test. A total of 66 serum metabolites were identified and integrated. Kruskal Wallis test or Mann-Whitney test were using to analyze metabolomics data, as indicated in the respective figure legend were applied to explore the origin of outcomes. Theses statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). Overall effects of changing photoperiod and the effect of diet and treatments on the serum metabolomic

profile were analyzed by principal component analysis (PCA) and the sparse Partial Least Squares Discriminant Analysis (sPLS-DA) using MetaboAnalyst v.4.0 (McGuill University, Montreal, Canada).

RESULTS

PAC administration to CAF-fed rats in conditions of photoperiod disruption improved the insulin sensitivity under L6 condition and the lipid profile under L18 condition.

Serum glucose of rats transferred to the new photoperiods showed no daylength-dependent changes, but an increase in this parameter was observed in CAF-fed animals under both photoperiod respect to their respective counterparts (Figure 2A). Although no changes in serum glucose were found, photoperiod-dependent changes in insulin levels were detected (Figure 2B). While CAF-fed rats transferred to L6 photoperiod showed a significant increase in this hormone compared to L6-STD fed rats ($p=0.002$), CAF fed rats under L18 conditions did not show changes in insulin values respect to the STD-fed. In fact, differences in the values of this hormone were detected between both CAF-fed groups ($p=0.005$, L18-CAF-VH vs L6-CAF-VH). The increased insulin levels observed in CAF-fed animals under L6 photoperiod were reversed by PACs administration ($p=0.005$), returning to similar values to those of healthy rats.

Regarding the lipid profile, total cholesterol of rats fed with CAF diet increased significantly under L18 conditions compared to the STD-fed animals ($p<0.001$) and PACs administration decreased their levels ($p<0.001$), returning to values of STD-fed rats. However, under L6 conditions CAF-fed rats showed similar values to their corresponding healthy rats (Figure 2C). In fact, a photoperiod effect in this parameter was observed in the CAF-fed rats since the effects of the diet was depending on light-dark conditions ($p<0.001$). In the case of triglycerides (Figure 2D), CAF-fed rats under L18 conditions showed also elevated levels compared to healthy rats ($p<0.001$), while GSPE-treated rats showed significant lower values ($p=0.004$). Under L6 photoperiod, CAF-fed rats

showed an increase in serum triglycerides compared to healthy rats ($p<0.001$), although this increase was lower than CAF-fed rats under L18 conditions, showing a photoperiod effect ($p<0.001$). Triglyceride values in CAF-VH and CAF-GSPE rats were similar. Regarding serum NEFAs levels (Figure 2E), no differences were found in the animals under L6 conditions. However, CAF-fed animals administered VH and GSPE showed tendency or significant differences, respectively, compared to their healthy counterparts under L18 conditions, although no differences were found between VH and GSPE in this photoperiod.

PAC administration in conditions of photoperiod disruption affected HPA, HPG and HPT axes

No changes in serum melatonin levels were observed in any photoperiod neither by diet nor by treatment (Figure 3A). For corticosterone and testosterone, no significant differences were found under L18 photoperiod, although the CAF diet in the animals administered VH showed a tendency to decrease respect to the healthy rats. Regarding corticosterone levels under L6 conditions, an interaction among photoperiod, diet, and treatment effect was observed since in healthy animals PACs administration resulted in an increase of this hormone levels compared to the rats administered VH ($p=0.004$) and also respect to the PACs administered animals under L18 condition ($p=0.004$) (Figure 3B). Regarding testosterone levels, the administration of PACs to STD-fed rats under L6 photoperiod resulted in a decrease compared to VH-administered rats ($p=0.047$) (Figure 3C). Thyroid hormones were also analyzed, and in the case of T3 hormone an interaction between the effect of photoperiod, treatment and diet was observed (Figure 3D). This hormone levels showed a tendency to increase in control CAF-fed rats under L6 photoperiod compared to control CAF-fed rats under L18 photoperiod ($p=0.092$). However, a significant reduction of these levels was observed after PACs administration since T3 levels of L6-CAF-GSPE group were lower than the obtained in L6-CAF-VH animals ($p=0.029$). In addition, these T3 levels of L6-CAF-GSPE group showed a tendency to decrease when were compared to the obtained for L18-CAF-GSPE group ($p=0.082$). For

T4 hormone, a significant effect of diet was observed under L18 conditions, in which CAF-fed rats showed a decrease in T4 concentrations ($p<0.001$) respect to the STD-fed animals (Figure 3E). In addition, an increase in T4 concentrations was observed in rats fed the CAF diet under L6 photoperiod compared to CAF-fed rats under L18 conditions ($p=0.001$). However, this increase was mitigated by PACs administration ($p=0.041$). These results were reflected in the T3-to-T4 ratio (Figure 3D), where an increase in the ratio was observed in CAF-fed and GSPE-treated rats under L18 conditions compared CAF-fed and VH-administered rats ($p=0.044$). Additionally, this effect was not observed under L6 condition, where GSPE treatment in CAF-fed rats resulted in a lower T3-to-T4 ratio than L18-CAF-GSPE ($p=0.043$).

PAC administration to CAF-fed rats in conditions of photoperiod disruption increased the expression of the clock gene Nampt under L18 conditions

The expression of Bmal1 was upregulated by CAF diet in both photoperiods respect their respective STD groups (Figure 4A), a tendency was observed under L18 condition ($p=0.074$) and significant differences under L6 photoperiod ($p=0.026$). However, no significant differences were found neither in Cry1 (Figure 4B) nor in Per2 (Figure 4C) expression, although a tendency to increase Cry1 expression in L18-CAF-GSPE rats respect to L18-STD-GSPE animals ($p=0.08$). Regarding to Nampt (figure 4D), a significant increase in gene expression was observed as a result of the CAF diet in both photoperiods compared to their respective STD controls ($p=0.039$ and $p=0.001$ for L18-CAF-VH and L6-CAF-VH respectively). Interestingly, in CAF-fed rats, PACs administration under L18 conditions resulted in an increase of the gene expression compared to the corresponding CAF-fed administered VH ($p=0.004$).

PAC administration to CAF-fed rats in conditions of photoperiod disruption modified the metabolomics profile in a photoperiod manner.

Firstly, in order to evaluate the effect of photoperiod change, the four VH groups of two photoperiods were analyzed by PCA and sPLS-DA (Figure 5). Although there was no clustering by PCA (Figure 5A), the STD-fed rats did show clustering by photoperiod with a clear separation between L6 and L18 conditions in sPLS-DA analysis (Figure 5B). The metabolomic profile was clearly different between CAF-fed and STD-fed groups by heatmap analysis (Figure 5C). Slight differences were observed between the L18-STD-VH and L6-STD-VH groups and more evident between the CAF-fed groups. Secondly, diet and treatment effect under 18 conditions were analyze (Figure 6) and only clustering by diet effect was observed using PCA (Figure 6A), however, in sPLS-DA (Figure 6B) samples did cluster by diet and treatment, showing a clear separation among L18-CAF-VH. L18-CAF-GSPE and STD-fed rats. The heatmap analysis also revealed a change in the serum metabolomic profile according to the diet composition (Figure 6C), clearly differentiating the 2 groups that were fed with CAF diet from the 2 groups that were fed with STD diet. Interestingly, L18-CAF-GSPE group showed a different metabolomics profile compared to VH rats. Finally, diet and treatment effects were analyzed under L6 conditions (Figure 7). PCA showed a clustering by diet effect between CAF-fed groups and STD-fed groups (Figure 7A). In fact, sPLS-DA showed the same clustering by diet and, in addition, a separation between L6-CAF-GSPE and L6-CAF-VH groups (Figure 7B). These effects were also observed by heatmap analysis (Figure 7C), where a clear different metabolomics profiles were observed between CAF-fed and STD-fed groups and between L6-CAF-VH and L6-CAF-GSPE.

Moreover, the evaluation by Kruskal Wallis test and Mann-Whitney test of the effects were carried out of 66 metabolites. Thus, statistical analysis between groups under L18 conditions found 16 metabolites whose concentration was significantly different due to diet and treatment effect (Table S1). Additionally, under L6 conditions, 18 metabolites were detected that varied by diet and treatment effect (Table S1). In this regard, remarkable changes were found in amino acid such as alanine, proline, phenylalanine and 4-hydroxyPhenyllactic

acid, as well as energy-metabolism metabolites such as succinic acid and citric acid (Figure 8). Particularly, CAF diet effect increased alanine levels under L18 (P=0.004) and L6 (p=0.065) conditions compared to STD-fed rats (Figure 8A). Interestingly, PACs treatment resulted in a lower alanine level only under L18 conditions (p=0.016). Regarding proline (Figure 8B), CAF diet resulted in a higher concentration under both L18 (p=0.016) and L6 (p=0.002) conditions and this effect was reversed by GSPE treatment under L18 (p=0.055) and L6 (p=0.009) photoperiod. In the case of phenylalanine levels (Figure 8C), The CAF diet promoted an increase in the concentration of this amino acid in serum only under L6 conditions (p=0.002), this increase was reversed by GSPE treatment (p=0.026). Furthermore, CAF diet reduced the levels of 4-hydroxyPhenyllactic acid only under the L18 photoperiod compared to its respective control group (p=0.004) (Figure 8D), however, the concentrations of this amino acid were reduced with GSPE treatment under both photoperiods (p=0.037 and p=0.041 for L18 and L6 conditions respectively). Succinic acid was also altered by diet in a photoperiod-dependent manner (Figure 8E). Thus, CAF-fed rats tended to show higher levels of this metabolite only under L18 conditions (p=0.055) compared to STD-fed rats, furthermore, these levels were also higher compared to the L6-CAF-VH group (p=0.004). The GSPE treatment tended to lower levels under L18 conditions (p=0.078). Finally, citric acid was only affected under L6 conditions CAF-fed rats compared to STD-fed rats (p=0.002) (Figure 8F), where it was lowered by the CAF diet and compared to the L18-CAF-VH group (p=0.002). In this case, GSPE treatment did not result in changes in levels.

DISCUSSION

Biological rhythms play a crucial role in the regulation and efficiency of metabolism, promoting several disorders when these rhythms are disrupted. Thus, shift or disturbances of normal light-dark cycle have been associated to metabolic disorders that could lead to development of metabolic syndrome [45]. In this context, different molecular mechanisms have been involved in the healthy properties of PACs on metabolism disorders, including their interaction

with the clock system [36,46]. Additionally, in a recent study, we have reported that PACs could mitigate in healthy and cafeteria-fed rats circadian disturbance caused by a sudden photoperiod change, restoring diurnal oscillation of some key metabolic regulators such as corticosterone, testosterone, or locomotor activity [Manuscript 4]. Therefore, the aim of this study was to evaluate the potential beneficial effects of PACs on the metabolism in circadian disruption conditions caused by an obesogenic diet and changes in the light-dark cycle.

Biochemical parameters were affected by the CAF diet in a photoperiod-dependent manner, showing worse lipid profile under L18 conditions, while rats fed a CAF diet under L6 conditions showed elevated insulin concentrations and glucose levels did not vary between photoperiods. In this sense, disturbances in lipid and glucose metabolism have been reported in a photoperiod disruption conditions, however these results clearly differ from other studies in which a worse lipid profile was reported in rats exposed to short photoperiods compared to those exposed to long photoperiods [47] or other studies that have reported no differences between photoperiods in lipid metabolism [39,48,49]. This discrepancy in the changes in these parameters could be explained by the short-term exposure of the photoperiods in our study compared to studies with chronic exposure to the different photoperiods. These results would be in agreement with the body weight loss in CAF-fed rats in the short photoperiod and lower body weight loss in rats under L18 conditions reported in the previous results of this study [Manuscript 4]. Interestingly, GSPE was able to reduce cholesterol levels to values of STD-fed rats. Additionally, GSPE treatment reduced triglyceride levels in this photoperiod although not to levels of STD rats. CAF-fed rats under L6 conditions showed no increase in serum cholesterol compared to STD-fed rats and triglycerides were not increased to the same magnitude as under L18 photoperiod, thus no effects of GSPE treatment were seen. In addition, Insulin levels dropped to the values of STD-fed rats with GSPE treatment under L6 conditions. In this regard, the results are in agreement with those reported in

other studies where beneficial effects of GSPE on lipid and glucose metabolism are observed [50–52].

The main marker of the light-dark cycle, melatonin, was not affected by diet or photoperiod, as expected since samples were obtained at a single time point during the light phase. This hormone increases in concentration during the dark phase, being an important signal for the organism to recognize the photoperiod to which it is exposed [53]. Furthermore, the results are in agreement with those obtained previously [Manuscript 4], where no melatonin variations were observed due to the change of photoperiod in the light phase, this hormone being the first to adapt to the new light-dark cycles. While melatonin signals the organism to adapt its metabolism to photoperiod changes, corticosterone is another hormone with important metabolic implications. This hormone plays a key role in the hypothalamic-pituitary-adrenal (HPA) axis [12,13] and its increase is related to stress [13,54]. Thus, its alteration could lead to diseases of glucocorticoid sensitivity or resistance [55]. In this context, STD-fed rats switched to L18 photoperiod tended to show higher corticosterone levels compared to CAF-fed rats. This result is in agreement with those reported in the previous study, where higher corticosterone values were observed in L18 during the light phase 5 days after the photoperiod change [Manuscript 4]. In contrast to the previous study, STD-fed rats in the L6 photoperiod that received GSPE showed highly increased corticosterone levels. The HPG and HPT axes are important in processes such as lipid and carbohydrate metabolism and are closely related to biological rhythms. Testosterone and thyroid hormones, respectively, act as modulators of these axes [14,15]. GSPE reduced testosterone levels in STD-fed rats. In this sense, GSPE could be interacting with the duration of daylight hours to act on testosterone levels in L6. Regarding thyroid hormones, a tendency to have higher T3 hormone levels has been observed in CAF-fed rats under L6 conditions, interestingly this is reversed with GSPE treatment, where a tendency to have higher T3 levels is observed in GSPE-treated rats under L18 conditions compared to GSPE-treated rats under L6

conditions. Elevated T3 levels have been associated with reduced cholesterol and triglyceride levels [14]. In addition, higher levels of thyroid hormones and the T3-to-T4 ratio are associated with a loss of BW [56]. These results are in accordance with those obtained for biochemical parameters, where an improvement in cholesterol and triglyceride levels is seen and could be mediated by the increase in T3 levels and thyroid hormone ratio levels that increase in the L18-CAF-GSPE group.

The most important controller of the circadian rhythm is the central clock, this pacemaker is in hypothalamus and maintains the synchronization of the peripheral clocks with the environment and its oscillators through hormone signals and neural connections [7]. To study the status of the SCN in these groups, we analyzed the gene expression of *Bmal1, Per2, Cry1* and *Nampt*. In this regard, *Bmal1* and *Nampt* were affected by the CAF diet, increasing their expression in both photoperiods. In contrast, no differences were found in the expression of Per2 and Cry1 genes. Although no significant effects were observed by GSPE treatment on Bmal1, it was observed that GSPE caused a slight increase in Bmal1 expression in CAF-fed rats at photoperiod L18. Interestingly, GSPE treatment caused an increase in *Nampt* expression under L18 conditions in CAF-fed rats compared to VH rats. This gene is an important clock and hypothalamus gene, and its expression is related to energy metabolism [19,57,58]. Thus, NAMPT is the rate-limiting enzyme in NAD biosynthesis through its salvage pathway [59]. NAD homeostasis is related to free radical-mediated reactive oxygen species production, which has been linked to innumerable pathologies, including metabolic diseases [60]. In addition, this metabolite activates several NAD-dependent deacetylases (SIRTs), controlling the activity of many cellular proteins. Thus, the improved lipid profile of CAF-induced obese rats could be modulated by the increased expression of *Nampt*.

Metabolomic data also showed a change in the profile of the groups in a diet- and photoperiod-dependent manner. Thus, the diet effect caused a strong

increase in 2-hydroxyisobutyric acid, glycolic acid, and proline concentrations under L18 conditions. In addition, CAF diet tended to increase levels of succinic acid, a metabolite that has been associated with mitochondrial stress in a diabetic condition when at prolonged elevated levels [24]. Interestingly, a significant increase in Krebs cycle metabolites, such as malic acid, succinic acid, citric acid or fumaric acid, was observed in rats fed the CAF diet under L18 conditions compared to those exposed under L6 conditions. It has been reported that the activity of Krebs cycle enzymes, including citric acid synthase, is reduced in mice under conditions of nutrient excess and this is associated with obesity [61,62]. The increase in these metabolites would explain the differences in the glycemic profile shown by the rats, with a better profile in CAF-fed rats under L18 conditions. Additionally, phosphoric acid, 4-hydroxyphenylactic acid, maltose and hippuric acid were significantly reduced in all CAF-fed rats compared to STD-fed rats. Interestingly, hippuric acid was negatively associated with BW gain [63]. In CAF-fed rats, increased alanine and phenylalanine concentrations were observed in both photoperiods. The increase of these amino acids, altering their metabolism, have been related to obesity and the development of metabolic syndrome [20]. Regarding L6 conditions, there was a significant increase in the levels of proline, glycerol, d-sucrose, and alanine due to the CAF diet, while the levels of d-mannitol, hippuric acid, 4-hydroxyphenyl lactic acid and citric acid, this metabolite involved in the Krebs cycle, were reduced. In this context, on the one hand, GSPE treatment under L18 conditions significantly mitigated these variations for 4-hydroxyphenylactic acid, alanine, and tended to restore normal values for glycolic acid, succinic acid, and proline. On the other hand, GSPE treatment mitigated the variations for proline, phenylalanine and 4-hydroxyphenylactic acid in CAF-fed rats, with no effects observed in STD-fed rats by GSPE treatment. Interestingly, obese rats in L6 conditions and treated with GSPE showed higher concentrations of dodecanoic acid, a metabolite that plays a key role in fatty acid oxidation [64] and that could explain the better lipid profile of these rats.

CONCLUSION

In conclusion, the results suggest that GSPE was able to modify the metabolic profile of cafeteria-induced obese rats with a disruption of the light-dark cycle in a photoperiod-dependent manner. CAF-fed animals showed an altered lipid profile under L18 conditions and reduced insulin sensitivity under L6 conditions. The difference in metabolic state between photoperiods was reflected in the serum metabolomic profile, where an increase in metabolites involved in the Krebs cycle was observed in rats under L18 conditions compared to rats exposed under L6 photoperiod. Regarding diet, a photoperiod-dependent increase of metabolites associated with obesity and metabolic syndrome such as alanine, phenylalanine or hippuric acid was observed. These disorders were mitigated by GSPE, changing serum metabolomic profiles. Furthermore, the results suggest that there could be an increase in metabolic rate through the regulation of the HPA, HPG and HPT axes, as GSPE was able to modify the concentration of hormones such as T3, T4 and testosterone. Regarding the gene expression of Nampt in hypothalamus, closely related to energy metabolism, was stimulated by GSPE treatment, this finding could suggest the ability as a modulator of biological rhythms to act on metabolism. However, further studies are needed to elucidate the metabolic pathways and processes involved in these events.

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FIGURES

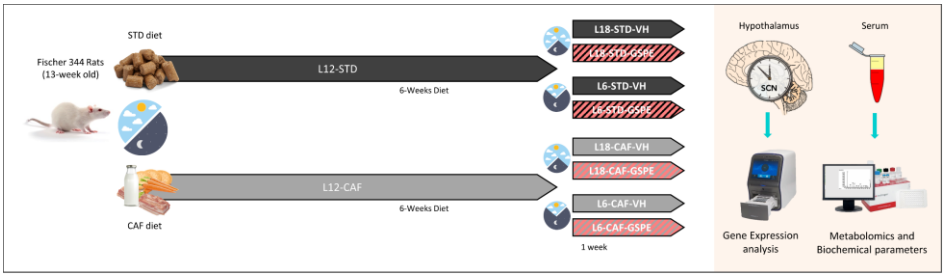

Figure 1. Experimental model*. Forty-eight rats were fed standard (STD) or cafeteria (CAF) diet for 6 weeks under L12 conditions. After this time, the animals were switched to L18 or L6 and administered vehicle (VH) or GSPE (25mg/kg) for 1 week.*

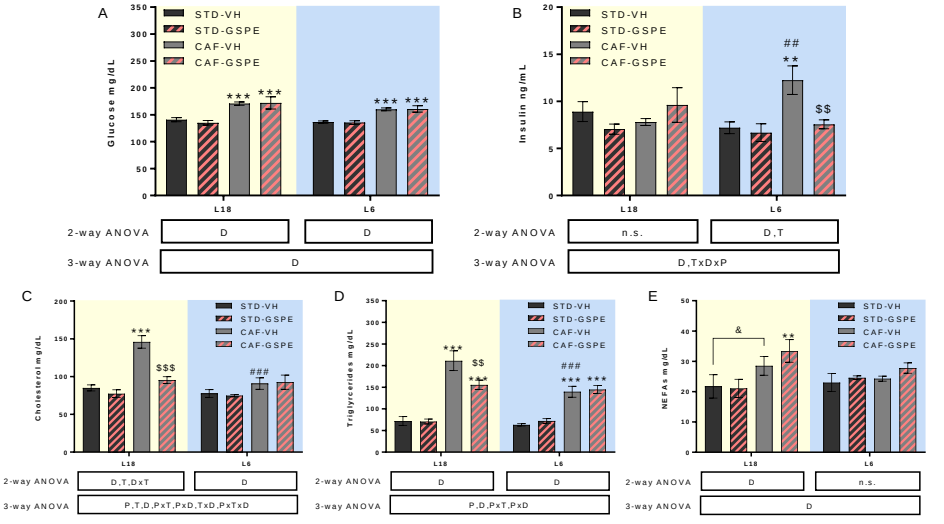

Figure 2. Serum biochemical parameters for L18 and L6 conditions. *Serum glucose (A), insulin (B), cholesterol (C), triglycerides levels (D) and NEFAs in mg/dL (E). Values are expressed as the mean ± S.E.M for L18 and L6 groups. D, diet effect; T, treatment effect; P, photoperiod effect; TxD, interaction between treatment and Diet. PxT interaction between photoperiod and treatment; PxD, interaction between photoperiod and diet effect; PxTxD, interaction among photoperiod, treatment and diet; n.s., no significant differences. * Indicates significant differences by diet effect, $ Indicates significant differences by treatment effect, # Indicates significant differences by photoperiod effect using 2-way and 3-way ANOVA followed by LSD post-hoc test (p ≤ 0.05). STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

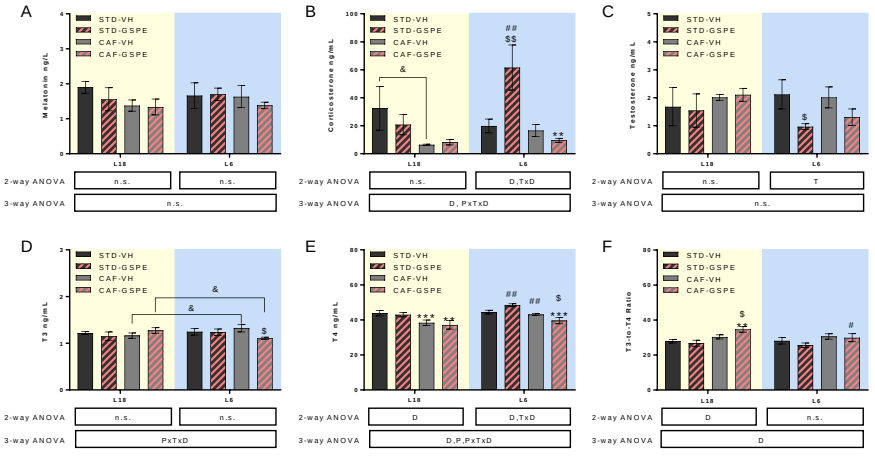

Figure 3. Serum hormones for L18 and L6 conditions. *(A) Melatonin in ng/L. (B) Corticosterona in ng/mL. (C) Testosterone in ng/L. (D) T3-to-T4 ratio. Values are expressed as the mean ± S.E.M (n=6)for L18 and L6 groups. D, diet effect; T, treatment effect; P, photoperiod effect; TxD, interaction between treatment and Diet. PxT interaction between photoperiod and treatment; PxD, interaction between photoperiod and diet effect; PxTxD, interaction among photoperiod, treatment and diet; n.s., no significant differences. * Indicates significant differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant dif-ferences by Photoperiod effect using 2-way and ANOVA followed by LSD post-hoc test (p ≤ 0.05). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

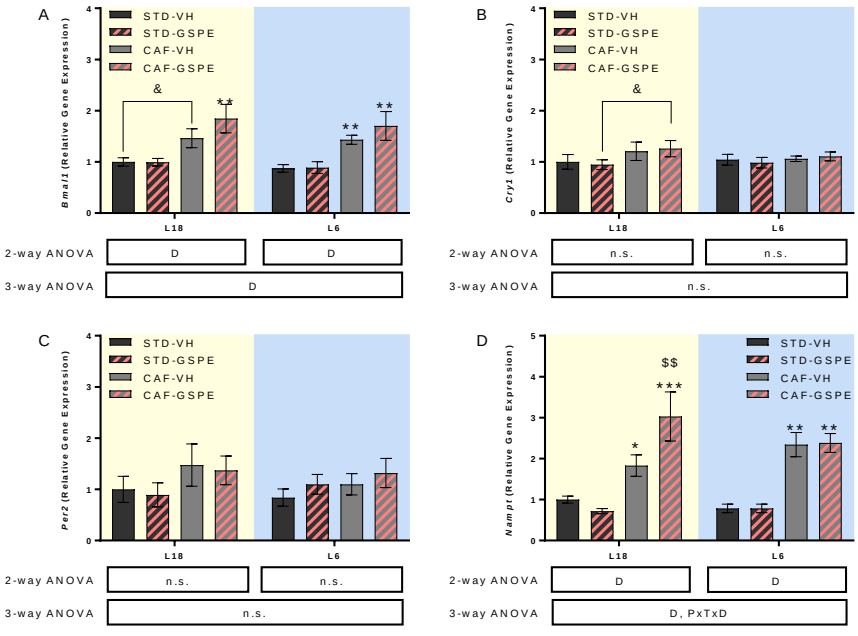

Figure 4. Circadian clock genes. *(A) Relative gene expression of Bmal1 (B) Relative gene expression of Cry1. (C) Relative gene expression of Sirt1 (D) Relative gene expression of Nampt. Values are expressed as the mean ± S.E.M (n=6)for L18 and L6 groups. D, diet effect; n.s., no significant differences. * Indicates significainteraction between photoperiod and diet effect; PxTxD, interaction nt differences by diet effect, $ Indicates significant differences by Treatment effect, # Indicates significant dif-ferences by Photoperiod effect using 2-way and ANOVA followed by LSD post-hoc test (p ≤ 0.05). & Indicates tendency using LSD post-hoc test (p=0.1-0.051). STD, Standard diet-fed rats; CAF, Cafeteria diet-fed rats; VH, rats administered vehicle; GSPE, rats administered 25 mg/kg grape seed proanthocyanidin-rich extract; L12, standard photoperiod 12 h light per day; L18, long pho-toperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

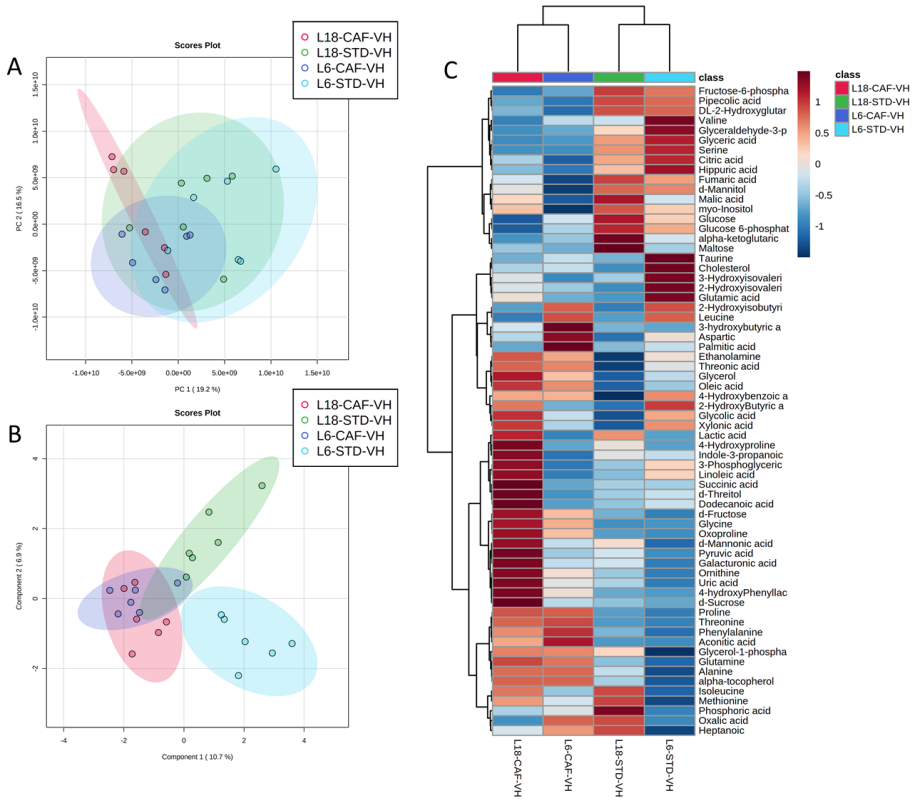

Figure 5. Metabolomic profiles for CAF-VH and STD-VH groups in L6 and L18 conditions*. Metabolomics analysis for groups under L18 conditions. A) Heatmap of abundance of Serum metabolites per experimental group. C) Relative metabolomics composition throughout principal component analysis (PCA) and the sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of serum samples of each group.*

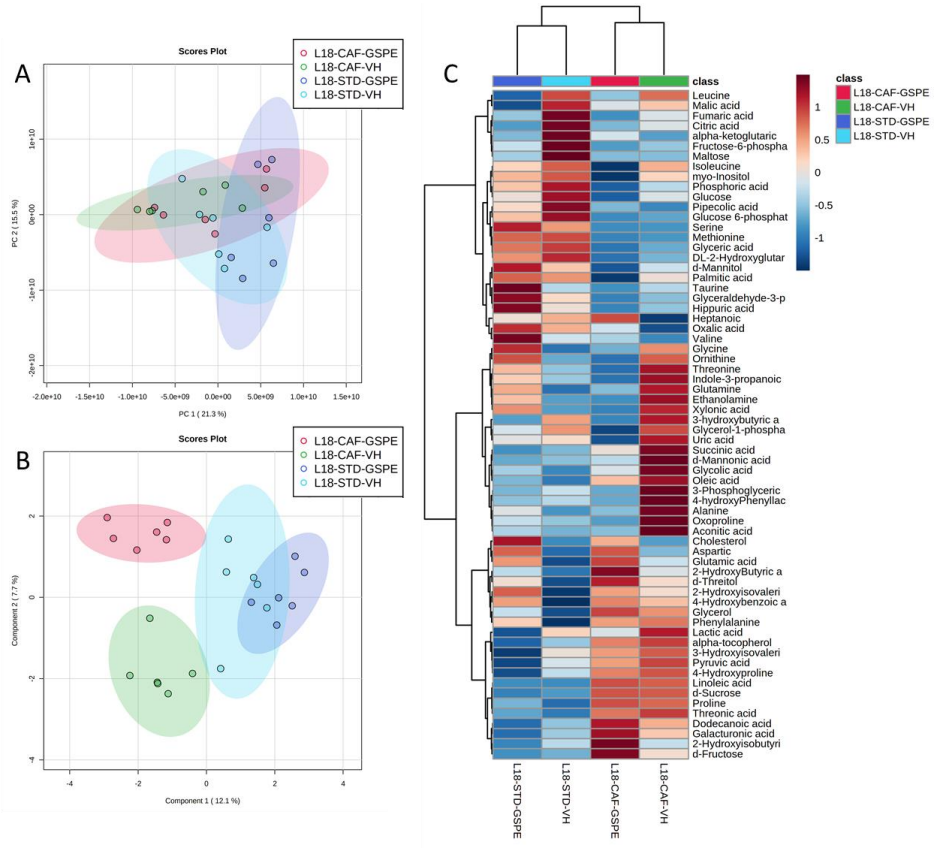

Figure 6. Metabolomic profiles for L18-photoperiod groups*. Metabolomics analysis for groups under L18 conditions. A) Heatmap of abundance of Serum metabolites per experimental group. C) Relative metabolomics composition throughout principal component analysis (PCA) and the sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of serum samples of each group.*

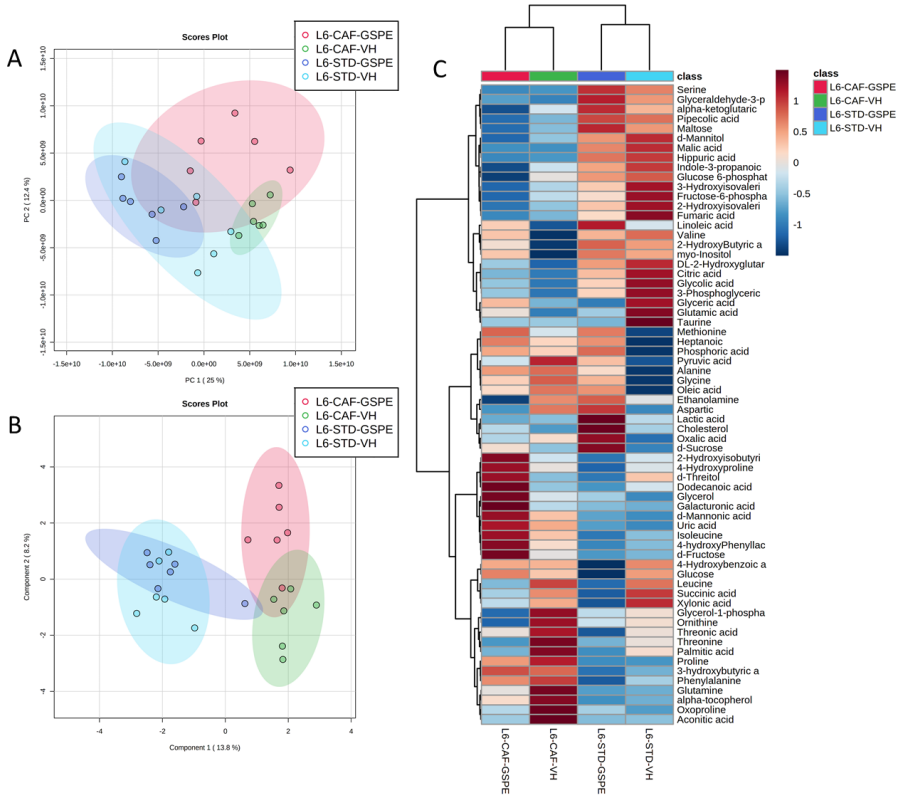

Figure 7. Metabolomic profiles for L6 photoperiod groups. *Metabolomics analysis for groups under L18 conditions. A) Heatmap of abundance of Serum metabolites per experimental group. C) Relative metabolomics composition throughout principal component analysis (PCA) and the sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of serum samples of each group.*

Table 1. ***Nucleotide sequences of primers used for PCR amplification in hypothalamus.***

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC
Cry1	TGGAAGGTATGCGTGTCCTC	TCCAGGAGAACCTCCTCACG
Per2	CGGACCTGGCTTCAGTTCAT	AGGATCCAAGAACGGCACAG
Sirt1	TTGGCACCGATCCTCGAA	ACAGAAACCCCAGCTCCA
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG
Ppia	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG

BMAL1, hydrocarbon receptor nuclear translocator-like 1; CRY1, cryptochrome 1; NAMPT, Nicotinamide Phosphoribosyltransferase; PER2, period 2; SIRT1, Sirtuin1; PPIA, Peptidylprolyl Isomerase A.

Table S1. Serum metabolites (Arbitrary units).

METABOLITES	L18-STD-VH (n=6) MEDIAN (IQR)	L18-STD-GSPE (n=6) MEDIAN (IQR)	L18-CAF-VH (n=6) MEDIAN (IQR)	L18-CAF-GSPE (n=6) MEDIAN (IQR)	L18 P-VALUE	L6-STD-VH (n=6) MEDIAN (IQR)	L6-STD-GSPE (n=6) MEDIAN (IQR)	L6-CAF-VH (n=6) MEDIAN (IQR)	L6-CAF-GSPE (n=6) MEDIAN (IQR)	L6 P-VALUE	CAF vs STD P-VALUE
2-HydroxyButyric acid	1.116 (0.874-1.217)	1.306 (0.999-1.709)	1.961 (1.372-2.155)	2.416 (2.008-3.351)	0.095	1.459 (0.895-3.036)	1.67 (1.325-1.999)	1.405 (0.952-1.737)	1.41 (1.077-2.749)	0.593	0.584
2-Hydroxyisobutyric acid	2.11 (2.001-2.297)	1.944 (1.798-2.547)	3.399 (2.997-4.409)**	3.603 (3.443-3.711)**	0.009	2.428 (2.165-3.148)	2.214 (2.084-2.288)	3.333 (3.03-4.083)	3.359 (2.955-3.489)	0.058	0.596
2-Hydroxyisovaleric acid	0.56 (0.503-0.596)	0.485 (0.411-0.635)	0.6 (0.445-0.687)	0.531 (0.481-0.572)	0.661	0.638 (0.534-0.734)	0.555 (0.425-0.673)	0.472 (0.443-0.519)	0.534 (0.474-0.572)	0.081	0.077
3-hydroxybutyric acid /3-hydroxyisobutyric acid	13.207 (10.9-14.59)	14.365 (10.708-21.853)	15.972 (9.802-19.917)	14.222 (11.414-18.044)	0.391	20.158 (13.675-32.971)	17.76 (14.127-24.17)	10.173 (9.019-14.099)	10.415 (8.886-13.833)	0.547	0.829
3-Hydroxyisovaleric acid	0.465 (0.428-0.511)	0.47 (0.45-0.571)	0.46 (0.4-0.583)	0.493 (0.424-0.531)	0.985	0.552 (0.453-0.669)	0.477 (0.463-0.515)	0.434 (0.4-0.504)	0.438 (0.417-0.509)	0.08	0.255
3-Phosphoglyceric acid	0.034 (0.029-0.038)	0.029 (0.022-0.034)	0.048 (0.039-0.056)	0.048 (0.041-0.054)	0.064	0.035 (0.03-0.043)	0.035 (0.032-0.04)	0.034 (0.032-0.041)x	0.031 (0.028-0.038)	0.202	0.045
4-Hydroxybenzoic acid	0.104 (0.1-0.106)	0.104 (0.102-0.105)	0.103 (0.099-0.105)	0.105 (0.102-0.106)	0.419	0.103 (0.102-0.104)	0.104 (0.103-0.106)	0.102 (0.1-0.104)	0.102 (0.097-0.106)	0.774	0.857
4-hydroxyPhenyllactic acid	18.936 (14.946-36.319)	17.479 (12.284-25.008)	7.505 (2.901-9.057)**	11.815 (9.026-13.967)$#	0.014	19.824 (3.534-28.675)	16.385 (13.276-17.492)	6.932 (4.908-7.705)	8.371 (7.355-10.211)$**	0.042	0.105
4-Hydroxyproline	3.488 (2.458-3.729)	2.941 (2.475-4.022)	3.594 (2.819-4.926)	3.422 (2.921-4.478)	0.5	2.287 (2.167-2.533)+	2.435 (1.982-2.967)	2.982 (2.396-3.393)	2.485 (2.013-3.154)	0.769	0.027
Aconitic acid	0.051 (0.041-0.084)	0.054 (0.033-0.117)	0.154 (0.073-0.317)	0.074 (0.06-0.093)	0.373	0.052 (0.024-0.07)	0.034 (0.027-0.053)	0.087 (0.073-0.406)#	0.055 (0.042-0.074)$#	0.018	0.069
Alanine	16.385 (15.152-16.919)	16.884 (15.81-18.24)	17.821 (17.167-19.033)**	16.571 (16.013-16.973)$	0.028	15.962 (14.955-17.133)	15.406 (14.533-16.171)	17.458 (16.541-20.436)#	17.19 (16.717-17.739)**	0.004	0.006
alpha-ketoglutaric acid	2.25 (1.712-3.723)	1.951 (1.398-3.112)	2.27 (1.968-2.606)	2.405 (1.909-2.728)	0.718	2.359 (2.009-3.611)	2.512 (1.99-3.202)	1.825 (1.548-2.259)	1.706 (1.49-1.966)	0.492	0.745
alpha-tocopherol	0.043 (0.039-0.049)	0.039 (0.032-0.041)&	0.066 (0.053-0.068)*	0.056 (0.05-0.069)**	0.001	0.029 (0.022-0.05)	0.034 (0.026-0.054)	0.074 (0.07-0.077)++	0.06 (0.052-0.07)	0.075	0.009
Aspartic	0.299 (0.223-0.418)	0.345 (0.283-0.384)	0.358 (0.281-0.407)	0.336 (0.325-0.347)	0.409	0.32 (0.278-0.43)	0.324 (0.284-0.389)	0.324 (0.285-0.363)	0.304 (0.299-0.347)	0.87	0.813
Cholesterol	11.455 (10.478-13.187)	9.564 (7.463-11.011)	12.476 (10.583-14.344)	11.793 (9.404-13.462)	0.101	10.482 (8.536-11.839)	9.569 (8.64-11.989)	13.437 (12.207-13.867)	12.14 (10.304-13.65)	0.188	0.373
Citric acid	32.587 (29.07-45.991)	32.052 (27.445-38.804)	36.627 (34-37.803)	32.683 (26.449-39.745)	0.52	37.319 (33.509-54.107)	35.811 (29.775-42.316)	25.074 (23.913-26.639)**++	25.822 (22.242-28.263)*	0.003	0.016
d-Fructose	0.054 (0.043-0.075)	0.053 (0.036-0.065)	0.083 (0.059-0.109)**	0.108 (0.095-0.132)**	0.007	0.05 (0.035-0.074)	0.052 (0.047-0.077)	0.071 (0.053-0.096)	0.099 (0.09-0.115)&**	0.015	0.47
DL-2-Hydroxyglutaric acid	0.154 (0.127-0.224)	0.151 (0.134-0.187)	0.147 (0.129-0.165)	0.135 (0.127-0.164)	0.381	0.167 (0.15-0.199)	0.159 (0.145-0.169)	0.121 (0.108-0.136)*++	0.122 (0.116-0.139)*	0.003	0.02
d-Mannitol	0.068 (0.014-0.075)	0.051 (0.045-0.067)	0.015 (0.008-0.046)	0.011 (0.006-0.018)	0.059	0.037 (0.016-0.057)	0.036 (0.02-0.049)	0.01 (0.007-0.02)*	0.006 (0.005-0.014)&*	0.005	0.222
d-Mannonic acid	0.03 (0.024-0.038)	0.029 (0.026-0.035)	0.037 (0.035-0.045)	0.033 (0.028-0.036)	0.117	0.03 (0.025-0.035)	0.025 (0.023-0.029)	0.034 (0.027-0.039)	0.033 (0.03-0.037)	0.216	0.087

Dodecanoic acid	0.235 (0.219-0.272)	0.228 (0.222-0.269)	0.307 (0.245-0.455)	0.388 (0.295-0.461)	0.114	0.256 (0.226-0.28)	0.244 (0.213-0.296)	0.276 (0.253-0.306)	0.324 (0.295-0.369)&*	0.028	0.612
d-Sucrose	0.028 (0.013-0.059)	0.012 (0.007-0.025)	0.345 (0.152-0.477)**	0.201 (0.12-0.332)**	0.008	0.014 (0.006-0.036)	0.037 (0.015-0.349)	0.055 (0.051-0.064)**++	0.116 (0.053-0.181)	0.042	0.041
d-Threitol	0.028 (0.025-0.03)	0.027 (0.024-0.029)	0.029 (0.025-0.036)	0.031 (0.03-0.035)	0.113	0.023 (0.022-0.026)	0.025 (0.023-0.026)	0.027 (0.025-0.029)	0.028 (0.026-0.031)	0.414	0.507
Ethanolamine	0.496 (0.472-0.564)	0.555 (0.463-0.608)	0.525 (0.48-0.729)	0.513 (0.44-0.56)	0.456	0.55 (0.395-0.579)	0.587 (0.455-0.628)	0.543 (0.508-0.58)	0.547 (0.407-0.584)	0.455	0.625
Fructose-6-phosphate	0.081 (0.068-0.272)	0.101 (0.048-0.142)	0.061 (0.041-0.086)	0.054 (0.038-0.066)	0.611	0.095 (0.057-0.193)	0.092 (0.071-0.109)	0.061 (0.054-0.112)	0.054 (0.047-0.061)	0.061	0.555
Fumaric acid	1.04 (0.634-1.458)	0.775 (0.567-1.233)	0.846 (0.729-1.046)	0.816 (0.649-1.127)	0.72	0.995 (0.8-1.447)	0.902 (0.695-1.12)	0.622 (0.568-0.827)+	0.641 (0.572-0.759)	0.191	0.196
Galacturonic acid	0.013 (0.011-0.016)	0.011 (0.01-0.017)	0.018 (0.018-0.021)*	0.021 (0.015-0.025)*	0.032	0.012 (0.011-0.013)	0.012 (0.01-0.014)	0.019 (0.016-0.022)**	0.02 (0.017-0.025)**	0.017	0.282
Glucose	39.681 (36.993-41.692)	35.717 (33.906-37.901)	37.453 (32.53-37.92)	38.204 (36.749-39.582)	0.191	37.57 (34.76-42.263)	38.532 (35.612-41.266)	37.556 (34.842-40.092)	38.59 (36.543-40.286)	0.917	0.384
Glucose 6-phosphate	0.039 (0.036-0.082)	0.035 (0.024-0.06)	0.033 (0.023-0.038)#	0.025 (0.016-0.034)	0.023	0.046 (0.027-0.057)	0.038 (0.036-0.052)	0.03 (0.025-0.052)	0.027 (0.023-0.03)	0.057	0.09
Glutamic acid	6.613 (5.409-7.89)	6.696 (6.167-7.395)	7.523 (6.872-8.062)	7.051 (6.287-7.789)	0.833	7.32 (6.546-8.254)	7.156 (6.54-9.266)	6.691 (6.38-6.957)	6.355 (5.656-7.387)	0.529	0.577
Glutamine	0.534 (0.433-0.855)	0.587 (0.457-1.663)	1.405 (0.747-3.522)	0.683 (0.55-0.909)	0.061	0.428 (0.27-1.203)	0.424 (0.294-0.492)	0.9 (0.727-6.487)#	0.654 (0.427-0.726)$#	0.003	0.004
Glyceraldehyde-3-phosphate	0.005 (0.003-0.005)	0.005 (0.004-0.007)	0.004 (0.004-0.005)	0.004 (0.004-0.004)	0.086	0.006 (0.005-0.006)	0.005 (0.005-0.006)	0.004 (0.003-0.004)**	0.004 (0.004-0.004)**	0.007	0.083
Glyceric acid	1.265 (0.794-1.849)	1.371 (0.76-1.521)	0.955 (0.861-1.054)	0.795 (0.714-1.368)	0.348	1.303 (0.624-2.434)	0.915 (0.649-1.547)	0.902 (0.746-1.46)	1.237 (0.939-1.47)	0.473	0.577
Glycerol	0.671 (0.536-0.965)	0.861 (0.544-1.062)	1.106 (0.857-1.477)*	1.225 (1.132-1.562)*	0.001	0.834 (0.706-0.887)	0.954 (0.768-1.01)	1.118 (0.88-1.235)*	1.227 (1.088-1.414)**	0.008	0.004
Glycerol-1-phosphate	0.201 (0.165-0.275)	0.188 (0.154-0.221)	0.211 (0.165-0.266)	0.183 (0.147-0.199)	0.284	0.213 (0.162-0.275)	0.167 (0.143-0.258)	0.21 (0.183-0.278)	0.165 (0.144-0.214)	0.142	0.951
Glycine	4.903 (4.716-5.021)	4.961 (4.726-5.151)	4.657 (4.276-4.934)	4.366 (4.133-4.547)	0.052	4.845 (4.725-5.139)	4.692 (4.611-4.801)	4.733 (4.685-5.176)	4.58 (4.297-4.933)	0.724	0.988
Glycolic acid	0.097 (0.094-0.114)	0.109 (0.097-0.133)	0.135 (0.121-0.148)**	0.116 (0.113-0.136)&	0.027	0.12 (0.097-0.146)	0.117 (0.109-0.125)	0.12 (0.115-0.134)	0.133 (0.127-0.137)	0.634	0.034
Heptanoic	0.434 (0.406-0.49)	0.411 (0.395-0.464)	0.427 (0.382-0.465)	0.441 (0.402-0.539)	0.405	0.483 (0.4-0.5)	0.452 (0.432-0.468)	0.408 (0.376-0.447)	0.45 (0.434-0.492)	0.392	0.79
Hippuric acid	0.342 (0.234-0.544)	0.631 (0.531-0.726)$	0.145 (0.121-0.287)*	0.106 (0.053-0.21)**	0.001	0.451 (0.312-0.559)	0.482 (0.407-0.585)	0.107 (0.076-0.154)**x	0.077 (0.056-0.146)**	0.004	0.006
Indole-3-propanoic acid	1.343 (0.97-1.683)	1.528 (0.955-1.645)	1.088 (0.812-3.042)	0.911 (0.562-1.414)	0.701	1.101 (0.572-1.539)	0.953 (0.774-1.192)	0.861 (0.545-1.047)	0.6 (0.374-0.934)	0.176	0.602
Isoleucine	4.477 (4.07-5.107)	4.47 (4.009-4.568)	4.355 (3.862-4.866)	4.198 (3.867-4.652)	0.563	4.364 (4.09-4.575)	3.938 (3.741-4.669)	4.236 (3.926-4.363)	4.252 (3.751-4.359)	0.483	0.378
Lactic acid	75.808 (67.974-85.853)	76.14 (72.71-89.479)	85.463 (74.452-95.224)	75.406 (71.158-79.759)	0.226	79.845 (73.389-102.516)	77.012 (75.487-87.892)	75.961 (72.358-88.192)	77.382 (69.064-79.441)	0.4	0.255
Leucine	7.4 (6.702-8.209)	7.387 (6.609-7.586)	7.18 (6.686-7.957)	7.124 (6.65-7.735)	0.919	6.999 (6.814-7.367)	6.524 (6.175-7.378)	7.022 (6.811-7.323)	7.044 (6.274-7.278)	0.664	0.901
Linoleic acid	1.1 (0.504-1.703)	1.245 (0.686-1.628)	1.473 (0.975-2.1)	1.872 (1.269-2.806)	0.613	0.992 (0.767-2.113)	1.461 (1.248-1.808)	1.001 (0.927-1.129)	1.34 (1.091-1.709)	0.1	0.56
Malic acid	0.587 (0.371-1.025)	0.501 (0.341-0.698)	0.567 (0.518-0.683)	0.602 (0.447-0.744)	0.217	0.539 (0.469-0.761)	0.503 (0.42-0.615)	0.406 (0.377-0.449)++	0.397 (0.348-0.469)	0.274	0.084
Maltose	0.021 (0.016-0.041)	0.012 (0.008-0.014)$	0.008 (0.004-0.012)*	0.009 (0.005-0.011)	0.019	0.008 (0.007-0.012)+	0.009 (0.008-0.016)	0.009 (0.006-0.017)	0.008 (0.005-0.011)	0.232	0.009
Methionine	3.496 (3.236-3.67)	3.443 (3.259-3.609)	3.139 (3.102-3.435)	3.189 (3.112-3.381)	0.166	3.372 (3.194-3.42)	3.292 (3.183-3.411)	3.439 (3.314-3.531)	3.285 (3.236-3.482)	0.348	0.272
myo-Inositol	1.13 (0.923-1.209)	1.074 (0.784-1.174)	0.928 (0.897-1.066)	0.801 (0.733-1.016)	0.106	0.947 (0.816-1.124)	1.02 (0.908-1.092)	0.888 (0.773-1.052)	0.908 (0.804-1.077)	0.397	0.182

Oleic acid	*2.925 (1.2-4.668)*	*3.241 (1.188-4.486)*	*6.258 (4.026-10.184)*	*9.893 (5.664-13.354)*	*0.294*	*2.748 (2.071-4.622)*	*3.811 (2.815-6.342)*	*5.37 (5.074-5.81)*	*7.469 (5.014-10.02)*	*0.613*	*0.113*
Ornithine	*2.685 (1.693-4.077)*	*2.208 (1.932-3.595)*	*2.284 (2.17-4.112)*	*1.709 (1.562-2.286)*	*0.268*	*2.401 (1.835-2.526)*	*1.713 (1.459-2.04)*	*2.185 (2.092-3.13)*	*1.777 (1.377-2.055)*	*0.208*	*0.78*
Oxalic acid	*24.293 (21.582-26.714)*	*26.491 (25.029-27.684)*	*24.139 (23.125-29.799)*	*22.728 (20.231-23.998)*	*0.099*	*26.4 (21.505-36.416)*	*27.622 (24.27-36.927)*	*27.249 (25.882-29.611)*	*33.773 (29.3-37.54)*	*0.454*	*0.552*
Oxoproline	*145.749 (136.603-166.354)*	*143.399 (132.564-167.096)*	*155.083 (123.075-183.659)*	*157.99 (142.17-165.27)*	*0.168*	*116.228 (105.816-175.582)*	*139.734 (122.876-145.385)*	*143.175 (111.038-179.911)*	*142.736 (121.332-153.167)*	*0.47*	*0.223*
Palmitic acid	*98.779 (77.763-108.3)*	*96.293 (75.619-106.847)*	*90.963 (73.175-105.392)*	*103.06 (84.162-113.351)*	*0.997*	*97.334 (84.965-118.958)*	*105.446 (98.11-114.62)*	*87.764 (81.965-91.883)*	*102.34 (75.426-107.523)*	*0.075*	*0.149*
Phenylalanine	*3.809 (3.607-4.009)*	*3.716 (3.456-3.877)*	*3.6 (3.516-4.176)*	*3.764 (3.616-3.831)*	*0.98*	*3.627 (3.538-3.706)*	*3.534 (3.428-3.698)*	*3.998 (3.873-4.051)***	*3.737 (3.589-3.909)$*	*0.001*	*0.076*
Phosphoric acid	*60.912 (57.252-67.868)*	*53.837 (51.97-57.999)$*	*50.315 (45.915-54.037)***	*49.091 (47.481-54.275)#*	*0.003*	*61.59 (59.219-70.695)*	*62.746 (60.781-67.589)*	*52.724 (47.416-56.343)*	*52.207 (49.76-56.746)*	*0.201*	*0.066*
Pipecolic acid	*0.096 (0.075-0.106)*	*0.088 (0.068-0.096)*	*0.054 (0.048-0.064)*	*0.06 (0.053-0.067)*	*0.134*	*0.09 (0.077-0.1)*	*0.083 (0.076-0.09)*	*0.054 (0.047-0.067)**	*0.061 (0.054-0.073)**	*0.01*	*0.068*
Proline	*26.014 (20.318-36.895)*	*26.863 (22.184-30.998)*	*45.239 (38.993-53.43)**	*39.705 (35.115-40.687)&***	*0.044*	*20.031 (13.587-27.608)*	*20.039 (17.055-23.79)*	*36.726 (35.376-38.474)**x*	*32.373 (29.917-35.205)$***	*0.0001*	*0.027*
Pyruvic acid	*24.766 (16.785-27.088)*	*19.627 (13.73-24.99)*	*27.456 (23.314-34.62)*	*29.068 (21.758-29.684)*	*0.082*	*19.781 (12.348-25.187)*	*21.118 (17.341-23.747)*	*26.343 (22.112-29.174)*	*19.131 (14.938-26.318)*	*0.838*	*0.269*
Serine	*9.355 (8.106-10.327)*	*9.605 (8.806-10.24)*	*13.718 (12.835-14.986)*	*13.062 (10.964-13.225)*	*0.509*	*9.04 (8.871-9.834)*	*9.033 (8.48-9.496)*	*13.246 (12.441-14.116)*	*12.433 (10.829-13.6)*	*0.115*	*0.854*
Succinic acid	*0.219 (0.185-0.321)*	*0.223 (0.194-0.243)*	*0.282 (0.268-0.292)#*	*0.224 (0.194-0.289)&*	*0.042*	*0.26 (0.218-0.29)*	*0.204 (0.179-0.257)*	*0.212 (0.184-0.243)++*	*0.24 (0.175-0.291)*	*0.454*	*0.055*
Taurine	*1.196 (1.092-2.008)*	*1.688 (1.173-2.916)*	*1.794 (1.373-2.587)*	*1.23 (1.041-1.535)*	*0.596*	*2.005 (0.923-4.912)*	*1.373 (1-2.538)*	*1.966 (1.695-2.372)*	*2.023 (1.636-2.278)*	*0.502*	*0.851*
Threonic acid	*0.443 (0.391-0.569)*	*0.466 (0.412-0.621)*	*0.701 (0.629-0.803)**	*0.676 (0.622-0.737)***	*0.012*	*0.516 (0.39-0.791)*	*0.463 (0.422-0.494)*	*0.683 (0.636-0.775)*	*0.672 (0.632-0.74)*	*0.061*	*0.07*
Threonine	*6.486 (6.054-7.463)*	*6.719 (6.138-7.427)*	*7.405 (7.065-8.515)*	*7.307 (6.639-7.902)*	*0.24*	*6.716 (6.066-7.299)*	*6.529 (6.201-7.063)*	*7.87 (7.506-8.348)***	*7.826 (7.189-8.6)**	*0.031*	*0.012*
Uric acid	*0.385 (0.353-0.423)*	*0.385 (0.345-0.423)*	*0.461 (0.411-0.505)*	*0.378 (0.355-0.464)*	*0.053*	*0.373 (0.305-0.437)*	*0.301 (0.273-0.533)*	*0.46 (0.432-0.522)*	*0.506 (0.476-0.665)*	*0.67*	*0.081*
Valine	*9.059 (8.376-10.22)*	*9.021 (8.137-9.48)*	*8.642 (7.902-9.741)*	*8.199 (7.647-8.887)*	*0.104*	*8.785 (8.143-9.291)*	*8.002 (7.697-9.028)*	*8.412 (8.152-8.577)*	*8.391 (7.495-8.672)*	*0.272*	*0.207*
Xylonic acid	*0.007 (0.006-0.008)*	*0.007 (0.006-0.012)*	*0.008 (0.007-0.013)*	*0.007 (0.005-0.008)*	*0.473*	*0.007 (0.007-0.012)*	*0.006 (0.005-0.008)*	*0.008 (0.007-0.01)*	*0.007 (0.006-0.008)*	*0.123*	*0.44*

*Data shown as Median (Interquartile Range) (IQR) in Arbitrary units. P-value by Kruskal-Wallis' test; * indicates significant differences between STD vs CAF; $ indicates significant differences between VH vs GSPE; + indicates significant differences between L6-VH vs L18-VH groups by Mann-Whitney p < 0.5. # Indicates tendency between STD vs CAF, & indicates tendency between VH vs GSPE conditions; x Indicates tendency between L6-VH vs L18-VH by Mann-Whitney p < 0.1*

Manuscript 6

Objective: to elucidate the effect of PACs on cardiovascular risk factors in CAF-induced obese Fischer 344 rats subjected to a sudden change of light/dark cycle.

Proanthocyanidin administration to obese rats after a sudden photoperiod change modifies their altered circadian locomotor activity and exhibit antihypertensive effect

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Manuscript in preparation for Hypertension

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Keywords: biological rhythms, clock genes, cosinor, chrononutrition, zeitgebers.

ABSTRACT

Light/dark cycles trigger changes in behavior and other physiological parameters, and its disruption can evoke metabolic diseases. Proanthocyanidins have demonstrated beneficial effects on hypertension and other cardiometabolic risk factors related to metabolic syndrome. In addition, modulating effects on the circadian rhythm has been reported for these polyphenols. Therefore, the aim of this study was to evaluate the effects of a grape seed proanthocyanidin-rich extract (GSPE) on circadian locomotor behavior, blood pressure and body temperature using cafeteria (CAF)-fed hypertensive rats transferred abruptly from a standard (12 h light/day, L12) to a long (18 h light/day, L18) or short photoperiod (6 h light/day, L6). Circadian rhythms were recorded by radiotelemetry in 24 CAF-fed Fischer 344 rats for 6 weeks under L12 conditions. After this time, animals were transferred to L18 or L6 and administered vehicle (VH) or GSPE (25mg/Kg) for 1 week. Results showed how photoperiod disruption produced alterations in locomotor activity and the circadian rhythm of blood pressure. PACs administration in L6 and L18 conditions improved the altered circadian locomotor activity and exhibit an antihypertensive effect, attenuating also the non-dipper pattern, i.e., the lack of blood pressure fall in the resting period, under L18 conditions. Although further research is needed, these results suggest that PACs could avoid the circadian locomotor disturbances and mitigate the changes in blood pressure caused by photoperiod disruption.

INTRODUCTION

The circadian rhythm regulates behavior and physiological processes to adapt the organism to the necessities required at each moment of the day in order to optimize the energy metabolism [1,2]. Processes such as feeding patterns, sleep-wake cycle, body temperature, or blood pressure (BP) have shown a rhythmic pattern marked by the light-dark cycle [3,4]. Locomotor behavior is also regulated by the circadian clock, and alterations in the rhythm of locomotor activity indicate modifications of circadian clock function. In addition, alteration of circadian system can impact on behavior and physiological processes and induce metabolic and physiological disorders that lead to the development of medical conditions characteristic of metabolic syndrome [5,6]. The main controller of this circadian rhythmicity is the master clock located in hypothalamic suprachiasmatic nucleus (SCN), which also synchronizes the peripheral clocks of the different tissues. In addition, feeding patterns and food composition can affect the circadian rhythm of these tissue oscillators, causing a misalignment or desynchronization between central and peripheral clocks. This misalignment is related to the development of metabolic disorders, including obesity and its derived diseases [7,8].

Rats fed cafeteria (CAF) diet is considered a robust model of human metabolic syndrome. This highly palatable, energy dense and unhealthy human food induces obesity, cardiovascular disorders, and hypertension (HTN) [9], and also causes, as other hypercaloric diets, circadian disturbances [8,10,11]. In this context, it has been reported that circadian rhythm disturbance can alter normal cardiovascular function and in BP, increasing the probability of cardiovascular accidents, atherosclerosis and other related cardiovascular diseases [12].

HTN is defined as a systolic blood pressure (SBP) ≥140 mmHg and/or a diastolic blood pressure (DBP) ≥90 mmHg [13] and is the leading preventable risk factor for cardiovascular disease (CVD) and the leading cause of premature death

worldwide [14]. As discussed above, BP presents variability throughout the day with a marked difference between the activity phase and the resting phase, which is related to better cardiovascular health when this difference is between 10 and 20% (pattern dipper). Alteration of the circadian rhythm of BP, reducing the difference between these two phases by less than 10% (non-dipper pattern), is associated with an increased risk of CVD, stroke, and death [15–17]. Thus, some causes of HTN were associated with circadian rhythm disturbance [18,19]. HTN and cardiovascular disorders require lifelong treatment, and most drugs have undesirable side effects, so the search for natural compounds that help to attenuate these disorders has increased. In this regard, evidence of the reduction of HTN by some fruits and vegetables, a rich source of phenolic compounds, has captured the interest of researchers. [20].

Grape seeds are one of the richest sources of proanthocyanidins (PAC) and their beneficial effects on different cardiometabolic disorders have been extensively investigated [21–23]. There are many beneficial effects associated with the intake of grape seed PACs which are related to cardiovascular health, such as anti-inflammatory properties [24], improvement of lipid and glucose profile [25,26] and, attenuation of HTN [9,11,27]. In addition, our group has also reported a role as modulators of circadian rhythms in obese and healthy rats [28–31]. Moreover, recent studies have shown that these phenolic compounds administered to rats after the alteration of their light-dark cycles, attenuated the disruptions caused by photoperiod changes on key circadian and metabolic control hormones and improved the metabolism of these animals in a diet- and photoperiod-dependent [Manuscript 4] [Manuscript 5].

Therefore, the aim of the present study was to evaluate the effect of a sudden change of the light-dark cycle on the rhythmicity of locomotor activity and other physiological processes as blood pressure and body temperature in CAF diet-induced obese rats and investigate the effects of the administration of PACs on the rhythmicity of these processes. For this purpose, photosensitive rats Fisher 344 (F344) were fed cafeteria diet and transferred abruptly from standard (STD)

(12 h light/day, L12) to long (18 h light/day, L18) or short (6 h light/day, L6) photoperiods. BP, body temperature, and locomotor activity were recorded using a telemetry system.

MATERIALS AND METHODS

Grape seed proanthocyanidin-rich extract

The grape seed proanthocyanidin-rich extract (GSPE) used in this study was obtained from white grape seed and is mainly composed of catechin, epicatechin, gallic acid, epicatechin gallate and dimers, trimers and tetramers of proanthocyanidins [32]. This extract was provided by Les Dérives Résiniques et Terpéniques (Dax, France).

Animal procedure

The animals used were 13-week-old male F344 rats from Charles River Laboratories (Barcelona, Spain) housed under STD laboratory conditions at 22 °C and 12 hours light/dark cycle with ad libitum access to food and drinking water. After two weeks of acclimatization period, rats were fed CAF diet for 6 weeks. After this time, animals were transferred from L12 to a L18 or L6 photoperiods and administered vehicle (VH), which was condensed milk 1/5 diluted, or GSPE (25 mg/kg) dissolved in VH for 1 week more. Thus, the animals were grouped into 4 different groups (n=6). CAF diet was prepared every day and contained bacon, cookie with paté, cookie with cheese, carrots, ensaïmada (pastry), STD chow and sweetened milk (22% sucrose w/v), and its caloric distribution was 56.43% carbohydrate, 45.72% lipid and 9.5% protein [9]. The onset of light was at 8:00 a.m. and defined as zeitgeber time 0 (ZT0). Both VH and GSPE treatments were administered to the rats at ZT0. Locomotor activity, SBP, DBP and body temperature were monitored for 24 hours, twice a week under L12 conditions from 2nd week and 3 times during the week of photoperiod change, by radiotelemetry using a Data Sciences International (DSI; St. Paul, MN) system. It consisted in the following components: MX2: 2.0, RPC-1 receptor for cages; HD-S10 sensor (catheter measurements: 8 cm x 6

mm), APR-2 ambient pressure detector, data acquisition and analysis system Powerlab 16/35 and LabChart Pro 8. Data were obtained with the LabChart Pro 8 Software and were recorded during all day in two different days during the week for L12 conditions, and 3 time during the seventh week. Dipper and non-dipper patterns were calculated as the percentage of difference between light and night periods as follows:

$$\% = \frac{SBP\ Dark\ phase\ (12h) - SBP\ Light\ phase\ (12h)}{SBP\ Dark\ phase\ (12h)}$$

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

Statistical Analysis

Data were represented as means ± standard error of mean (SEM) of all data by weeks for each group. In addition, the mean of the week was also made by period of light (ZT0 to ZT12) and period of darkness (ZT12 to ZT24). For all 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 two-way ANOVA with factorial designs photoperiod (P) and treatment (T). One-way ANOVA followed by LSD as a post-hoc test or Student's t-test, as indicated in the respective figure legend were applied in order to explore the origin of outcomes of two-way ANOVA analyses. Theses statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

RESULTS

Grape seed proanthocyanidins modify the locomotor activity disrupted by cafeteria diet and a sudden photoperiod change

Locomotor activities from whole-day and ZT0 to ZT12 and ZT12 to ZT24 periods were analyzed (Figure 2A-F). Locomotor activity was affected by CAF diet since a progressive decrease in the activity was observed in the animals fed this diet under L12 conditions (Figures 2A and 2D). This decrease was due to a decrement in the locomotor activity of L12 animals during the ZT12 to ZT24 phase (Figures 2C and 2F), while no changes in the activity were observed from ZT0 to ZT12, period of resting phase in rats under L12 photoperiod (Figures 2B and 2E). Interestingly, after the alteration of photoperiod, a change in the activity pattern of the animals was observed, since the rats that received the VH, both in L18 and L6, showed an increase in their activity in the ZT0 to ZT12 photoperiod one week after the light/dark shift (Figure 2B and 2E). The administration of GSPE resulted in a decrement of locomotor activity compared to the CAF-fed animals administered VH. This effect was observed in both photoperiods when the activity was analyzed along the day (Figure 2A and 2C), and at the period from ZT0 to ZT12 (Figure 2B and 2D). However, when were analyzed the activity data from ZT12 to ZT18, GSPE administration caused a decrement of the activity in the animals switched to L6 photoperiod (Figure 2F), but no differences were found in the locomotor activity between GSPE and VH administered animals in L18 conditions (Figure 2C). Figure 3A-D include representative actograms of the rats during the last 2 weeks before the sudden photoperiod change, animals under L12 conditions, and the seventh week of experiment in which the rats were switched to L18 or L6 conditions and administered vehicle (VH) or grape seed proanthocyanidins extract (GSPE).Although CAF diet decreased locomotor activity, did not alter circadian rhythmicity since activity rhythms were preserved during the six weeks in which the animals were under L12 conditions. However, this rhythm was altered with the photoperiod change.

Blood pressure lowering effects of grape seed proanthocyanidins in cafeteria fed rats after a sudden photoperiod change

Under L12 conditions CAF-fed rats showed a progressive increase in SBP values from week 2 to week 6 (Figure 4). This effect was observed in both whole day average values (Figure 4A and 4D) in both light phase (Figure 4B and 4E) (ZT0 to ZT12) and dark phase (Figure 4C and 4F) (ZT12 to ZT24) values. After switching to a new photoperiod, rats receiving VH continued the progressive rise in both L6 and L18 conditions. GSPE treatment resulted in a progressive decrease in SBP values on the whole day mean, with the second day being significant and trend on the last day of monitoring. In the case of rats under L6 conditions, they showed a downward trend on the second day of monitoring. Interestingly, SBP values during the night phase under L18 conditions showed a significant decrease on the last two days of monitoring with GSPE treatment, while the VH group continued the progressive rise. In the case of the rats under L6 conditions, they showed a downward trend with treatment on the second monitoring day. However, during the dark phase, no differences were found between treated and VH groups. Regarding hourly monitoring, blood pressure showed the marked oscillation dependent on the light-dark cycle during the weeks under L12 conditions (Figure 5A and 5B). A significant increase in SBP values was also observed between weeks 2 and 6 of experimentation and CAF feeding. The change of photoperiod involved the alteration of this oscillation, especially under L6 conditions where no differences between light and dark phases were observed (Figures 5E and 5D). Interestingly, GSPE treatment resulted in the maintenance of this oscillation during the days of photodiode switching under L18 conditions (Figures 5C and 5D). These results are reflected in the dipper/non-dipper pattern. Thus, all groups showed a BP difference between day and night of less than 10%, however, the group treated with GSPE under L18 conditions is significantly higher than the rest of the groups (Figure 6A - 6D). DBP values increased with the CAF diet progressively under L12 conditions, while after the photoperiod change no differences were found between treated

and untreated groups (data no show). The circadian oscillation of this parameter is also affected, as no differences are seen between day and night (data no show).

Grape seed proanthocyanidins did not modify the changes in body temperature caused by a sudden photoperiod change

In addition, the body temperature of the rats was modulated by the change in photoperiod. Thus, when the rats were transported to the L18 photoperiod, the temperature rise was shifted by a few hours to adapt to the new light-dark cycle (Figure 7A - 7C). Nevertheless, rats transported to L6 conditions did not show a shift in body temperature to adapt to the new light-dark cycle (Figure 7D and 7F). GSPE treatment had no effect on body temperature in any of the photoperiods.

DISCUSSION

Several behaviors, physiological and metabolic processes are regulated by circadian rhythms and the loss of their rhythmicity is related to metabolic disorders [33]; one of these processes is BP, that shows a notable oscillation marked by light-dark cycle, increasing during the activity phase and decreasing during the resting phase. PACs have shown beneficial effects on the different comorbidities of MetS. Recently, the modulatory effect of PACs on biological rhythms has been reported [36,46]. In addition, previous studies demonstrated the mitigating effect of PACs on the disruption of light-dark cycles, improving the metabolic and physiological profile of rats [Manuscript 4] and [Manuscript 5]. Therefore, the aim of this study was to evaluate the potential beneficial effects of GSPE on locomotor activity, body temperature, and BP under conditions of circadian disruption caused by an obesogenic diet and changes in the light-dark cycle.

Locomotor activity was analyzed, and it was observed that CAF diet promoted sedentariness, as locomotor activity was reduced throughout the experiment. Decreased activity has been reported in obese rats fed CAF under L12

conditions and in rat models of jet lag and shift work [10,34], but the mechanisms leading to this reduction are unclear. Moreover, HTN has been associated with reduced locomotor activity [35–37]. Recently, in a previous study, we observed how CAF diet decreases vertical activity [Manuscript 4]. This reduction in activity could be explained by the drop in activity in the dark phase, between ZT12 and ZT24, where all groups show lower locomotor activity. The sudden change in photoperiod, activity increased in CAF-fed rats under L6 and L18 conditions between ZT0 and ZT12. In this period the rats under L18 conditions are in the light phase, so they would be in their resting phase and should not show an increase in activity. The change of photoperiod could be causing an alteration of the rhythmic patterns of locomotor activity. In the case of L6, the rats have 6 hours of darkness, this would explain the increased activity during this period. Interestingly, GSPE kept locomotor activity low during the first 12 hours of the day, preventing the loss of locomotor activity rhythmicity. Thus, GSPE may be mitigating the impact on locomotor activity in CAF diet-induced obese rats by disruption of the light-dark cycle. However, these results differ from those obtained in the previous study [Manuscript 4], in which GSPE tended to increase the activity of obese rats compared to VH rats in both photoperiods, but only in the dark phase under the L6 photoperiod and L18 conditions. This discrepancy in the results could be due to the sensitivity of the methodology and the better conditions of the procedure.

BP showed a oscillation marked by light-dark cycle, with an increase during the activity phase and a decrease during the resting phase. The alteration of this BP rhythmicity may be due to several factors, including HTN. In this regard, obesity and HTN, comorbidities characteristic of the MetS, are two of the most important risk factors for CVD events [13,14,38]. Thus, BP control is fundamental to prevent CVD [39]. It has been shown that rats fed with CAF diet are a solid model to study MetS and its comorbidities such as HTN [10,40]. In this regard, our group reported that after 10 weeks of CAF diet, rats developed the most representative disorders of MetS, including HTN [9]. In the present

study, after 6 weeks of CAF diet feeding, rats showed HTN with SBP values of 155.69 ± 1.22 mmHg, which are similar to those reported by our group in other studies [27]. In this context, the difference between the activity phase and the resting phase was related to a better cardiovascular health when this difference is between 10 and 20% (pattern dipper). Alteration of the circadian rhythm of BP, reducing the difference between these two phases by less than 10% (non-dipper pattern), is associated with an increased risk of CVD, stroke, and death [15–17].

As has been previously reported, 6 weeks of CAF feeding led to the development of STD. It was observed that over the weeks, both in the all-day mean and in the mean separating the light and dark phases, there was a progressive rise in BP that stabilizes with the passage of weeks. After the photoperiod change, the groups again showed a tendency for BP to rise, however this rise cannot be attributed to the photoperiod change alone. The circadian rhythm of BP was altered since the non-dipper profile worsened after the photoperiod change. Regarding PACs treatment, a decrease in SBP was observed with GSPE treatment, especially in the light phase, and in L18 conditions compared to VH rats. These results were not reproduced in L6 conditions, where the effect of GSPE was not as noticeable, tending to reduce BP but not significantly. In previous studies our research group demonstrated that alteration of the light-dark cycle with exposure to more hours of light causes a worsening of the lipid and physiological profile in CAF-fed rats and an improvement of these parameters with GSPE treatment for one week under L18 conditions [manuscript 4 and manuscript 5]. Other studies have demonstrated reduction of HTN by GSPE under L12 conditions [9,27], in this study the abrupt alteration of circadian rhythm causes an effect of GSPE in a photoperiod-dependent manner. Moreover, this alteration led to a drastic reduction in the difference between dark-phase and light-phase BP. Interestingly, GSPE increased this difference under L18 conditions. The effect of GSPE on the circadian oscillation of BP has not been studied previously,

however, in our study integrating a state of metabolic disturbance and with disruption of the light-dark cycle, it has been observed that there is an enhancement of the photoperiod-dependent oscillation as this enhancement does not occur in L6 conditions. Our group also reported reduction in DBP with GSPE treatment in CAF diet-induced obese rats [9,27], however in the present study no DBP reduction was observed. The discrepancies in the results could be due to the strong alteration of the abrupt change of the light-dark cycle and the short treatment time.

After the photoperiod change, rats showed modifications of body temperature by the new light-dark cycle. Thus, their rhythmicity pattern of body temperature, especially in L18 conditions, were modified. The circadian rhythm of body temperature is generated by an endogenous component controlled by a circadian clock and an exogenous component mainly due to motor activity variations [41,42]. These two functions are intimately related metabolically and temporally [41,43]. In fact, while the rats were under L12 conditions, temperature and activity showed a similar circadian rhythmicity. In this regard, the synchrony of locomotor activity and body temperature following a change in the light-dark cycle has been reported [44]. Thus, our results showed an adaptation of both parameters to the new photoperiod, however, no effects were observed by GSPE treatment.

CONCLUSIONS

This study demonstrated that the effect of GSPE on obesity-induced HTN in a context of light-dark cycle disruption was in a photoperiod-dependent manner. The worsened obesogenic conditions entailed by the photoperiod shift to an L18 condition, reported in previous studies, was palliated by GSPE improving BP values, in addition to increasing the difference between the SPB of the night phase versus the day phase, bringing the treated rats closer to a dipper profile. In addition, locomotor activity is reduced with GSPE treatment during the first

12 hours, reducing the disorder in the activity pattern of VH rats, maintaining the circadian rhythm of locomotor activity.

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FIGURES

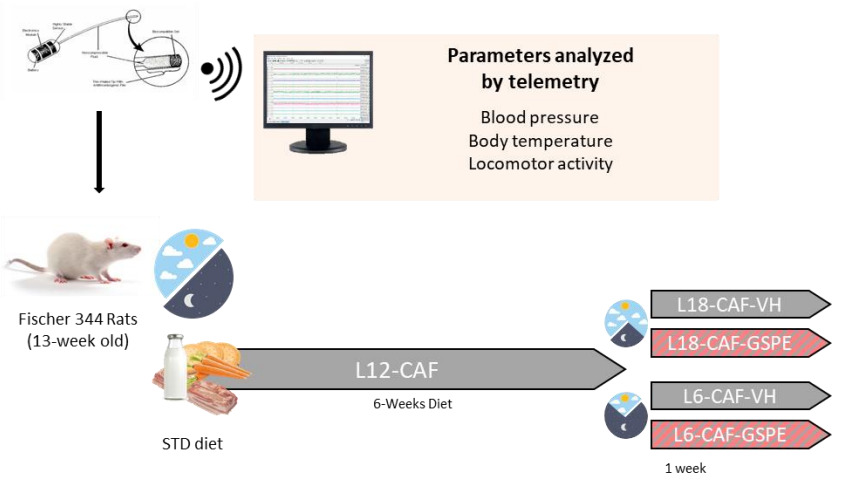

Figure 1. Experimental design to evaluate the effect of GSPE after an abrupt disturbance of photoperiod. *CAF, Cafeteria diet fed rats; VH, CAF-diet rats administered vehicle; CAF-GSPE, CAF-diet rats administered 25 mg/kg GSPE; L12, normal photoperiod 12 h light per day; L18, long photoperiod 18 h light per day; L6, short photoperiod 6 h light per day.*

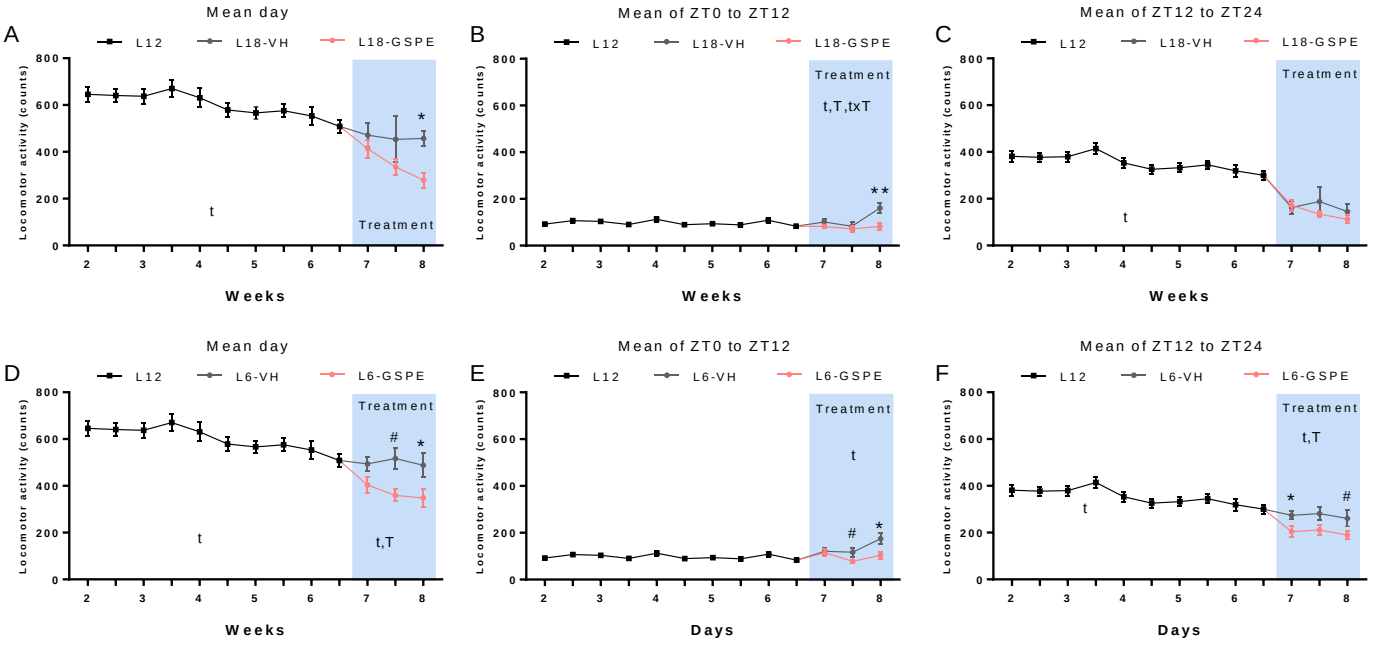

Figure 2. *A) Locomotor activity for rats switched to L18 conditions during long-term of experiment (weeks 2-7), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). Figures B and C represent the mean SBP in darkness and light periods respectively. D) Locomotor activity for rats switched to L16 conditions during long-term of experiment (weeks 2-7), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). Figures B and C represent the mean Locomotor activity in darkness and light periods respectively. p value was estimated using repeated measured-ANOVA followed by Student´s t test between VH vs GSPE. (*) and (**) were used to identify significant differences between groups in the same week for $p < 0.05$ or $p < 0.01$, respectively. .*

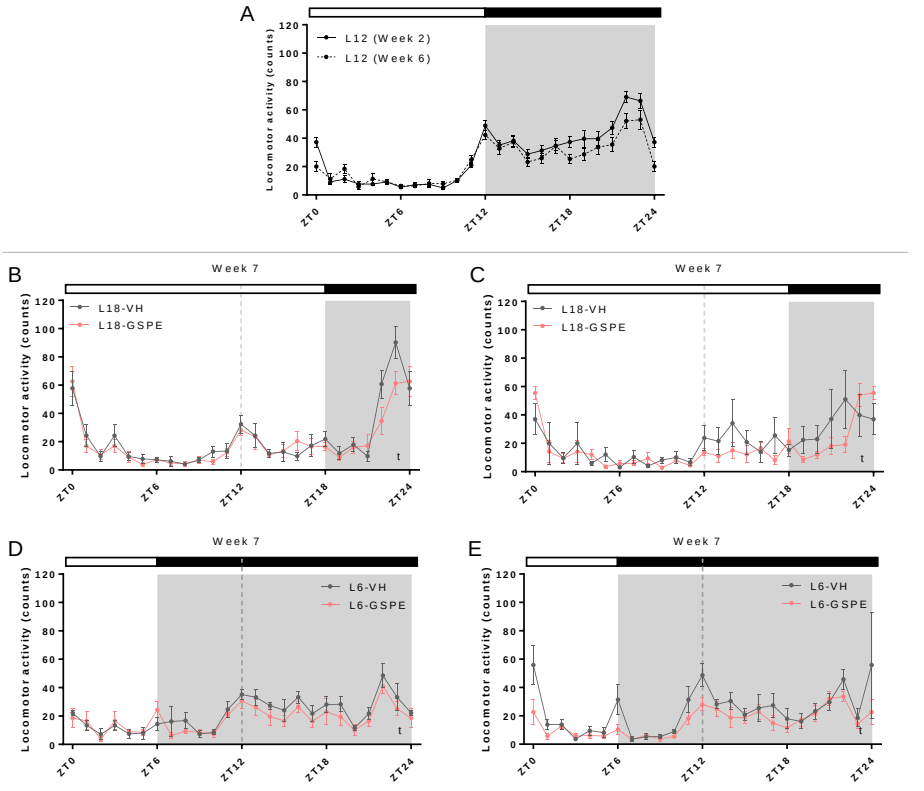

***Figure 3**. Locomotor activity recorded during 24 h. ZT0 and ZT12 is when the light period and the darkness period initiate respectively. A and B represent the L12 conditions. C and D represent the locomote activity during 24h in the last week of experiment (week 7) for L18 conditions with 25 mg/kg/day bw grape seed proanthocyanidins extract (GSPE) or vehicle (VH). E and F represent the Locomotor activity during 24h in the last week of experiment (week 7) for L6 conditions with 25 mg/kg/day bw grape seed proanthocyanidins extract (GSPE) or vehicle (VH).*

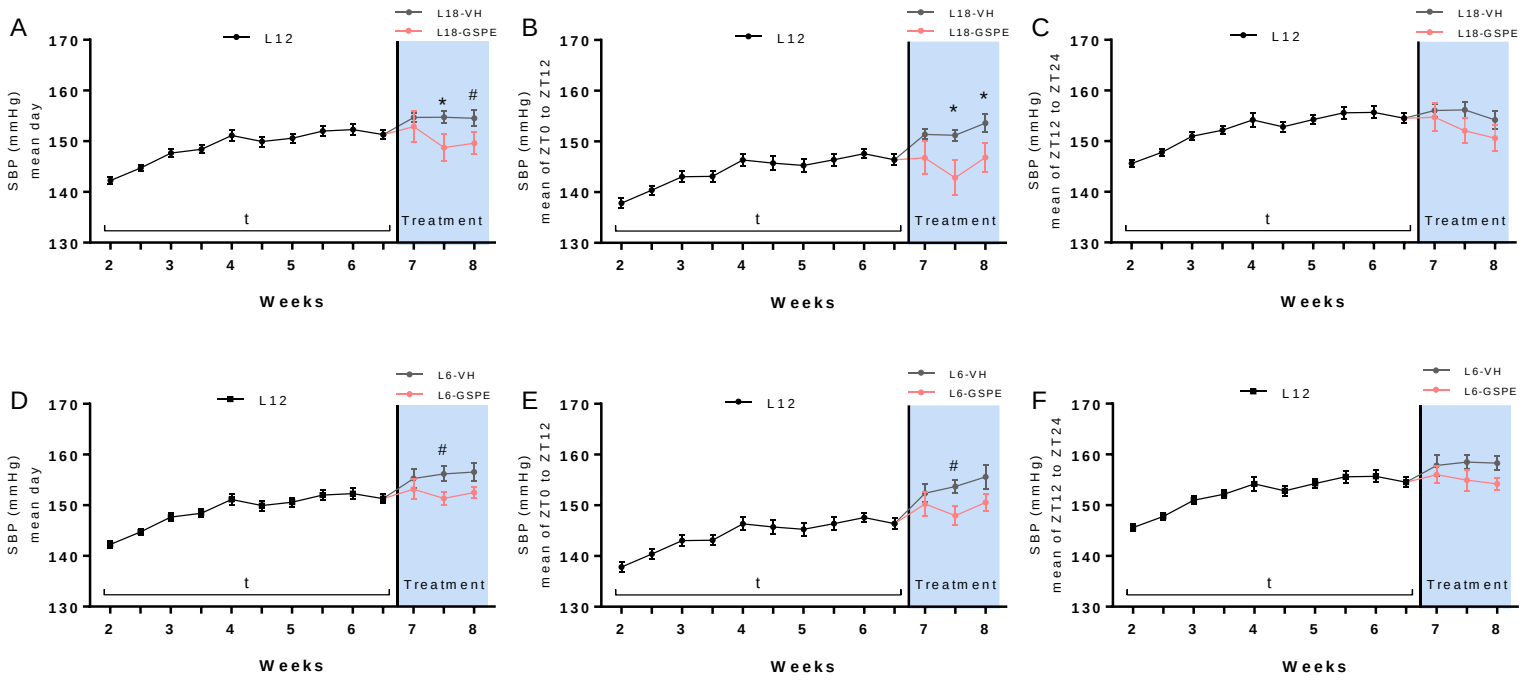

Figure 4. *A) Systolic Blood Pressure (SBP) for rats switched to L18 conditions during long-term of experiment (weeks 2-7), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). Figures B and C represent the mean SBP in darkness and light periods respectively. D) Systolic Blood Pressure (SBP) for rats switched to L16 conditions during long-term of experiment (weeks 2-7), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). Figures B and C represent the mean Locomotor activity in darkness and light periods respectively. p value was estimated using repeated measured-ANOVA followed by Student´s t test between VH vs GSPE. (*) and (**) were used to identify significant differences between groups in the same week for $p < 0.05$ or $p < 0.01$, respectively.*

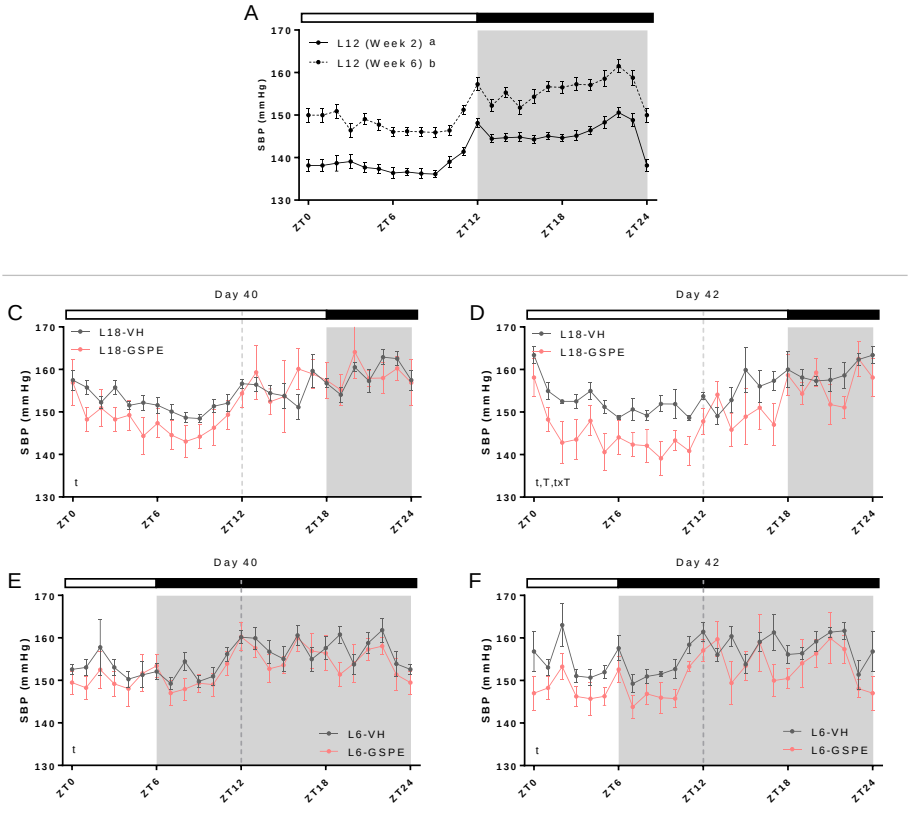

***Figure 5**. Systolic Blood Pressure (SBP) recorded during 24 h. ZT0 and ZT12 is when the light period and the darkness period initiate respectively. A and B represent the L12 conditions. C and D represent the locomote activity during 24h in the last week of experiment (week 7) for L18 conditions with 25 mg/kg/day bw grape seed proanthocyanidins extract (GSPE) or vehicle (VH). E and F represent the Locomotor activity during 24h in the last week of experiment (week 7) for L6 conditions with 25 mg/kg/day bw grape seed proanthocyanidins extract (GSPE) or vehicle (VH).*

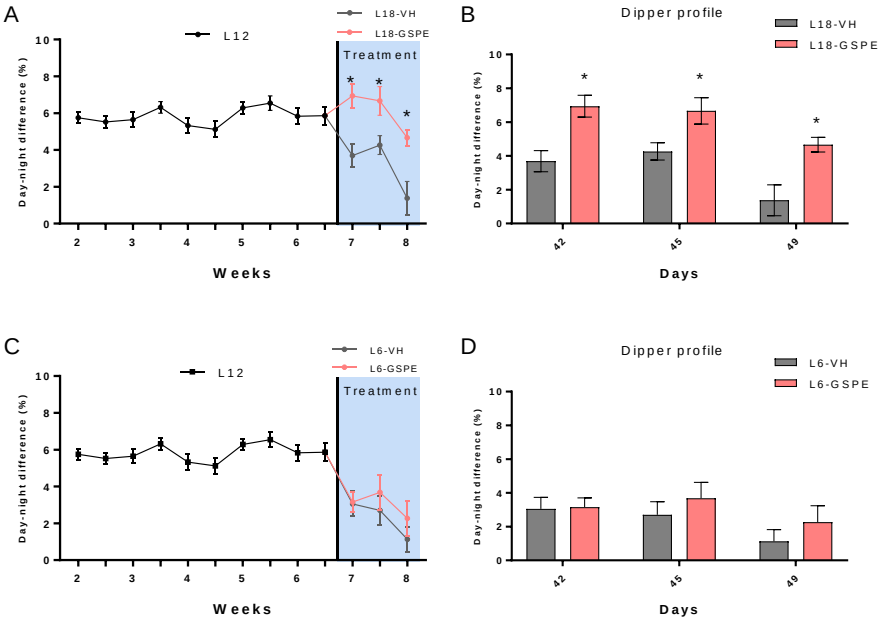

***Figure 6**. Difference between dark phase and light phase recorded during long-term of experiment (weeks 2-7, 49 days), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). A for L18 conditions and B for L6 conditions C represent the mean of dipper pattern..*

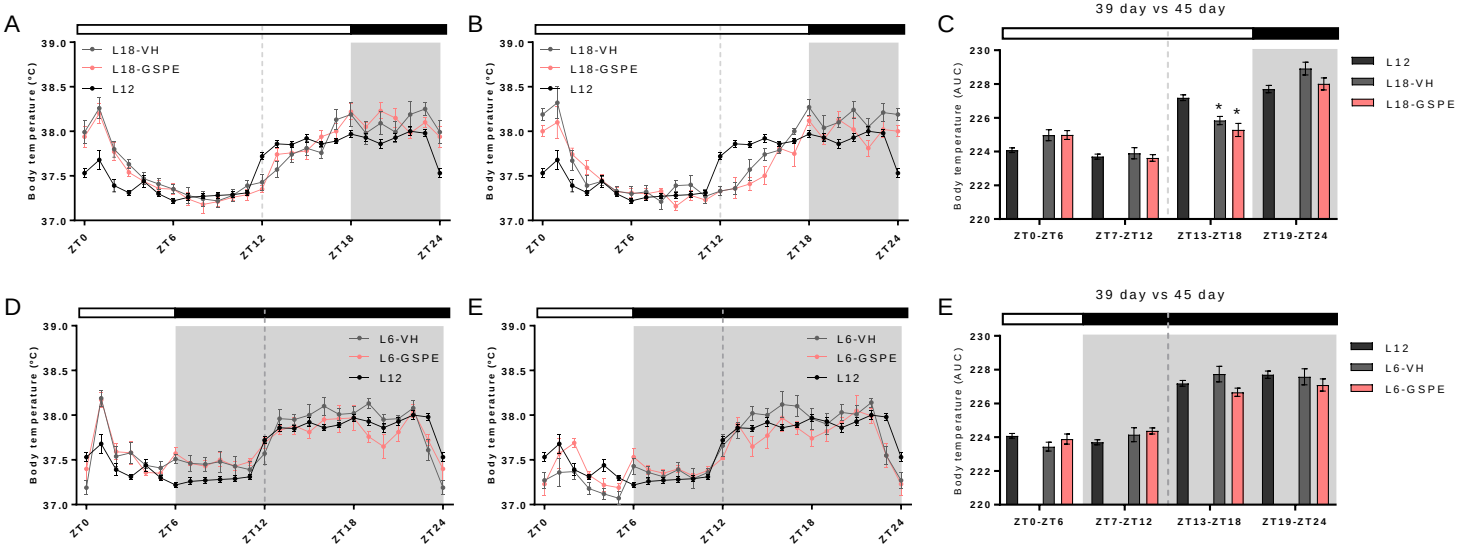

Figure 7. *A) Locomotor activity for rats switched to L18 conditions during long-term of experiment (weeks 2-7, 49 days), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). Figures B and C represent the mean SBP in darkness and light periods respectively. D) Locomotor activity for rats switched to L16 conditions during long-term of experiment (weeks 2-7, 49 days), seventh week with vehicle (VH) or grape seed proanthocyanidins extract (GSPE). Figures B and C represent the mean Locomotor activity in darkness and light periods respectively. Significant differences ($p < 0.05$) between treatments are represented by different letters in the legend. p value was estimated by two-way ANOVA and Tukey test was used as post hoc. (*) and (**) were used to identify significant differences between groups in the same week for $p < 0.05$ or $p < 0.01$, respectively. p was estimated by Student's T-test.*

GENERAL DISCUSSION

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Biological rhythms play an important role in the physiological and metabolic adaptation of the organism to the time of day (circadian rhythm) or year (seasonal rhythm) via external cues. Many physiological processes such as feeding patterns, sleep-wake cycle, temperature, or blood pressure (BP) show a rhythmic pattern marked by the light-dark cycle (1,2). Light, the most important external signal, is captured by the retina through the retinohypothalamic tract (RHT) and resets the central regulatory system. This central clock is placed in the hypothalamic suprachiasmatic nucleus (SCN) and is responsible for synchronizing the peripheral clocks of the organism (3,4). Another important signal capable of reprogramming or altering biological clocks is food composition. Thus, the misalignment of the circadian rhythm has been reported with the consumption of calorie-dense foods such as in mice fed high-fat diet. These animals showed alterations in the expression of circadian rhythm genes, nuclear receptors that regulate clock transcription factors, and clock-controlled genes involved in fuel utilization in the hypothalamus, liver, and adipose tissue. Furthermore, it has been observed that the timing of food intake has also an important impact on the circadian rhythm of the organism, causing changes in behavior, metabolism, and some physiological processes (5–7). In this sense, the importance of the timing pattern of food intake on liver gene transcription in wild-type mice a study has been reported (6,8). Changes in brown adipose tissue and skeletal muscle metabolism dependent on feeding time have also been showed in Wistar rats (9,10). Thus, not only diet composition modulates peripheral clocks, but also the timing of food intake is gaining importance. However, the interaction between metabolic diseases and circadian rhythms is bidirectional, and the disruption of hepatic circadian rhythms has been related to non-alcoholic fatty liver disease (NAFLD) (11).

The circadian rhythm disturbance due to modern lifestyles (shift work, artificial light, fast food, eating time, etc.) has been also related to the development of

metabolic disorders that in the long term could lead to type 2 diabetes, cardiovascular diseases, overweight and obesity (12–14). Indeed, "Circadian Syndrome" is emerging as a new terminology which would include metabolic disorders typical of the metabolic syndrome, in addition to other comorbidities such as depression, sleep disturbances, cognitive dysfunction and steatohepatitis.

Grape seed proanthocyanidins (PACs) are phenolic compounds which have shown beneficial effects on several metabolic processes. In previous studies our group has shown that chronic administration to rats fed CAF diet of a grape seed extract-enriched in PACs (GSPE) ameliorated metabolic disorders presents in these animals. Specifically, this extract significantly counteracted dyslipidemia (15–17), inflammation (18), insulin resistance (19), mitochondrial dysfunctionality (20), hypertension (HTN) (18, 20) and oxidative stress (21). Studies about the molecular mechanisms by which grape seed PACs exert these activities have allowed us to define some of the metabolic pathways by which their effects are mediated (15), which involved to nuclear receptors (22) and epigenetic modifications (17). In addition, different studies have demonstrated that PACs can modulate both central and peripheral biological rhythms in healthy animals under jet-lag conditions, but also in CAF fed obese rats (23–26). Other phenolic compounds have also demonstrated to regulate the circadian rhythms. In this context, the interaction of phenolic compounds with the clock system has been recently suggested by our group as other potential mechanism involved in phenolic beneficial effects [**Manuscript 1**].

Moreover, the interaction between phenolic compounds and biological rhythms seems be bidirectional since the bioavailability of these compounds is affected by the time of day and season of the year in which they are consumed (27,28). The differences in circulating content of phenolic metabolites depending on when they are consumed, suggest a circadian regulation of the mechanisms of absorption and metabolization of these compounds. This fact is

relevant as phenolic bioactivities are dependent on their bioavailability, and these results show circadian oscillation of this process. According to these finding, the effects of phenolic compounds, including PACs, have shown differential effects on clock genes and bioactivities depending on when they are consumed [**Manuscript 1**].

Then, the aim of this thesis was to elucidate whether PACs can act as synchronizers of clock system and investigate if these phenolic compounds can restore biological rhythms in different administration times and in different situations of circadian disruption such as changes in light-dark cycles or in feeding patterns.

Photoperiod-sensitive Fischer 344 (F344) rats are a promising animal model with which to study biological rhythms. However circadian rhythms of this animals have not been so studied as in other animal models. Therefore, to evaluate and better understand the circadian rhythm of F344 rats, a study was conducted to determine circadian rhythms of these animals and investigate the effects that food composition presents on this rat model [**Manuscript 2**]. For this purpose, F344 rats were fed a standard chow diet and the animals were supplemented at the beginning of their activity or resting phase (ZT12 and ZT0, respectively) with diluted condensed milk, which is used as vehicle in many studies in which effects of PACs are investigated. Phenolic compounds are generally very astringent, ant the use of sweet vehicles masks their taste and can be consumed voluntarily by rats. Specifically, in this study, F344 rats were administered a low dose of low-fat condensed milk diluted in water (diluted 1/5) and its composition was 60.8% glucose, 8.9% protein, 0.2% fat. This amount of condensed milk corresponds to a minimal dose of sugar and negligible amount of protein. In fact, using a translation of animal to human doses (29), and estimating the daily intake for a 70 kg human, the condensed milk administered corresponds to an intake of 2.3 g/day of sugar (half teaspoon) and 0.3 g/day of protein. Nevertheless, the results of this study showed

differences in the animals depending on the time in which they were supplemented and a greater increase in BW gain was observed when the condensed milk was administered at ZT0. In addition, the low amount of sugar administered at ZT0 led to the loss of the circadian rhythm of serum glucose and leptin, and a total reversal of the oscillation of insulin and the anorexigenic gene Cart compared to ZT12-administered animals. Although no differences were observed in food intake depending on timing of administration, these changes suggest an alteration of the feeding pattern caused by the minimal amount of sugar consumption at the beginning of the rat resting phase. Additionally, the serum metabolomic profile was differed between healthy rats that received the condensed milk at ZT12 from those that received at ZT0. While 17 metabolites displayed circadian oscillation when the low dose of sugar was administered in the evening, only 6 metabolites with circadian rhythm were observed in the rats receiving condensed milk in the morning. According to this, hypothalamic clock genes were affected when the minimal amount of sugar was consumed at ZT0. In this regard, Bmal1 showed an acrophase shift toward the light phase, and *Rev-erbα,* which showed an increase in the amplitude of its circadian rhythm, reduced its expression during the dark phase. The changes in the metabolic profiles and in circadian expression of these genes in healthy rats, highly related to the regulation of energy metabolism and food intake signaling, showed the importance of minimal changes in dietary patterns and the bidirectionality of the interaction of circadian rhythm and metabolic alterations. Several studies have reported the importance of meal timing and its impact on the body's metabolism and physiology (6). In this sense, according to our findings, chocolate administration to rats at the beginning of their light phase was related to greater weight gain than when it was administered at the beginning of the dark phase (30). However, our results highlight in healthy rats the importance of minimal changes in feeding pattern and the timing of administration since the amount of sugar present in condensed milk was really low. In addition, this study allowed us to test the script, which was developed

based on the cosinor method to carried out the studies of circadian rhythms and investigate the circadian rhythms of the photosensitive F344 rats.

Subsequently, the effects of PACs and the timing of their administration on the circadian rhythm was studied in an obesogenic context [**Manuscript 3**]. As was expected, CAF-fed animals increased their BW gain and presented other cardiometabolic risk factors associated to metabolic syndrome. No differences were found in the BW gain between the animals administered the VH in the morning or in the evening. According to other studies, CAF diet administration resulted in a disruption of the circadian rhythms (26,31–33) and a clear disturbance of circadian rhythm for serum biochemical parameters and hormones were observed in the F344 rats fed CAF diet. Indeed, CAF diet affect the rhythmicity of serum glucose, insulin, leptin, and hormones, such as testosterone, T3, T4 or corticosterone levels, key in the regulation of axes that modulate the circadian rhythm such as hypothalamic-pituitary-adrenal axis (HPA), Hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) (34,35). Interestingly, circadian rhythm alterations were observed in the expression of clock genes in the hypothalamus, like Bmal1 or Rev-erbα, and genes of appetite signaling, such as *Cart*. Moreover, some changes in the rhythmicity of some parameters were observed as a consequence of the VH administration at different time. Specifically, the rhythmicity of serum triglycerides and the gene expression of *Bmal1, Cart, Pomc* and *Nr3c1* were modified depending on the time of VH administration. However, these changes were much less prominent than those observed in healthy rats [**Manuscript 2**]. Nevertheless, since both VH groups did not present the same rhythmicity in some parameters animals administered GSPE were always compared to their respective control groups.

Regarding GSPE effects, a more pronounced decrease in BW gain was observed in the ZT12-treated rats than in the ZT0-treated rats and according to this, a higher energy expenditure was observed than in the GSPE administered rats at

ZT12. Moreover, GSPE restored the circadian rhythmicity of most of the parameters that loss their rhythms by CAF diet. Interestingly, while some biochemical parameters such as glucose and leptin recovered their rhythmicity when GSPE was administered at ZT12, insulin displayed circadian rhythm when the administration of GSPE was at ZT0. Remarkably, melatonin, whose rhythmicity was strongly altered by the CAF diet, recovered its circadian rhythm only when GSPE was administered at ZT12. The effect of PACs on melatonin has been previously reported by our group. Nevertheless, the ability of GSPE to increase melatonin levels in healthy rats was at ZT0, elongating melatonin circulation beyond dawn, whereas this effect was not seen when treatment was at ZT12 (24). In addition, the results showed that GSPE acted on the central clock by recovering the rhythmicity of the expression of genes such as Bmal1, Rev-erbα and Rorα. The effects of GSPE on Bmal1 expression has also been described (24). Indeed, modulation of Bmal1 was observed when PACs were administered to ZT0. In addition, modulation of *Nampt* was also reported upon administration of GSPE to ZT0, whereas regulation of Rev-erbα was found only when PACs were administered to ZT12. In the present study, although *Nampt* rhythm was not recovered, gene expression of *Bmal1, Rev-erbα* and *Rorα*, was restored with treatment at ZT0, whereas when treatment was at ZT12, Bmal1 and Rev-erbα showed circadian rhythmicity. The recovery of key central clock genes such as *Bmal1*, *Rev-erbα* or *Rorα* after GSPE administration support its role as a zeitgebers of the circadian rhythm (24,26,36). Other phenolic compounds have also demonstrated their activity as modulators of clock system [**Manuscript 1**]. Regarding the effect of time administration on gene expression, *Rorα* recovered their circadian rhythmicity at ZT0 but not at ZT12 and contrary *Argp* showed circadian rhythm at ZT12 but not at ZT0. The differential PACs effects observed at the different times of administration could be related to the changes on bioavailability of these phenolic compounds according to the time of day (27).

As discussed previously, circadian rhythm disruption and metabolic disorders have a bidirectional interaction (12). Therefore, the second objective of this thesis was to evaluate the effect of GSPE in a simulated jet lag context in healthy and CAF-induced obese rats. The first step was to investigate the effects of GSPE on physiological and circadian modulators [**Manuscript 4**] in healthy and CAF-induce obese F344 rats subjected to a sudden change of light/dark cycle. For this purpose, rats were treated with GSPE for one week after an abrupt change of photoperiod, causing a disturbance of the biological rhythm of the animals by changing the light-dark cycle. Prior to the photoperiod change, half of the rats were fed with CAF for 6 weeks to induce obesity. In this study, the sudden light/dark shift caused the loss of BW in a photoperiod and diet-dependent manner. Interestingly, it was observed that CAF-fed rats switched to L6 photoperiod showed a greater loss of BW than their counterparts in L18 photoperiod, demonstrating a diet-photoperiod interaction effect. Regarding PACs effects, GSPE administration caused a BW loss in CAF-fed rats under L18 conditions, showing an interaction between photoperiod, diet and GSPE since the same effect was not observed in the rest of groups. A change in feeding pattern was observed as GSPE administration increased fat intake compared to the corresponding CAF-fed VH groups. Moreover, this increase was photo-period dependent since it was significantly higher under L18 than under L6 photoperiod. Disruption of the light/dark cycle combined with CAF diet has previously been shown to potentiate BW gain and other cardiometabolic risk factors (37). The results of this study showed that this effect appears to be mitigated by GSPE administration in a photoperiod-disturbance context. Furthermore, it was observed that the CAF diet promoted sedentariness in rats under L12 conditions, as activity was drastically reduced. When the rats were switched to L18 or L6 conditions, a generalized drop in activity was observed in all groups. In this sense, a decrease in activity has been reported in CAF-diet obese rats under in L12 conditions and in rat jetlag and shift-work models (30,38). However, after the sudden change in photoperiod GSPE reduced

activity in STD-fed rats under L6 conditions from ZT6 to ZT12 (light phase in L12 conditions), that was shown to be increased compared to STD-fed rats under L18 conditions. Interestingly, GSPE tended to increase activity in obese rats compared with VH rats in both photoperiods, but only in the dark phase under L6 photoperiod and from ZT12 to ZT18 in L18 conditions (dark phase in L12 conditions). Regarding hormone status, the increase in corticosterone levels observed in STD rats when they were switched to L18 photoperiod, which were reversed by GSPE administration, could be due to the sudden change of photoperiod and would relate to the increased stress of these animals (39,40). Interestingly, the CAF-VH group under L6 photoperiod showed high corticosterone levels in the light phase and did not follow their normal rhythm between ZT3 and ZT15. However, this effect was reversed by GSPE administration, recovering its rhythm. Therefore, GSPE could be attenuating the stress induced by the sudden change of photoperiod. The rhythm of testosterone and the T3-to-T4 ratio were lost when the photoperiod was changed, which could lead to metabolic disturbances since these hormones are key modulators, as previously mentioned (34,35). Despite of GSPE administration reduced testosterone in L18 at ZT15 in CAF-fed rats, the rhythm was not recovered. Regarding the T3-to-T4 ratio, the rhythm was lost in practically all groups, however, T3-to-T4 ratio was restored their day/night oscillation by GSPE treatment. Loss circadian parameter of this hormone could be leading to metabolic disorders produced by changes in feeding patterns since this ratio has an established circadian rhythm dependent on diet (41,42). In addition, metabolomic data also showed a change in the profile of the groups in a diet- and photoperiod-dependent manner [**Manuscript 5**]. Thus, increased alanine and phenylalanine concentrations were observed in both photoperiods. The increase of these amino acids, altering their metabolism, have been related to obesity and the development of metabolic syndrome (43). CAF diet tended to increase levels of succinic acid; a metabolite that has been associated with mitochondrial stress in a diabetic condition when at prolonged elevated levels

(44). GSPE administration under L18 conditions significantly mitigated these variations in alanine, and tended to restore normal values for glycolic acid, succinic acid, and proline. On the other hand, GSPE treatment mitigated the variations for proline, phenylalanine and 4-hydroxyphenylactic acid in CAF-fed rats, with no effects observed in STD-fed rats by GSPE treatment. These results suggested that grape seed PACs might be mitigating, in a photoperiod- and diet-dependent manner, the metabolic disorders induced by an alteration of the light/dark cycle by suddenly changing the photoperiod. This photoperiod change could be inducing disorders in the HPG and HPT axes as their main modulators such as testosterone and T3-to-T4 ratio, respectively, lost the rhythm set by the light/dark cycle.

Considering these results, the next step was to evaluate the metabolic status and central clock disturbances of this abrupt change of photoperiod and the GSPE treatments [**Manuscript 5**]. For this purpose, hypothalamus and serum from the previous experiment were analyzed. In this analysis a photoperiod-dependent GSPE effect was observed. On the one hand, the change to L6 photoperiod caused an improvement of the lipid profile in CAF-fed rats, with cholesterol values similar to STD-fed rats, and triglyceride levels considerably lower than CAF-fed rats under L18 conditions, this was in agreement with what was described in the previous study, where we see that L6-fed rats present a greater BW drop than rats under L18 conditions [**Manuscript 4**]. In addition, the change of photoperiod caused an increase in the expression of *Bmal1* and *Nampt*, which are closely related to the regulation of metabolism at the circadian level (45–47). Despite having a better lipid profile, these rats presented worse insulin sensitivity, since they presented much higher serum insulin levels than the rest of the groups, in addition to presenting lower concentrations of metabolites related to the Krebs cycle. Treatment with GSPE reduced insulin levels to levels similar to STD-fed rats and showed a change in metabolomic profile compared to VH rats. No major changes were observed in STD-fed rats after the photoperiod change, however, GSPE treatment resulted

in a reduction in testosterone levels and an increase in corticosterone. On the other hand, under L18 conditions the lipid profile of CAF-fed rats was strongly affected, showing higher cholesterol and triglyceride levels than STD-fed rats under the same conditions and, than CAF-fed rats under L6 conditions. This increase in cholesterol and triglycerides was reduced with GSPE treatment, recovering healthy condition levels in the case of cholesterol. In addition, thyroid hormone showed a tendency to have higher T3 hormone levels in CAF-fed rats under L6 conditions, interestingly this is reversed with GSPE treatment, where a tendency to have higher T3 levels is observed in GSPE-treated rats under L18 conditions compared to GSPE-treated rats under L6 conditions. Elevated T3 levels have been associated with reduced cholesterol and triglyceride levels (34). In addition, higher levels of thyroid hormones and the T3-to-T4 ratio are associated with a loss of BW (48). These results are in accordance with those obtained for biochemical parameters, where an improvement in cholesterol and triglyceride levels is seen and could be mediated by the increase in T3 levels and thyroid hormone ratio levels that increase in the L18-CAF-GSPE group. In accordance with to our results those obtained in the previous study [**Manuscript 4**], where a greater weight loss and the tendency to increase locomotor activity with GSPE treatment were observed in this group. Finally, Nampt expression in the hypothalamus increased with treatment compared to VH rats. Interestingly, the fact that GSPE treatment in CAF-fed rats under L18 conditions, with a worsened metabolic profile, involved an increase in Nampt expression in the hypothalamus supports the hypothesis that GSPE has a modulatory effect on circadian rhythm and that part of its effect on metabolism is mediated by its action as a zeitgeber.

As previously mentioned, different metabolic and physiological processes are cyclically regulated by biological rhythms, mainly marked by the light-dark cycle (1,2). In this sense, BP is strongly influenced by the circadian rhythm, with an increase during the activity phase and a decrease during the resting phase. The alteration of this rhythmicity may be due to several factors, including CAF-

induced HTN in the context of a metabolic syndrome. Thus, the difference between the activity phase and the resting phase between 10 and 20% is related to better cardiovascular health (dipper pattern). Alteration of the circadian rhythm of BP, reducing the difference between these two phases by less than 10% (non-dipper pattern), is associated with an increased risk of cardiovascular diseases (CVD), stroke, and death (49–51). The last objective was to elucidate the effect of PACs on the rhythmicity of physiological parameters in CAF-induced obese F344 rats subjected to an abrupt change in the light/dark cycle [**Manuscript 6**]. To this end, BP, temperature, and locomotor activity of CAF-fed rats were monitored for 7 weeks, the last of which involved a change in photoperiod to cause an alteration of the light-dark cycle and the administration of GSPE. A decrease in systolic BP (SBP) was observed with GSPE treatment, especially in the light phase, and under L18 conditions. In addition, the dipper profile was lost with the CAF diet and worsened with the change of photoperiod. However, GSPE treatment increased the percentual difference between light and dark phase in SBP. The same effects were not found for GSPE-treated rats switched to L6 conditions, where a less pronounced reduction in BP was observed compared to rats under L18 conditions. In addition, no increase in the difference between light-phase and dark-phase BP was observed. These results showed concordance with those obtained in previous studies, where a better metabolic profile and BW reduction were observed in CAF-fed rats under L18 conditions [**Manuscript 4** and **manuscript 5**]. The results of locomotor activity showed a reduction of locomotor activity by CAF diet, results in concordance with our previous study [**Manuscript 4**] and GSPE treatment resulted in a reduction of activity in the dark phase under L18 conditions. This fact would indicate a better adaptation to the new photoperiod with respect to VH rats which showed an altered activity pattern throughout the 24 hours. In the case of the switch to L6, a drop in the activity was observed with GSPE treatment in both the dark and light phases.

These results also showed the antihypertensive effect of GSPE, a property already widely reported by our group (52–54), and its ability to act in a photoperiod-dependent manner in a context of light-dark cycle disruption. The increase in the difference between light-phase and dark-phase SBP with GSPE treatment suggests an effect on circadian rhythm-mediated mechanisms in reducing SBP values in a cyclic manner under L18 conditions.

The results of this thesis demonstrated how modifications in the light-dark cycle [**Manuscript 4** and **Manuscript 6**], in the composition of the diet [**Manuscript 3**], and in the timing of food intake [**Manuscript 2**] alter the circadian system. These circadian disruptions modify, to a greater or lesser extent, metabolic pathways that could lead to metabolic syndrome. In addition, circadian rhythms have been shown to interact with the PACs effects since their effects are affected by the timing of their administration and modulate central clock and regulate the rhythmicity of physiological parameters. Thus, GSPE restored the circadian rhythm of several biochemical parameters and modulating hormones of the main axes that regulate energy metabolism. Interestingly, GSPE was able to modulate or restore the circadian rhythm of key core clock genes, an indication that the effects of GSPE may be mediated by its role as a zeitgeber regardless of the disruptive situation to which it is subjected. Thus, the restoration of rhythms of genes such as *Bmal1*, *Rev-erbα, Rorα* in a disruption by diet composition [**Manuscript 3**] or the increased expression of *Nampt* in a situation of photoperiod change [**Manuscript 5**] suggest that GSPE acts on metabolic disorders by regulating the circadian rhythm.

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CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
INTERACTION BETWEEN CIRCADIAN RHYTHM AND GRAPE SEED PROANTHOCYANIDINS IN HEALTHY AND OBESE RATS: POTENTIAL ROLE AS A CENTRAL AND PERIPHERICAL CLOCK SYNCHRONIZER
Jorge Ricardo Soliz Rueda

CONCLUSIONS

- The daily intake of a minimal amount of sugar impact on circadian rhythms and show time-of-day-dependent effects on metabolism in healthy rats, causing higher circadian metabolic disruption when it is consumed in the morning instead of the beginning of the rat activity phase. This impact is lower in cafeteria-induced obese rats than in healthy rats.

- The consumption of cafeteria diet alters the central clock and the circadian rhythmicity of serum biochemical parameters, hormones and expression of appetite signaling gene in hypothalamus.

- Body weight-lowering effects of proanthocyanidins in cafeteria diet-induced obese rats are more prominent when the phenolic compounds are administered at the beginning of the rat activity phase.

- Proanthocyanidins administration restore the circadian rhythm of central clock, serum biochemical parameters, hormones and appetite signaling genes in a time-of-day-dependent manner. Remarkably, rhythmicity of melatonin is only restored by these phenolic compounds when they are consumed at the beginning of the rat activity phase.

- The disruption of the light-dark cycle by an abrupt change in photoperiod alters the rhythmicity of serum hormones, locomotor activity and blood pressure. These changes occur in a photoperiod-dependent manner, causing metabolic disorders unevenly between long and short photoperiods.

- Long photoperiod exposure impairs lipid profile and body weight more markedly than short photoperiod exposure in an obesogenic context, while short photoperiod exposition worsens glucose metabolism.

- The consumption of proanthocyanidins mitigates the impact of the disruption of the light-dark cycle, restoring the rhythmicity of

parameters such as locomotor activity, serum hormones and blood pressure in a photoperiod-dependent manner.

- Proanthocyanidins modify metabolomic profile and improve serum lipid profile and insulin levels, reduce body weight gain, and exhibit an antihypertensive effect in a photoperiod-dependent manner improving the cardiometabolic state of the animals in both photoperiods.
- Although proanthocyanidins exhibit beneficial effects under both photoperiods these are more prominent under long day conditions.

The findings obtained in this thesis demonstrated that circadian rhythms impact on proanthocyanidins activity, since differential effects are found depending on their time of administration. Thus, their beneficial effects are more prominent when the animals are supplemented at the beginning of their activity phase. In addition, the results suggest that the effects of proanthocyanidins could be mediated in part by the modulation of the circadian rhythm. Thus, it was observed proanthocyanidins can modulate central clock genes while improving cardiometabolic risk factors in different scenarios of circadian rhythm disruption, from disruption by diet composition to disruption of the normal light-dark cycle. Therefore, this thesis contributes to understanding the bidirectional interaction between the beneficial effects of phenolic compounds and biological rhythms in different contexts characteristic of the current lifestyle, in which the circadian rhythms are strongly altered by artificial light, work shifts, jet lag, among others.

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Biological rhythms play an important role in the physiological and metabolic adaptation of the organism to the time of day, circadian rhythm, or year, seasonal rhythm. External cues or modulators called zeitgebers, such as light or food, play a crucial role in the reprogramming or altering these oscillations. Circadian rhythm disruption due to modern lifestyles such as changes in light/dark cycles or dietary patterns has been related to the development of metabolic disorders. In this context, phenolic compounds such as proanthocyanidins (PACs) have demonstrate to exert beneficial effects on metabolic disorders. Moreover, polyphenols have shown their capacity to modulate some components of the clock system. Indeed, the interaction of phenolic compounds with biological rhythms has been recently pointed out by our group as a potential mechanism involved in their beneficial effects. Then, the aim of this thesis was to elucidate whether PACs act through biological rhythms and to investigate whether these phenolic compounds can restore biological rhythms in different perturbation situations, such as changes in light-dark cycles or in dietary patterns. For this, circadian rhythms of healthy and cafeteria (CAF) diet obese Fischer 344 rats, the effects of different situations of perturbation of these rhythms and the properties of PACs to restore these disruptions were studied. CAF diet caused disruption of central clock genes and alteration of metabolism circadian rhythms. PACs demonstrated to act as zeitgebers mitigating the circadian disruption, notably the restoration of the circadian rhythm of melatonin. In addition, in a jet lag situation caused by a sudden light/dark shift, PACs mitigated the circadian disruption in a photoperiod-dependent manner. Thus, PACs have an important role as modulators of central and peripheral circadian rhythm, which may mediate the beneficial effects of these phenolic compounds on metabolic syndrome.

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