UNIVERSITAT ROVIRA I VIRGILI

PHYSIOLOGICAL AND METABOLIC ADAPTATIONS TO DIFFERENT PHOTOPERIODS: EFFECTS OF OBESITY AND SEASONAL FRUIT CONSUMPTION

Roger Mariné Casadó

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Physiological and metabolic adaptations to different photoperiods: effects of obesity and seasonal fruit consumption

Roger Mariné Casadó

Doctoral Thesis
Department of Biochemistry and Biotechnology

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Directed by Prof. Lluís Arola Ferrer and Dr. Antoni Caimari Palou

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Tarragona 2018

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FEM CONSTAR que aquest treball, titulat "**Physiological and metabolic adaptations to different photoperiods: effects of obesity and seasonal fruit consumption**", que presenta **Roger Mariné Casadó** per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat, i que compleix els requeriments per optar a la Menció Internacional.

HACEMOS CONSTAR que el presente trabajo, titulado "**Physiological and metabolic adaptations to different photoperiods: effects of obesity and seasonal fruit consumption**", que presenta **Roger Mariné Casadó** para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de esta universidad, y que cumple los requerimientos para optar a la Mención Internacional.

WE STATE that the present study, entitled "**Physiological and metabolic adaptations to different photoperiods: effects of obesity and seasonal fruit consumption**", presented by **Roger Mariné Casadó** for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university, and fulfil the demanded requirements to get the International Mention.

Reus, 5 de Setembre de 2018 / Reus, 5 de Septiembre de 2018 / Reus, 5th September 2018

Els directors de la tesi doctoral
Los directores de la tesis doctoral
Doctoral Thesis Supervisors

Dr. Lluís Arola Ferrer

Dr. Antoni Caimari Palou

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A la
meva família
i amics

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“Nothing in life is to be feared,

it is only to be understood.

Now is the time to understand more,

so that we may fear less.”

Marie Curie (1867-1934).

Polish physicist and chemist.

Winner of two Nobel Prize (Physics, 1903; Chemistry, 1911).

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SUMMARY

Through evolutionary history, the coexistence of animals and plants have led to the development of an adaptive phenomenon that has been recently explained by the xenohormesis theory. This theory postulates that the phytochemicals synthesized by stressed plants could be recognized as signals by the heterotrophs that consume them, being informed about the external conditions in which plants were harvested and allowing them to favorably adapt to unpredictable changes in the environment. Thus, each plant contains a distinctive phytochemical composition informing about the environmental status. In this framework, the main aim of the present thesis was to evaluate whether fruit consumption out of season would induce an erroneous signaling, leading to detrimental effects on physiology and metabolism of normoweight and cafeteria-fed obese Fischer 344 rats, by analyzing glucose and lipid metabolism-related parameters in blood, liver and skeletal muscle. To achieve this objective, we firstly characterized the physiological and metabolic adaptations to the chronic exposure to different photoperiods, which resembled seasonal variations in day length, in normoweight and obesogenic conditions. Once characterized, we evaluated the effects of the consumption of sweet cherry, a popular anthocyanin-rich fruit harvested in spring/summer, in short and long photoperiods resembling winter and summer, respectively. Firstly, we reported that the chronic exposure to different photoperiods induces several variations in physiological and metabolic parameters in normoweight and diet-induced obese rats, mainly affecting glucose and lipid metabolism and insulin signaling. Secondly, we revealed that cherry intake induces marked photoperiod-dependent effects, promoting more pronounced and, to some extent, more negative effects concerning glucose metabolism and insulin signaling in normoweight and diet-induced obese F344 rats when it was consumed out of

season. These findings could contribute to highlighting the importance of the consumption of seasonal fruits to maintain an optimal health.

RESUM

Històricament, la coexistència d'animals i plantes ha comportat el desenvolupament d'un fenomen adaptatiu recentment definit per la teoria de la xenohormesi. Aquesta, postula que els fitoquímics sintetitzats per plantes en estat d'estrès podrien ser reconeguts pels heteròtrofs que els consumeixen, sent informats sobre les condicions ambientals en les quals han crescut aquestes plantes i permetent una adaptació favorable als canvis no predictius en l'ambient. Per tant, cada planta conté una composició distintiva de fitoquímics que informa sobre l'estat de l'entorn. L'objectiu principal d'aquesta tesi va ser avaluar si el consum de fruita fora de temporada induiria una senyalització errònia, promovent efectes perjudicials en la fisiologia i el metabolisme en rates Fischer 344 normopès i obeses, mitjançant l'anàlisi de paràmetres relacionats amb el metabolisme glucídic i lipídic en sang, fetge i múscul esquelètic. Per assolir aquest objectiu, vam caracteritzar les adaptacions fisiològiques i metabòliques a l'exposició crònica a diferents fotoperíodes, els quals simulen les variacions estacionals en la durada del dia, en condicions de normopès i obesitat. Posteriorment, vam avaluar els efectes del consum de cirera, una fruita rica en antocianines i cultivada a la primavera/estiu, en un fotoperíode curt i llarg, simulant hivern i estiu, respectivament. Els resultats van revelar que l'exposició crònica a diferents fotoperíodes indueix canvis en diferents paràmetres fisiològics i metabòlics en rates normopès i obeses, afectant majoritàriament al metabolisme glucídic i lipídic i a la senyalització d'insulina. D'altra banda, vam concloure que el consum de cirera indueix efectes dependents del fotoperíode, promovent efectes més pronunciats, i en certa mesura, més deleteris sobre el metabolisme de la glucosa i la senyalització d'insulina en rates normopès i obeses quan es consumeix fora de temporada. Aquests resultats podrien contribuir a destacar la rellevància del consum de fruites de temporada en el manteniment d'una salut òptima.

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ABBREVIATIONS

4E-BP1	Eukaryotic initiation factor 4E-binding protein
AANAT	Arylalkylamine N-acetyltransferase
ACC	Acetyl-coenzyme A carboxylase
ADP	Adenosine diphosphate
AgRP	Agouti-related peptide
Akt	Akt serine threonine kinase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AS160	Akt substrate of 160 kDa
ATGL	Adipose triglycerides lipase
ATP	Adenosine triphosphate
BMAL1	Brain and muscle Arnt-like 1
CAF	Cafeteria diet
CAMKKβ	Ca^{2+}/calmodulin-dependent protein kinase kinase beta
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine-regulated transcript
CBS	Cystathionine β-synthase
CD36	Fatty acid translocase, homolog of CD36
CH	Cherry
CHGA	Chromogranin A
CLOCK	Circadian locomotor output cycles kaput
CPT1	Carnitine palmitoyltransferase 1
CREB	cAMP response element binding protein
CRP	C-reactive protein
CRY	Cryptochrome
CS	Citrate synthase

CVD	Cardiovascular disease
DAG	Diacylglycerol
DAT	Dopamine transporter
DGAT2	Diacylglycerol O-acyltransferase 2
DIO2	Type II iodothyronine deiodinase
DIO3	Type III iodothyronine deiodinase
DRD5	Dopamine receptor D5
EE	Energy expenditure
eEF2K	Eukaryotic elongation factor 2 kinase
EYA3	Eyes absent 3
F344	Fischer 344
FA	Fatty acid
FABPm	Fatty acid binding protein
FASN	Fatty acid synthase gene
FATP1/5	Fatty acid transporter 1/5
FBP1	Fructose-1,6-biphosphatase 1
FOXO1	Forkhead box protein O1
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GHSR	Ghrelin receptor
GK	Glucokinase
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GNG	Gluconeogenesis
GnRH	Gonadotropin-releasing hormone
GP	Glycogen phosphorylase
GPAT	Glycerol-3-phosphate acyltransferase
GPCR	G protein-coupled receptor
GSK3	Glycogen synthase kinase 3

GSPE	Grape seed proanthocyanidin extract
GSV	GLUT4 storage vesicle
HAD	Hydroxyacyl-CoA dehydrogenase
HDL	High-density lipoprotein
HFD	High-fat diet
HK	Hexokinase
HMGCR	HMG-CoA reductase
HSL	Hormone-sensitive lipase
IGF1	Insulin-like growth factor 1
IKKβ	IκBα kinase beta
IL6	Interleukin 6
IML	Intermediolateral cell column
IMP	Inosine monophosphate
ipRGCs	Intrinsically photosensitive retinal ganglion cells
IRS1/2	Insulin receptor substrate 1/2
JNK	c-Jun N-terminal kinase 1
Kp	Kisspeptin
L6	6 hours of light/day
L12	12 hours of light/day
L18	18 hours of light/day
LD	Long day
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LFD	Low-fat diet
LKB1	Liver kinase B1
MAFbx	Muscle atrophy Fbox
MCP1	Monocyte chemoattractant protein 1
MetS	Metabolic syndrome
miR	MicroRNA

mTORC1/2	Mammalian target of rapamycin complex 1/2
MuRF1	Muscle ring finger 1
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
ND	Normal day
NEFAs	Non-esterified free fatty acids
NF-κB	Nuclear factor kappa B
NMR	Nuclear Magnetic Resonance
NPY	Neuropeptide Y
NR1D1	Nuclear receptor subfamily 1, group D, member 1
OBRB	Leptin receptor
p	Phosphorylated
p70S6K	p70S6 kinase
PA	Proanthocyanidins
PBMCs	Peripheral blood mononuclear cells
PCA	Principal component analysis
PCK1	Phosphoenolpyruvate carboxykinase 1
PER	Period
PFK1	Phosphofructokinase 1
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLS-DA	Partial least squares discriminant analysis
POMC	Proopiomelanocortin
PT	Pars tuberalis
PVN	Paraventricular nucleus
Raptor	Regulatory-associated protein of mTOR
RFRP	RFamide-related peptides
RHT	Retinohypothalamic tract

RORα	RAR-related orphan receptor alpha
RORE	ROR/REV-ERB-response element
ROS	Reactive oxygen species
RQ	Respiratory quotient
SAD	Seasonal affective disorder
SCN	Suprachiasmatic nuclei
SD	Short day
SIRT1	Sirtuin 1
SREBP1	Sterol regulatory element-binding protein 1
STD	Standard diet
T3	Triiodothyronine
T4	Thyroxine
TAG	Triacylglycerol
TBC1D1	TBC1 domain family member 4
TCA	Tricarboxylic acid
TH	Thyroid hormone
TNF-α	Tumor necrosis factor alpha
TSC2	Tuberous sclerosis complex 2
TSHβ	Thyroid stimulating hormone beta
TTFL	Transcription-translation feedback loops
VE	Vehicle
VLDL	Very low-density lipoproteins
ZT	Zeitgeber time

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I. INTRODUCTION

1. BIOLOGICAL RHYTHMS

Living organisms are constantly exposed to predictable variations in the geophysical environment, being forced to adapt themselves in order to ensure their survival. These periodic changes, which are consequence of Earth's rotation around its axis (photoperiod), its tilt (day length variations) and its simultaneous revolving around the sun (seasonal changes), are responsible for major evolutionary changes in life [1]. In this sense, most species evolved in order to internalize and predict environmental variations through the development of a time-measuring system, which allows them to anticipate daily and annual changes and favorably adapt their physiology and metabolism. The resulting periodic variations of these functions are known as circadian (from the Latin *circa*, about; *dian*, day) or circannual (about a year) rhythms [2,3].

Both circadian and circannual rhythms are conducted by two principal mechanisms: a) an intrinsic endogenous timing system that persists in the absence of environmental cues, with oscillating periods of approximately 24 hours or 365 days, respectively, driven by endogenous central and peripheral clocks, and b) environmental cues (Zeitgebers) that entrain these rhythms to the external conditions, such as the day length (photoperiod) [4,5].

1.1. Day length as the primary *zeitgeber*

Daily changes in dietary patterns, physical activity and temperature are basic zeitgebers, coordinating several physiologic and metabolic responses in the whole body [6]. Similarly, seasonal variations in rainfalls, barometric pressure, food availability and climate can act as environmental signals to synchronize circannual clocks [7]. Nevertheless, these cues are not so predictable, since they are not consistent in time and magnitude. Hence, the main signal used by most organisms in order to establish the astronomical daytime or season is the

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photoperiod (light/darkness phases) [7]. The ability to use the light as an anticipatory cue to environmental changes is known as photoperiodism [8], and it involves several cells and tissues forming a complex photoreception signalling pathway.

Photic information is detected in the retina by the complementary action of the rods and cones photoreceptors together with the intrinsically photosensitive retinal ganglion cells (ipRGCs) located in the ganglia cell layer, which express the photopigment melanopsin [9]. These signals are then transmitted via the retinohypothalamic tract (RHT) to the suprachiasmatic nuclei (SCN). This bilateral structure located in the ventral periventricular zone of the anterior hypothalamus contains approximately 10,000 neurons and it is considered the master circadian pacemaker in mammals, as it synchronizes peripheral clocks and body rhythms [10].

Under darkness conditions, the SCN sends the photoperiodic information via a multisynaptic projection that initiates in the nucleus and travels through the paraventricular nucleus (PVN), the preganglionic sympathetic neurons in the intermediolateral cell column (IML) of the thoracic spinal cord and the superior cervical ganglia, whose projections ends in the postganglionic adrenergic fibers that innervate the pineal gland [11,12]. These sympathetic nerves produce norepinephrine, which stimulates postsynaptic β1 and α1 adrenergic receptors on the pinealocytes, increasing the intracellular levels of 3’-5’-cyclic adenosine monophosphate (cAMP). The rise of cAMP levels stimulates the activity of arylalkylamine N-acetyltransferase (AANAT), which is considered the rate-limiting enzyme in melatonin production from serotonin [7,11,13]. Melatonin synthesis and secretion into the circulation is regulated on a 24-h basis by the SCN, exhibiting a 150-fold increase at night and being inhibited during the daytime, in which light exposure interrupts the transmission of signals from the

SCN to the pineal gland, repressing the release of norepinephrine [13] **(Figure 1)**.

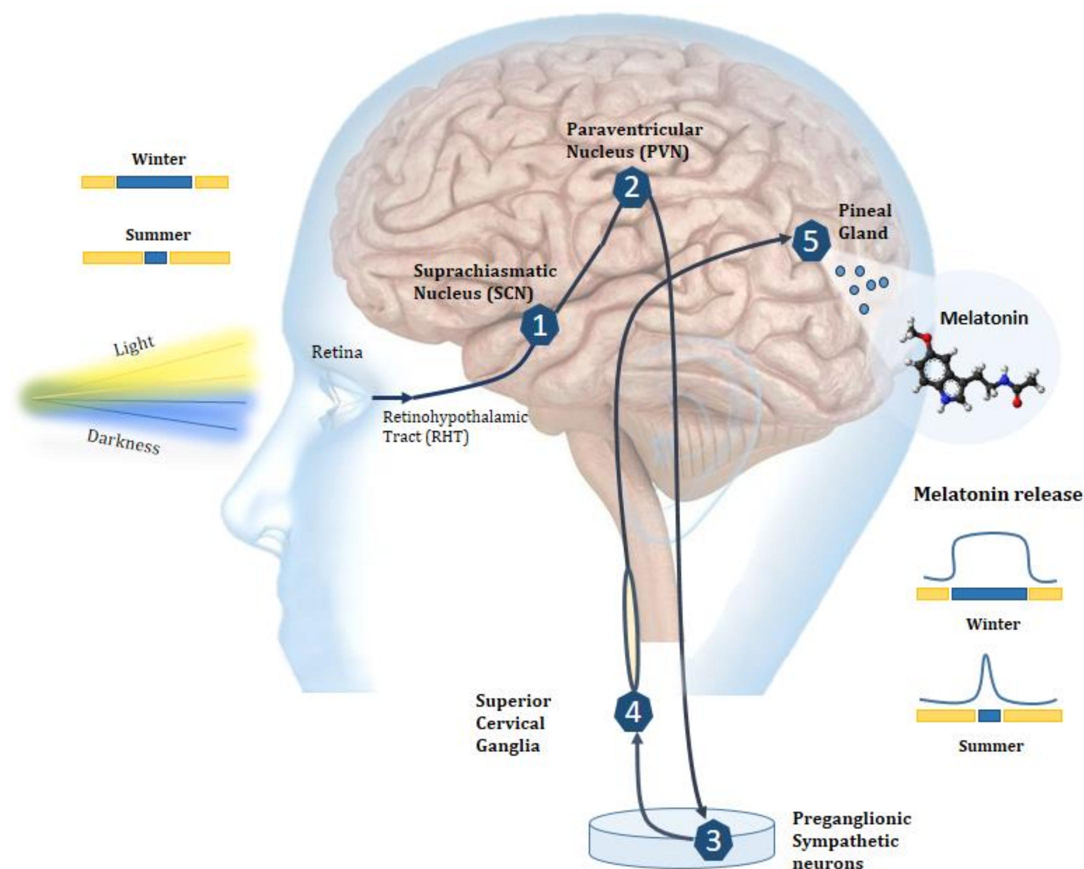

Figure 1. Photoreception signaling pathway regulating melatonin release. Adapted from [11].

The rhythmic secretion of melatonin functions as a crucial indicator of photoperiod variations, informing the brain with an internal hormonal representation of the external day length [14].

1.2. Circadian rhythms

As it has been briefly above-commented, circadian rhythms are evolutionary endogenous and self-sustained timing systems that modulate the oscillation of

several physiological and behavioral processes in a 24-hour timescale, in order to guarantee the maintenance of a robust homeostatic balance. Among these processes, feeding patterns, hormonal regulation, the activity/rest cycle and several metabolic functions are highly regulated by these rhythms [15]. In mammals, this complex and coordinated system is composed by a central clock in the brain's SCN, which accurately warrants a correct phase alignment of the peripheral clocks present in most organs and tissues' cells, via neural connections and hormonal signaling [16].

1.2.1. Molecular machinery of the circadian clock

These endogenous circadian rhythms are generated by a series of interconnected positive and negative transcription-translation feedback loops (TTFL), which have been described in all organisms. However, the several genes and proteins involved in these TTFL differ among species [17]. In mammals, the most important core clock components are the circadian locomotor output cycles kaput (CLOCK) and the brain and muscle Arnt-like 1 (BMAL1), two transcription factors which form a heterodimer complex in the nucleus and activates the transcription of Period (PER) and Cryptochrome (CRY) by binding to the E-box elements of these genes [18,19]. The accumulation of both resulting proteins in the cytoplasm induces the formation of a complex (PER-CRY) that enters the nucleus and represses CLOCK-BMAL1, constituting a cyclic expression pattern of 24 hours in which CLOCK and BMAL1 dimerize in the morning and the transcription of PER and CRY peaks at midday [18,20]. The fluctuation of these core-clock genes is increased and stabilized by a secondary loop in which *Bmal1* expression is activated by the nuclear receptor RAR-related orphan receptor alpha (RORα) and inhibited by the nuclear receptor subfamily 1, group D, member 1 (NR1D1, also known as RER-ERBα) through binding the ROR/REV-ERB-response element (RORE) in the promoter region of BMAL1 [21] **(Figure 2)**.

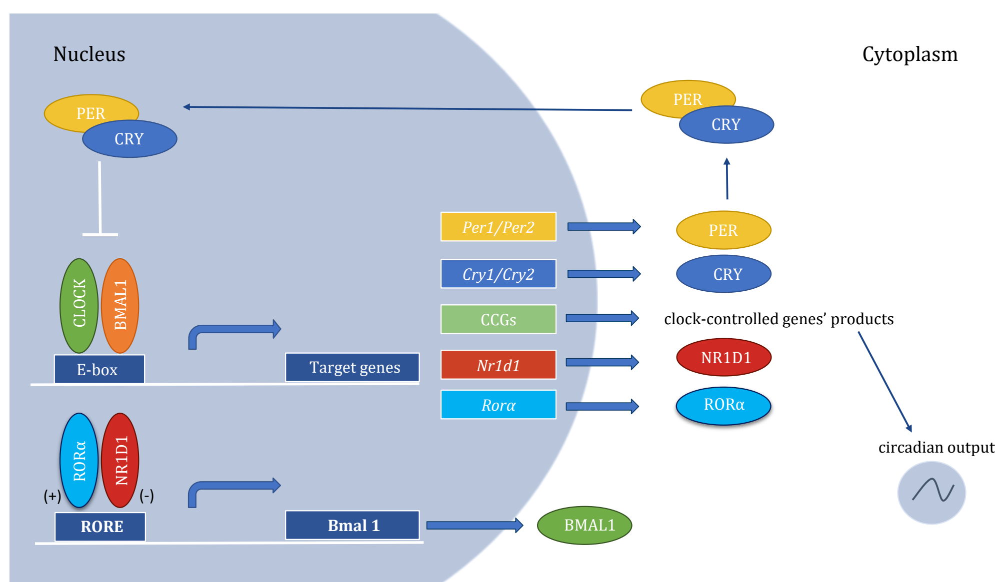

Figure 2. Circadian core-clock transcriptional machinery in mammals. Adapted from [22].

Although this TTFL system is mainly modulated by transcriptional and translational processes, there are several posttranslational regulators that ensure the correct phase and periodicity of this rhythmic system. As an example, it has been reported that CLOCK is a histone acetyltransferase highly regulated by sirtuin 1 (SIRT1), a histone deacetylase dependent on the intracellular levels of nicotinamide adenine dinucleotide (NAD+). Relevantly, this substrate is controlled by the enzyme nicotinamide phosphoribosyltransferase (NAMPT), which catalyzes the first step in NAD synthesis and whose promoter is activated by CLOCK:BMAL1 dimer [23]. It has been proposed that adenosine monophosphate-activated protein kinase (AMPK), a key metabolic energy sensor, can stimulate NAMPT transcription through the phosphorylation and inhibition of CRY, which reinforces the fact that circadian rhythms are directly associated with metabolism [24].

These positive and negative feedback loops control several circadian-regulated output genes, which are involved in the regulation of several physiological and metabolic functions [25–27].

1.2.2. Circadian physiological and metabolic outcomes

The circadian clock machinery is also accountable for the regulation of several tissue-specific rhythmic programs that permit an easiest adaptation to external demands. Thus, sleep/wake and feeding/fasting cycles, as well as other physiological parameters such as body temperature, blood pressure and hormone secretion, possess 24-hour rhythms [28–31] **(Figure 3)**.

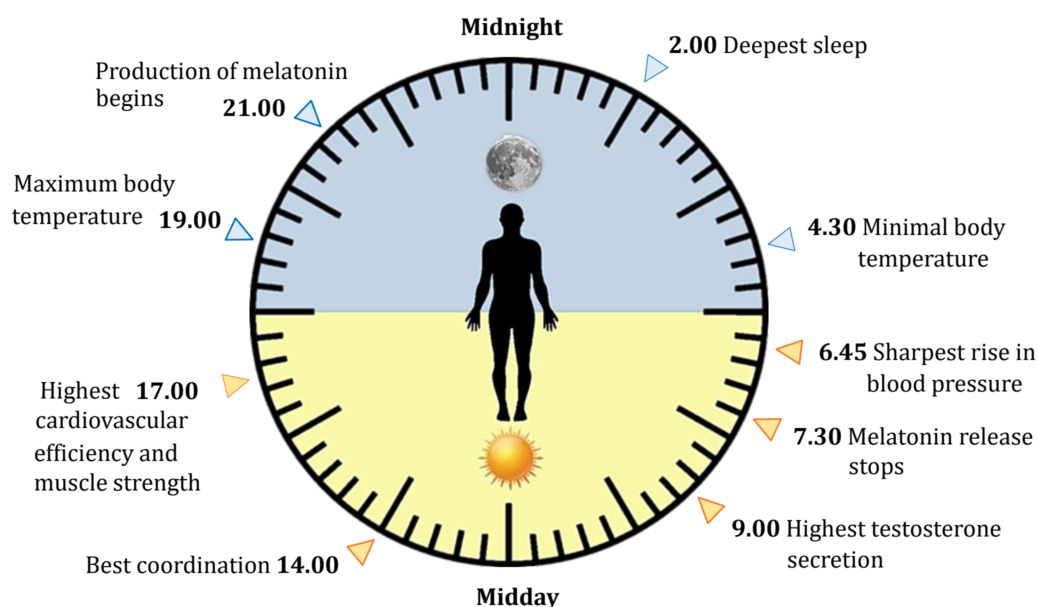

Figure 3. 24-hour rhythms of several physiological processes in humans. Adapted from [32].

Over the past decades, the study of how circadian rhythms modulate the metabolic homeostasis has gained interest for many reasons. For instance, the study of this area, known as chronobiology, is crucial for human's health, since daily oscillations of several biochemical and metabolic parameters clearly determine in which timing of administration a specific drug will be more effective [33]. For example, as illustrated in ***Figure 3***, the study of the daily fluctuations in blood pressure has contributed to detect its sharpest peak in the morning and its decline during the night [34]. In accordance, several drugs, such as the calcium channel blocker Verapamil, have been adjusted in order to

display the maximum plasma levels in the morning after their administration at night [35].

It has been described that approximately up to the 10% of all mammalian genes in the whole organism display circadian rhythms, involving a high number of interactive specialized cells, tissues and organs, such as the liver and the skeletal muscle, which are crucial in the regulation of the whole-body metabolic homeostasis [36,37]. Relevantly, by carrying out studies with genetic knockout mice, there is a large number of evidences about the direct or indirect modulation of several metabolic processes by the components of the circadian core-clock machinery **(Table 1)**.

Table 1. Metabolic processes modulated by the mammalian circadian core-clock components in the liver and the skeletal muscle.

Tissue	Clock component	Metabolic effect	References
Liver	CLOCK	Enhances glycogen synthesis (↑ *Gys2*)	[38]
	BMAL1	Improves insulin sensitivity (↑ Akt) and promotes lipogenesis *de novo* (↑ *FASN*)	[39]
	PER	Promotes glucose storage to glycogen (↑ *Gys2*)	[40]
	CRY	Inhibits gluconeogenesis (↓ *G6Pase*, *Pck1*)	[41]
	NR1D1	Stimulates bile acid synthesis (↑ CYP7A1)	[42]
Skeletal muscle	CLOCK	Increases glucose uptake (↑ *Glut4)*	[43]
	BMAL1	Enhances glucose uptake (↑ *Glut4)* and glycolysis (↑ *Hk and Pfk1*)	[44,45]
	RORα	Stimulates fatty acid β-oxidation and cholesterol efflux (↑ *Cpt1 and Cav3*)	[46]
	NR1D1	Improves mitochondrial biogenesis and oxidative function (↑ AMPK)	[47]

Akt, Akt serine threonine kinase; AMPK, adenosine monophosphate-activated protein kinase; *Cav3*, caveolin 3; *Cpt1,* carnitine palmitoyltransferase 1; CYP7A1, cholesterol 7 alpha-hydroxylase; *FASN*, fatty acid synthase; *G6Pase*, glucose-6-phosphatase; *Glut4*, glucose transporter 4; *Gys2*, glycogen synthase 2; *Hk*, hexokinase; *Pck1*, phosphoenolpyruvate carboxykinase 1; Pfk1, phosphofructokinase 1.

Relevantly, although all the circadian molecular mechanisms regulating these metabolic processes can act autonomously, the perturbation of the environmental cues that act as synchronizers of this system can alter its rhythmicity and consequently, contribute to the development of several pathologies.

1.2.3. Circadian rhythms disruption

Biological rhythms are the result of an evolutionary adaptation to unfavorable environmental conditions, such as food insufficiency and continuous variations in day length and climate. Despite the fact that circadian rhythms still persist in modern societies, the extreme variation of the external cues that synchronize these rhythms, as a consequence of the appearance of artificial light and air conditioning, as well as sedentary lifestyles and the continuous food availability, can critically disrupt their function, altering all the aforementioned physiological and metabolic outcomes [6].

Apart from all the research performed in order to describe the effects of the depletion of core-clock genes on metabolism in cell cultures and rodents, several epidemiological and observational studies have evidenced that the disruption of these circadian rhythms in humans (e.g. rotating night shift work or jet lag) can produce hyperphagia, hyperinsulinemia, body weight gain and hypertriglyceridemia [27], and can increase the risk for coronary heart disease [48], obesity [49], diabetes [50], cancer [51]) and psychiatric disorders [52]. Indeed, an interventional study carried out with healthy young men reported that sleep restriction (4 hours in bed) induced significant changes in the regulation of appetite, leading to reduced levels of the anorexigenic hormone leptin, increased levels of the orexigenic hormone ghrelin and consequently, increased hunger [53]. Moreover, in another study performed with 26 healthy subjects, it was described that after shifting the time of sleep, individuals

displayed a reduced insulin sensitivity and higher inflammation in comparison with those who maintained a regular nocturnal bedtime [54].

In addition, several studies have evidenced that the misalignment between circadian rhythms and feeding can also induce detrimental effects in energy metabolism, emphasizing the relevance of the time of nutrient intake. Thus, in a study performed with healthy obese women submitted to a weight-loss program for 12 weeks, the authors reported that the intake of a high-energy diet at lunch induced more beneficial effects in terms of body weight loss and insulin sensitivity than those who consumed that diet at dinner [55].

1.3. Circannual rhythms

The persistent annual variations of the environment (e.g. day length, temperature and food abundance), especially in those zones located far from the equator, pressed the different species to develop specific long-term mechanisms in order to ensure their survival throughout the year. These mechanisms are known as circannual clocks, which are defined as self-sustained physiological rhythms that exhibit a periodicity of approximately a year [56]. In natural conditions, as previously commented, these rhythms are set by light, responding to the absolute seasonal day length and also to the direction of the photoperiod variations [57]. Regarding this photoperiodic response, two different types of circannual rhythms have been described. Type I rhythms are those that cannot persevere more than one cycle in the absence of light stimuli, typically observed in animals with a short lifespan (e.g. Syrian and Siberian hamsters). Differently, type II rhythms have evolved in order to persist more than two cycles without light resetting throughout the year, as it has been reported in animals with a longer lifespan, such as hibernators [58].

Behind the strong responses of several physiological, behavioral and reproductive functions through the changing seasons, there is a complex and

highly regulated hormonal coordination that shapes the seasonal molecular mechanisms.

1.3.1. Seasonal molecular mechanisms

Although the molecular mechanisms underlying these circannual rhythms and their involvement to circadian clocks are still not fully elucidated, there is growing evidence that the transcriptional coactivator eyes absent 3 (EYA3), a clock-controlled gene product, exerts a crucial role in the regulation of seasonal responses entrained by the photoperiod [59]. This protein acts in the thyrotroph cells, located in the pars tuberalis (PT) region of the anterior pituitary gland, which is considered the master regulator of seasonal biology in mammals [14].

Being modulated by CLOCK and BMAL1, EYA3 protein exhibits a circadian pattern in which its expression rises 12 h after the darkness onset [59]. Thus, in long day (LD) seasons, EYA3 peaks in light conditions and coactivates the transcription of the gene encoding the thyroid stimulating hormone beta (TSHβ) subunit in the thyrotroph cells of the PT. PT-derived TSHβ operates locally, enhancing the transcription of type II iodothyronine deiodinase (DIO2) in the tanycytes, which are specialized ependymal cells of the third ventricle and whose processes reach toward the hypothalamic parenchyma [60,61]. The higher DIO2 enzymatic activity induces the activation of thyroid hormones (TH), converting the inactive form thyroxine (T4) to the active triiodothyronine (T3), which stimulates other hypothalamic signalling that modulates the seasonal metabolic and reproductive adaptation to an LD state [62]. As an example, it has been evidenced that T3 regulates the expression of the hypothalamic RFamide-related peptides (RFRP) and kisspeptin (Kp) peptides, which are described to be involved in seasonal breeding through the regulation of the gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus, being able to synchronize reproduction with a specific season [63,64].

Differently, in short day (SD) seasons, EYA3 rises in dark conditions, being markedly reduced due to melatonin's repression of cAMP release. The consequent decrease of TSHβ and DIO2 activity, and the upregulation of the type III iodothyronine deiodinase (DIO3), which deiodinates the biologically active T3, cause a significant decline of T3 availability in the hypothalamus, inducing a different seasonal response to SD [62] **(Figure 4)**.

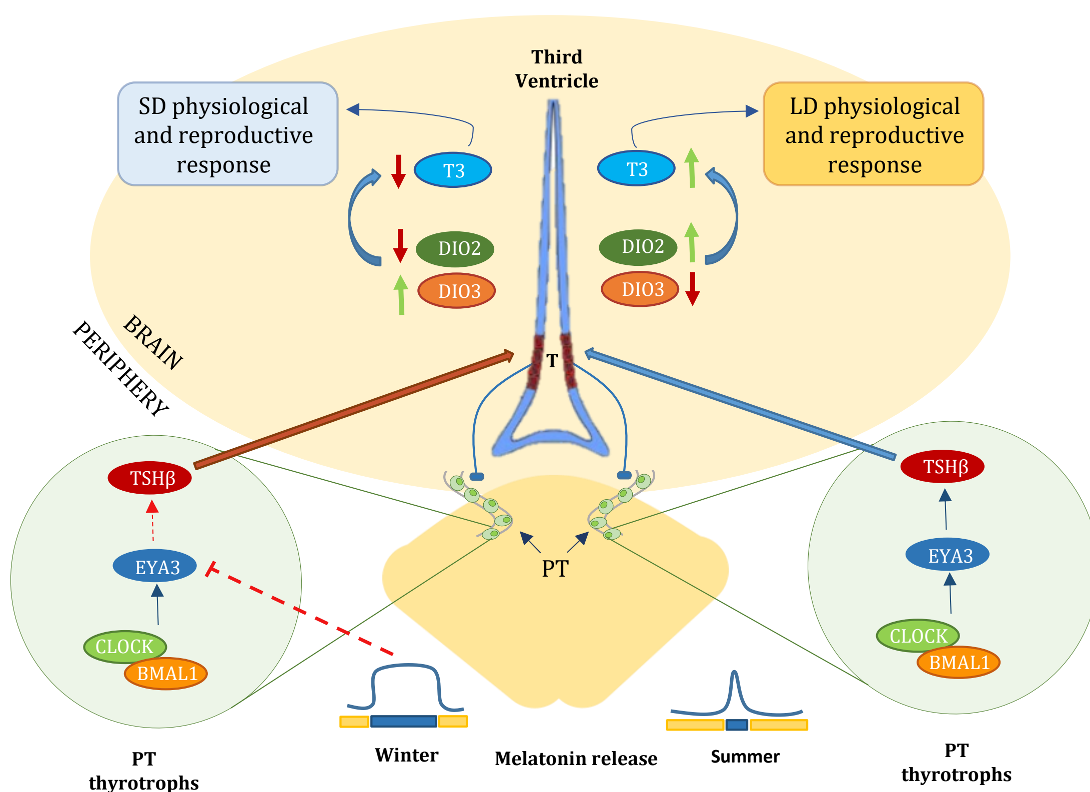

Figure 4. Molecular mechanisms involved in the physiological response to seasonal day length variations. Adapted from [62]. T, tanycytes.

In addition, it has recently been described that several proteins, genes and cells involved in these seasonal photoperiodic responses can be also associated with the endogenous circannual timing, which can persist under constant conditions [65,66]. Briefly, in a study performed in sheep, it was observed that PT thyrotrophs could act as calendar cells defining the phase of the circannual

rhythms by displaying a specific proportion of two different states: cells expressing TSHβ/EYA3 (LD state) or chromogranin A (CHGA) (SD state) [65]. The SD marker CHGA is a prohormone involved in the production of secretory granules in neuroendocrine tissues, which are associated with several inhibitory actions that could account for the quiescent state of PT during SD seasons [66]. Moreover, the same author reported a marked seasonal-dependent morphogenic cycle of these cells, in which animals exposed to the LD photoperiod exhibited a higher size of PT thyrotroph and a higher number of junctional contacts between these cells compared to those submitted under an SD one, suggesting an enriched cell-to-cell communication [65]. In this study, after 27 weeks of being exposed to an LD photoperiod, sheep's phenotype was reverted to an SD state without being exposed to any day length variation [65]. This phenomenon is known as photorefractoriness, and it is promoted by the endogenous circannual rhythms, which ensure the adaptation of the organism to the upcoming season, anticipating seasonal day length variations [80].

It is widely evidenced that the resulting switch of these seasonal responses stimulates a differential remodeling of physiological, reproductive and metabolic pathways, which characterize the adaptive process toward seasonal variations [62].

1.3.2. Seasonal physiological and metabolic variations in humans

Despite the fact that humans living in industrialized and modern societies are isolated from seasonal variations due to man-made alterations in the environment, such as the appearance of artificial light, heating and air conditioning systems [67], an extensive variety of events and biological processes with seasonal rhythms have been reported in numerous observational studies [68,69]. Body fat mass increases during winter in

latitudes far from the equator, in which higher changes in day length and temperature are recorded, as well as physical activity and energy expenditure levels, which are reported to be lower in winter than in summer [70,71]. In addition, the amount of injuries in some field sports are higher in autumn regardless of the state of the pitch, being the month of the year a relevant factor influencing injury risk [72].

In a study performed with geographically and ethnically diverse individuals, Dopico and collaborators found more than 5,000 genes related with the immune system with seasonal-dependent expression patterns in peripheral blood mononuclear cells (PBMCs) [73] **(Figure 5)**. In addition, this study reported a more inflammatory status in Europeans during the northern hemisphere winter, which was illustrated by higher circulating levels of the soluble receptor of interleukin 6 (IL6) and C-reactive protein (CRP), which are biomarkers for autoimmune, cardiovascular and psychiatric diseases [73].

This winter-like responsiveness has also been described for different metabolic parameters in several populations [74]. In this sense, a 12-months longitudinal study conducted with 517 healthy volunteers reported a marked seasonal variation in circulating lipid levels, in which men and women showed a peak of total cholesterol in December and January, respectively [75]. In another study, diabetic treated patients displayed a higher systolic and diastolic blood pressure, pre-prandial glucose and low-density lipoprotein (LDL) cholesterol in winter than in summer [76]. Similarly, seasonal-dependent differences have been reported in insulin secretion and/or in its glucose lowering effects, which were illustrated by a faster and bigger insulin response in autumn than in spring [77,78] **(Figure 5)**.

In another study carried out with 1,202 middle-age Japanese males, Kamezaki *et al.* described increased fasting circulating glucose levels and higher systolic and diastolic blood pressure in winter than in summer, reporting significant

seasonal variations in the prevalence of metabolic syndrome (MetS) [79], which is described as a group of interconnected risk factors —obesity, insulin resistance, hypertension and dyslipidemia— that induces a higher risk of cardiovascular disease (CVD) [80,81]. In a similar study, these authors also reported that the higher incidence of MetS in winter was linked with an increase in insulin resistance [82].

The importance of these seasonal metabolic changes on human's health is illustrated by the significant higher rates of cardiovascular mortality during winter in both northern and southern hemispheres [83]. Related with this, it was reported that 20,000 deaths per year were caused by the winter peak of coronary and cerebrovascular diseases in England and Wales [74].

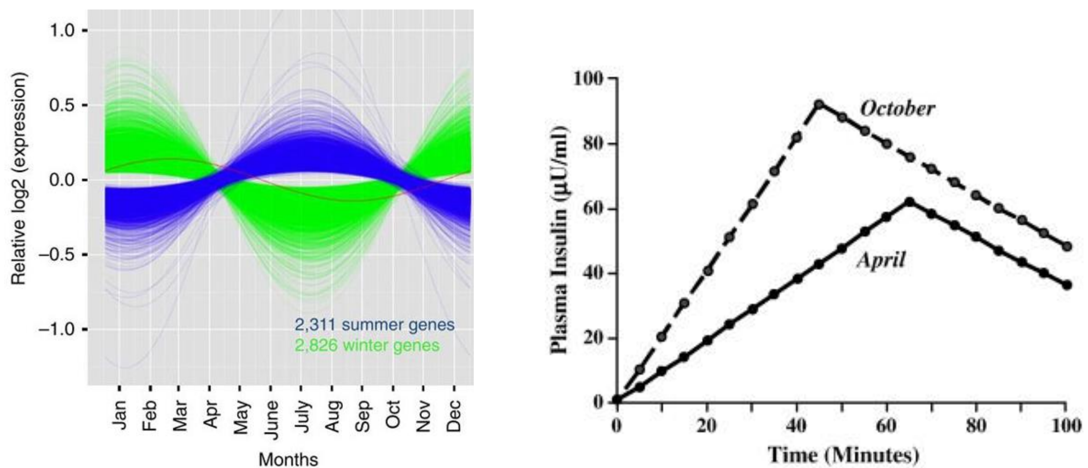

Figure 5. Seasonal variations in human's physiology and metabolism. On the left, Dopico and collaborators identified more than 5,000 immune system-related genes showing seasonal variations in children. Among these seasonal genes, two anti-phasic expression patterns were markedly differentiated: winter- (green) or summer- (blue) upregulated genes. On the right, the insulin response to an oral glucose load in healthy young men showed a marked seasonal variation, being faster and stronger in October than in April. Adapted from Dopico *et al.* [73] and Haus [77], respectively.

In addition, depressive episodes with seasonal patterns have been reported mostly in young adults and women, exhibiting a major decline during fall and

winter with a remission in spring and summer seasons [84]. Known as seasonal affective disorder (SAD), it is defined as a mood disorder in which people show depressive states symptoms (sadness, lack of concentration and decreased activity levels), hyperphagia, carbohydrate cravings and a greater weight gain and fat mass caused by overeating [84,85].

The aforementioned scientific evidences and the strong negative correlation found between CVD mortality and the hours of sunshine [86], strongly suggest that human health is placed more at risk in winter than in summer. Nevertheless, human variability (age, sex and lifestyle conditions) and external environmental factors other than the photoperiod that can be risk factors for disease, such as low temperatures and sedentarism, makes it difficult to stablish the relevance of seasonal variations in day length on health. In this sense, the use of animal models, which can be maintained under constant temperature and social input, have emerged as a useful strategy to shed more light on how seasonal changes in day length can impact on human's health.

1.3.3. Seasonal physiological and metabolic responses in mammalian animal models

The use of animal models has helped to elucidate the basics of the seasonal physiologic, behavioral, metabolic and reproductive responses to day length variations to a greater extent. Most of these studies have been focused in mammals and birds, since they exhibit a robust seasonal rhythmicity in several functions [3]. Among the different laboratory mammalian models, there have been differentiated two kind of groups, depending on their seasonal responsiveness: seasonal or non-seasonal animals.

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1.3.3.1. Seasonal mammalian animal models

Seasonal mammals exhibit circannual rhythms that allow them to temporarily distribute several energy-demanding processes in order to ensure reproduction and survival among different seasons [87]. In terms of reproductive responses toward seasonal variations, two different kinds of seasonal breeding mammals have been described: SD or LD breeders. SD breeders, which include goats, sheep and deer, mate in autumn and have a gestation period of six months. Differently, LD breeders, such as hamsters and voles, mate in spring and early summer and gestate only for a few weeks. In both SD and LD breeders, progeny is born in LD seasons due to the optimal conditions of temperature and food availability [88,89].

The LD breeder Siberian hamster (*Phodopus sungorus*), is considered one of the most optimal mammalian models in the study of seasonality in laboratory facilities, since they exhibit large-amplitude circannual cycles in several parameters in response to changes in the photoperiod, such as food intake, body weight, energy expenditure and reproduction [90]. As well as other seasonal species, Siberian hamster is characterized for exhibiting two clear phenotypes depending on seasonal day length: a stimulatory or inhibitory response to LD or SD photoperiods, respectively [89]. However, the critical day length that induces each response is species-dependent [91]. In winter-like SD photoperiods, these animals display decreased food intake, body weight (10-20%) and adiposity, as well as reduced size and function of their reproductive system, illustrated by gonadal regression and diminished spermatogenesis and testosterone levels [92]. In addition, it has been reported that after a prolonged exposure of ≈20–30 weeks to an SD photoperiod, these animals display a photorefractory response switching to a spring/summer-like LD phenotype and stimulating somatic and reproductive regrowth [93].

However, one of the main disadvantages to using these animal models is the scarce genetic information available in data repositories and limited molecular and biochemical tools to study them. For this reason, in recent decades, the inbred Fischer 344 (F344) rat strain, which has been reported to display a clear photo-responsiveness without any kind of manipulation [94], has emerged as an interesting model in the study of photoperiodism and circannual rhythms.

1.3.3.2. Fischer 344 rats: an alternative model in the study of seasonal responsiveness in physiology and health

Among all the studies that have been performed by using F344 rats, strong physiological and reproductive responses to photoperiod variations have been reported [94–98].

Similarly to what was observed in other seasonal mammalian animals, several studies have described two different phenotypes in response to the exposure to an SD or LD photoperiods in this rat strain. In this sense, Tavolaro and collaborators showed a decreased food intake, body weight gain, lean mass and testis size in 4-week-old F344 rats exposed to photoperiods of ≤10 h of light/day for 4 weeks, as well as a downregulation in the mRNA levels of *Dio2*, which is considered a LD photoperiod marker, as previously explained [98]. Furthermore, Ross *et al.* reported a strong photoperiod-dependent response in the hypothalamic gene expression profile in F344 rats. They observed that animals exposed to an SD photoperiod (10 h of light/day) for 28 days displayed a significant downregulation of hypothalamic *Tshβ* and *Dio2* mRNA levels, both considered LD markers, and an upregulation of *Dio3* gene expression. Moreover, as previously reported by other authors, SD animals exhibited decreased cumulative food intake and body weight after approximately 20 days of photoperiod exposure [99].

Relevantly, different studies have also observed a refractory response after the continuous exposure to a specific photoperiod in this rat strain, although the time required to develop this adaptive phenomenon is controversial. Shoemaker and collaborators found that after 18 weeks of treatment, F344 rats submitted to the SD photoperiod (8 h of light/day) adopted the characteristic LD phenotype regarding body weight, food intake and testis volume [96]. Differently, in another study carried out with the same rat strain and light schedules, animals exposed to the SD photoperiod exhibited a refractory response only after 8 weeks of day length exposure, increasing their testis volume and no longer differing from LD animals [94].

Recent studies have focused in the study of the susceptibility of this rat strain to obesogenic conditions. Studies in diet-induced obese rats fed highly caloric palatable diets, which reflect the variety of energy-rich foods in Western diets that are associated with the development of obesity and other metabolic alterations [100], have shown a clear interaction between diet and photoperiod [101,102]. For example, Togo *et al.* described that the exposure to an LD photoperiod (16 h of light/day) for 3 weeks increased the preference for high-carbohydrate, low-fat diet (LFD) more than for a high-fat diet (HFD), which was accompanied by higher total energy intake, body weight and epididymal adipose tissue than SD animals, which did not display any preference between both diets [101]. In another study, both normoweight and HFD-fed animals exhibited clear photoperiod-dependent responses regarding TH metabolism (increased hypothalamic *Tshβ* and *Dio2* and decreased *Dio3* mRNA levels in LD animals) and food intake regulation (increased gene expression of the orexigenic agouti-related peptide (*Agrp*) mRNA levels in the hypothalamus in LD animals). Nevertheless, these authors also reported that the combination of a HFD and the exposure to different day lengths resulted in the loss of LD photoperiod stimulation of fat mass, contrary to what was observed in normoweight rats [102].

1.3.3.3. Non-seasonal mammalian animal models

Except for F344, most laboratory mice and rats have been considered unsuitable for the study of seasonal responses, since they are not seasonal breeding animals [88]. However, several studies have shown a significant metabolic and affective response of these animals towards seasonal day length variations [103–106]. As an example, Otsuka and collaborators reported that 4-week-old C57BL/6J male mice exposed to an SD photoperiod (8 h of light/day) during 3 weeks displayed season-dependent changes in different behavioral and physiological parameters, such as increased depression-like behaviors and sucrose intake, which are the main symptoms of SAD [104]. In a similar study, Tashiro *et al.* described a decreased insulin sensitive-phenotype in those animals exposed to a short photoperiod, attributed to the downregulation of the glucose transporter 4 (GLUT4) protein and gene expression levels in the gastrocnemius muscle [106].

In addition, it has also been described a marked interaction between photoperiod exposure and obesity. As an example, Larkin *et al.* reported that genetically obese Zucker rats exhibited a more evident response to seasonal-like photoperiods than lean rats, as it was illustrated by the increased food intake, total and lean body mass and epididymal adipose tissue weight in animals exposed to an LD photoperiod (14 h of light/day) for 9 weeks in comparison with those exposed to a SD one (10 g of light/day), effects that were not observed in lean rats [107].

2. LIVER AND SKELETAL MUSCLE HOMEOSTASIS

Living organisms have adapted to persistent variations in nutritional conditions as an evolutionary mechanism toward food availability changes. These metabolic adaptations are possible due to a complex and interconnected network that involves physiological and molecular mechanisms, which control the mobilization of energy substrates through the different energy-demanding tissues and organs, such as the liver and the skeletal muscle. Hence, through a robust metabolic and hormonal modulation, each tissue displays specific molecular responses that are integrated with the whole-body in order to maintain a correct energy homeostasis.

2.1. The liver

The liver is an essential metabolic organ that plays a central role in energy homeostasis through the synthesis, storage and redistribution of carbohydrates, lipids and proteins, regulating the availability of metabolic substrates in the organism [108]. Having first access to most consumed nutrients because of their absorption into the hepatic portal vein, the liver is the most relevant organ in terms of postprandial metabolism due to its ability to transform these dietary nutrients into ready-to-use energetic substrates and provide them to energy-demanding tissues, such as the skeletal muscle, brain and heart [109]. This organ is mainly characterized by its metabolic flexibility to operate under the constant variations that occur in the feeding and fasting states in order to maintain the homeostatic balance in the whole-body metabolism [108–110].

In postprandial conditions, which are characterized by the rise of circulating insulin levels, glucose enters hepatocytes through the glucose transporter 2 (GLUT2) and is phosphorylated by glucokinase (GK) to produce glucose-6-

phosphate (G6P). Then, G6P can be transformed into glycogen in order to be stored in the liver (glycogenesis) or degraded to pyruvate and acetyl-CoA (glycolysis) so as to a) generate adenosine triphosphate (ATP) within the tricarboxylic acid (TCA) cycle, or b) synthesize fatty acid (FA) *de novo*, which, together with dietary FA, are stored as triacylglycerol (TAG) in the liver (lipogenesis) or exported to the adipose tissue as very low-density lipoproteins (VLDL) particles. In addition, dietary amino acids are metabolized as precursors of hepatic and other tissular protein synthesis, as well as for the synthesis of FA, hormones and nucleotides [111,112].

In contrast, in the fasting state, the release of glucagon from the pancreatic α cells together with the decreased circulating insulin levels, stimulates the synthesis of hepatic glucose and its release to the systemic circulation. Glucose production can derive from a) glycogen hydrolysis (glycogenolysis), which is associated with short-term fasting periods, and b) *de novo* synthesis of glucose (gluconeogenesis (GNG)) in long-term fasting periods in which glycogen stores are exhausted. The main precursors in the GNG process are the muscular lactate, TAG-derived glycerol, alanine and other glucogenic amino acids derived from protein degradation. In addition, hepatocytes degrade TAG to FA (lipolysis), which through its β-oxidation produce energy and ketone bodies (ketogenesis), such as acetoacetate and β-hydroxybutyrate, which are sent to other extrahepatic tissues as metabolic fuel [108,111–114].

2.2. The skeletal muscle

The skeletal muscle is the single largest organ of the body, comprising up to the 50% of total body mass and containing 50-75% of all body proteins. Differently from other muscle types, the skeletal muscle contributes to generate the voluntary movement by transforming chemical to mechanical energy. This function is possible due to a complex display of muscle cells, known as

myocytes or fibers, and connective tissue. These skeletal muscle cells are very heterogeneous in structure and role among the different kinds of skeletal muscle. There have been described three types of fibers: type I or red fibers, which are characterized by a slow contraction, high fatigue-resistance and high mitochondrial content, being the most oxidative muscle cells (predominant in the soleus muscle); type IIa or intermediate fibers, which structure have the characteristics of both I and IIb fibers; and type IIb or white fibers, characterized by a fast contraction, low fatigue-resistance and low mitochondrial content (predominant in the gastrocnemius muscle) [115,116].

Besides being specialized in ATP production as energy source, these cells also contribute to the whole-body metabolic homeostasis, regulating the protein synthesis and degradation balance and storing relevant substrates, such as carbohydrates, depending on the nutritional status or physical activity level, among other factors. Similar to the liver, in the postprandial state, glucose enters the myocytes through GLUT4 and is phosphorylated by hexokinase (HK) to produce G6P, which can generate ATP through the glycolytic pathway or polymerize in order to be stored as glycogen. These anabolic pathways are also observed in protein and TAG synthesis from circulating and dietary amino acids and FA [117,118]. However, in energy-demanding conditions due to fasting or physical activity, TAG and glycogen stores are degraded, as well as proteins, and the resulting substrates are used as energy sources in the skeletal muscle or sent to the liver as GNG precursors [111,115].

2.3. Metabolic integration at a molecular level

The metabolic integration of organs and tissues that ensures a robust metabolic homeostasis and their flexibility to adapt to persistent changes in the nutritional status are mediated by the activation and inhibition of several

intracellular signaling pathways that involve genes and proteins with key metabolic roles.

In the regulation of glucose, lipid and amino acid metabolism and, consequently, in the maintenance of the homeostatic robustness in metabolic tissues, there are two proteins that play a key role: Akt serine threonine kinase (Akt) and AMPK.

2.3.1. Akt: the downstream post-receptor target of insulin

As it has been aforementioned, insulin secretion from the pancreatic β-cells in the postprandial state induces several cellular processes in different insulin-sensitive organs and tissues in order to maintain glucose and other metabolites homeostasis. To accomplish this goal, this hormone requires a multifaceted network of metabolic pathways in order to transduce its signal into cells in different context-dependent responses [119]. Akt, also known as protein kinase B (PKB), has been described as a key intermediate in the insulin signalling pathway [120]. It includes three mammalian isoforms with a similar structure but different function and tissue-specific expression: Akt1/PKBα, ubiquitously expressed and highly related with cell survival; Akt2/PKBβ, mainly expressed in insulin-responsive tissues such as liver, skeletal muscle and adipose tissue, involved in the regulation of energy homeostasis; and finally Akt3/PKBγ, expressed in the brain and associated with postnatal brain development [121].

Akt is activated by the phosphorylation cascade stimulated by insulin's union with its receptor and also by the G protein-coupled receptor (GPCR), as illustrated in **Figure 6**. Once activated, Akt phosphorylates protein targets on serine and threonine residues, triggering the activation or inhibition of a wide range of downstream substrates related with several metabolic functions.

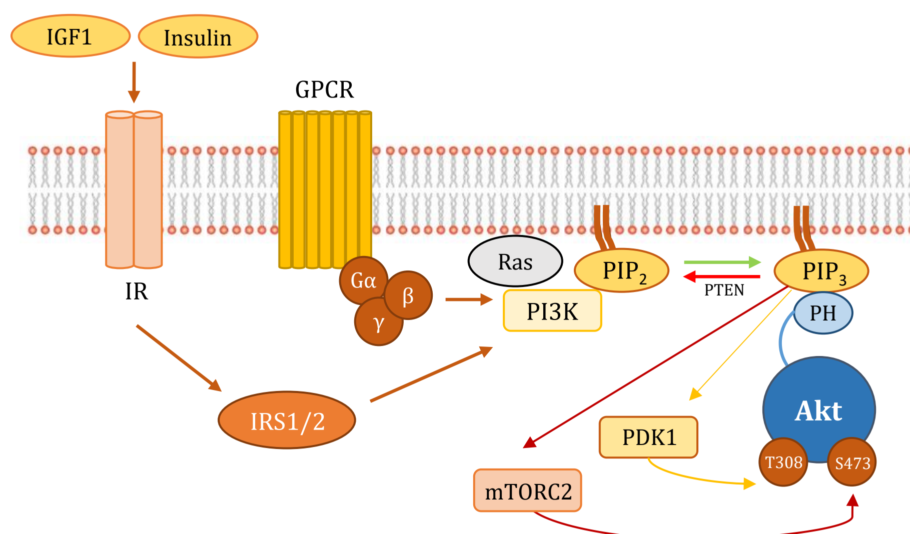

Figure 6. Molecular mechanisms involved in Akt activation. Insulin or IGF1 binds the insulin receptor (IR) and activates the IR tyrosine kinase (IRTK), which induces the recruitment and Tyr phosphorylation of the IR substrate 1/2 (IRS1/2) protein. This protein interacts with phosphatidylinositol 3-kinase (PI3K), activating it and inducing the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) from phosphatidylinositol-4,5-bisphosphate, (PIP_2) by the action of the phosphatase and tensin homolog (PTEN). PIP_3 serves as a docking site for several PH domains engaging recruited Akt Ser and Thr kinases. Akt is fully activated by the phosphorylation of T308 and S473 residues by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), respectively, which are also activated by PIP_3. Adapted from [119].

Glucose metabolism

Akt1 has been found to act in the pancreatic insulin-secreting β-cells, increasing the expansion of pancreatic islets and insulin production through the activation of the mammalian target of rapamycin complex 1 (mTORC1), a protein considered the master regulation of cell growth and metabolism [121].

In the liver, insulin-mediated Akt2 activation exerts a markedly suppressive effect on glucose output through two different mechanisms. Firstly, Akt2 inhibits the GNG process by phosphorylating the forkhead box protein O1 (FOXO1), which is a key transcription activator of gluconeogenic key enzymes,

such as phosphoenol pyruvate carboxykinase (PCK) and glucose 6-phosphatase catalytic subunit (G6Pase). By being phosphorylated in three conserved residues, FoxO1 is excluded from the nucleus and sequestered in the cytosol, repressing its activity [122,123]. Secondly, Akt2 can inhibit glucose production by redirecting the synthesized G6P to glycogen. Nevertheless, the mechanism involved in Akt2-mediated hepatic glycogen synthesis has not been fully elucidated, since it has been described to be independent of the essential glycogen synthase kinase 3 (GSK3) phosphorylation and inhibition observed in the skeletal muscle [124].

In the adipose tissue, and especially, in the skeletal muscle, Akt2 promotes insulin-stimulated glucose uptake by increasing the GLUT4 content in the plasmatic membrane. Specifically, it has been described that Akt2 phosphorylates and inhibits two RabGTPases (Akt substrate of 160 kDa (AS160) and TBC1 domain family member 4 (TBC1D1)) present in GLUT4 storage vesicle (GSV), promoting its redirection and translocation to the plasma membrane in these tissues [125]. Furthermore, Akt2 also directs G6P toward glycogen synthesis, phosphorylating and inhibiting the GSK3 and allowing the action of the glycogen synthase [126].

Lipid metabolism

Akt2 is involved in insulin-mediated lipogenesis *de novo* in the liver, skeletal muscle and adipose tissue. In hepatocytes, Akt2 activates mTORC1 by phosphorylating and inhibiting the tuberous sclerosis complex 2 (TSC2), which results in the activation of the lipogenic sterol regulatory element-binding protein 1 (SREBP1). Subsequently, this protein is translocated to the Golgi complex and proteolyzed, activating several lipogenic enzymes, such as fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACC) and glycerol-3-phosphate acyltransferase (GPAT), involved in the synthesis of FA and TAG [127]. In muscle and adipose tissue, SREBP1 is also induced by the activation of

PI3K/Akt, but the role of muscular mTORC1 in this process remains unknown [128,129]. In addition, mTORC1 has been described to suppress the lipolysis in the adipose tissue, mainly affecting the activation of the lipolytic adipose triglycerides lipase (ATGL) and hormone-sensitive lipase (HSL) [130].

In the skeletal muscle, Akt2 is critically associated with insulin-mediated FA uptake, regulating FA transport through promoting the translocation of the FA translocase, homolog of CD36 (CD36) and the FA transporter 1 (FATP1). Regarding the molecular mechanisms involved in this process, it has been suggested that the Akt2 downstream target AS160 could also account for the internalization of CD36 [131].

Protein metabolism

Akt2 also plays a key role in the insulin-like growth factor 1 (IGF1) and insulin regulation of the development of skeletal muscle growth, by controlling the muscular protein turnover [132]. mTORC1 activation by Akt2, stimulates protein synthesis by activating p70S6 kinase (p70S6K) and inhibiting eIF4E-binding protein (4E-BP), which possess downstream targets involved in the enhancement of protein translation and elongation [133]. Moreover, Akt2 inhibition of FoxO1, downregulates the expression of the E3 ubiquitin ligases muscle atrophy Fbox (MAFbx) and muscle ring finger 1 (MuRF1), both involved in protein degradation [133].

The actions of Akt on glucose, lipid and protein metabolism are summarized in ***Figure 7***.

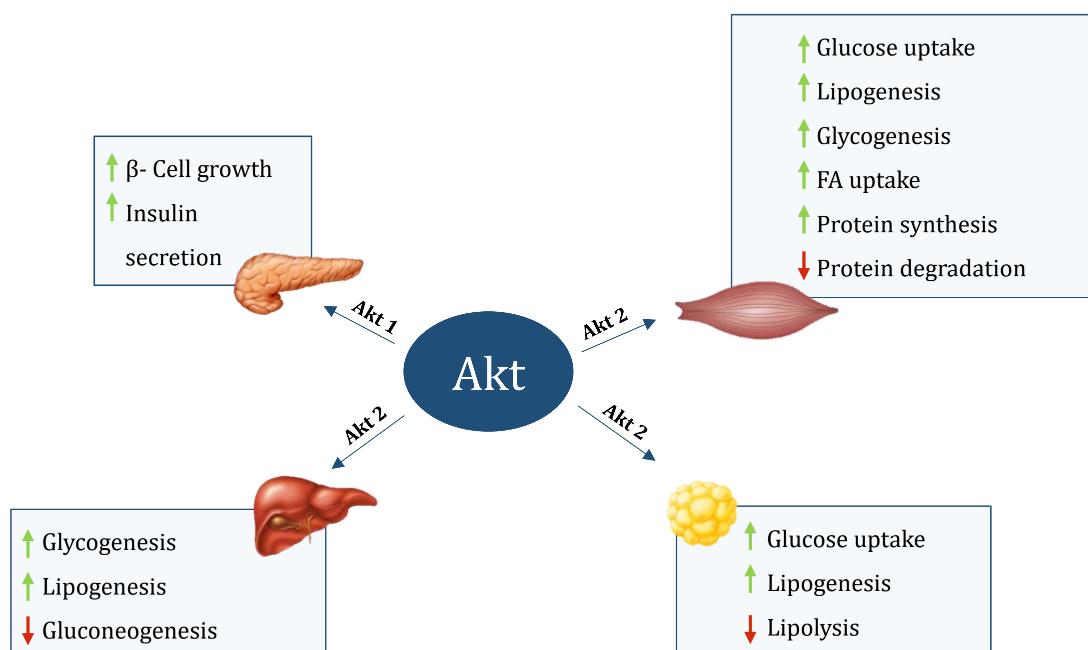

Figure 7. Summary of the metabolic actions of Akt in different organs and tissues.

2.3.2. AMPK: the major regulator of energy homeostasis

AMPK is a sensor of the cellular energy status that modulates several metabolic pathways to balance energy supply and demand, being considered the major regulator of energy homeostasis in the organism. This protein is allosterically activated by the increase of intracellular adenosine monophosphate (AMP) levels, which are associated with energy demanding conditions (e.g. fasting or physical activity). Hence, AMPK activates catabolic pathways that produces ATP and inhibits ATP-consuming anabolic pathways, such as cell proliferation or biosynthetic processes [134].

This protein is a heterotrimeric Ser/Thr kinase constituted by three different subunits (α, β, γ) with specific functions. The α is the catalytic subunit containing the Thr^{172} residue, whose phosphorylation fully activates AMPK. The β subunit contains a carbohydrate-binding module, allowing glycogen and other oligosaccharides to interact and inhibit AMPK. Finally, the γ subunit comprises 4 tandem repeats known as cystathionine β-synthase (CBS), which

create binding sites for two adenosine molecules including AMP, adenosine diphosphate (ADP) or ATP in an exchangeable fashion, a third one that permanently binds AMP and a fourth one that remains empty. As aforementioned, although AMP binding to this subunit promotes a 2 to 5 fold increase in AMPK activity (nucleotide-dependent regulation), this protein is completely activated when phosphorylated. It has been reported that the two main kinases involved in AMPK phosphorylation are the liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin-dependent protein kinase kinase beta (CAMKKβ), both acting independently of variations in AMP [135,136].

Being activated by energy suppression and inhibited by an over-nutrition, AMPK regulates energy balance in a wide range of organs and tissues by targeting crucial metabolic substrates, ranging from key enzymes to transcriptional regulators.

Glucose metabolism

AMPK effects on glucose metabolism are focused in the production of cellular ATP through stimulating glucose degradation and suppressing its synthesis and storage [137,138].

This protein promotes glucose uptake in the skeletal muscle by stimulating GLUT4 translocation to the plasma membrane. Although this process is produced by phosphorylating the same target proteins described in Akt action, AMPK acts through an insulin-independent pathway [139]. Moreover, it has been reported that AMPK can stimulate the glycolytic process by activating phosphofructokinase 1 (PFK1), the rate-limiting enzyme of glycolysis in cardiomyocytes and macrophages [138].

In order to reduce energy-wasting processes, AMPK inhibits glycogen synthesis by phosphorylating hepatic and muscular GYS isoforms and stimulates its degradation, by activating glycogen phosphorylase (GP) [140]. In addition, this

protein suppresses hepatic GNG by phosphorylating and inhibiting several co-activators of the cAMP response element binding protein (CREB) and FOXO1, the two main regulators of the gluconeogenic process [141].

Lipid metabolism

AMPK exerts a marked inhibitory effect on *de novo* synthesis of FA, promoting the phosphorylation and inhibition of two different targets. Firstly, it suppresses ACC1 and ACC2, which catalyze the reaction that converts acetyl-CoA to malonyl-CoA, which is the rate-limiting step of FA synthesis; and secondly, it also inhibits SREBP1c, a transcription factor that stimulates the expression of ACC1 and FASN in the liver and adipose tissue [142,143]. In addition, it has been described that AMPK can also inhibit hepatic synthesis of TAG and cholesterol, by directly suppressing the rate-limiting enzymes GPAT and HMG-CoA reductase (HMGCR), respectively [144,145]. Although the role of AMPK in lipogenesis is clear, its regulation of the lipolytic process is controversial. While it has been described an anti-lipolytic effect mediated by AMPK in the adipose tissue in mice [146], recent studies performed in similar animal models have evidenced a clear activation of the lipolytic ATGL and HSL by this protein [147].

AMPK also increases FA uptake by triggering the translocation of the FA transporters CD36 and plasma membrane fatty acid binding protein (FABPm) in the skeletal muscle [148]. Moreover, AMPK inhibition of ACC2 consecutively induce the activation of β-oxidation of FA. This simultaneous action is produced by the activation of carnitine palmitoyltransferase 1 (CPT1), which is suppressed by malonyl-CoA at the mitochondria outer membrane [145]. CPT1 is a key enzyme in the carnitine-dependent transport from the cytosol to the mitochondrial matrix, allowing the initiation of the FA oxidation process, which involves other key enzymes such as citrate synthase (CS) and hydroxyacyl-CoA dehydrogenase (HAD) [111].

Protein metabolism

AMPK inhibits protein synthesis by modulating mTORC1 through two different mechanisms. It can phosphorylate and activate TSC2, which negatively regulates mTORC1, or inhibit a subunit of this protein known as regulatory-associated protein of mTOR (Raptor) [135]. Moreover, AMPK can also inhibit the elongation process, by activating the eukaryotic elongation factor 2 kinase (eEF2K), which is also a downstream of mTOR protein [149]. Indeed, it has been evidenced that AMPK can be negatively regulated by Akt, being involved in Akt-mediated activation of protein synthesis through the activation of mTOR [150]. As one of the main modulators of skeletal muscle turnover, AMPK stimulates protein degradation by phosphorylating FOXO3a, which increases the expression of the E3 ligases MAFbx and MuRF1 [151].

The actions of AMPK on glucose, lipid and protein metabolism are summarized in ***Figure 8***.

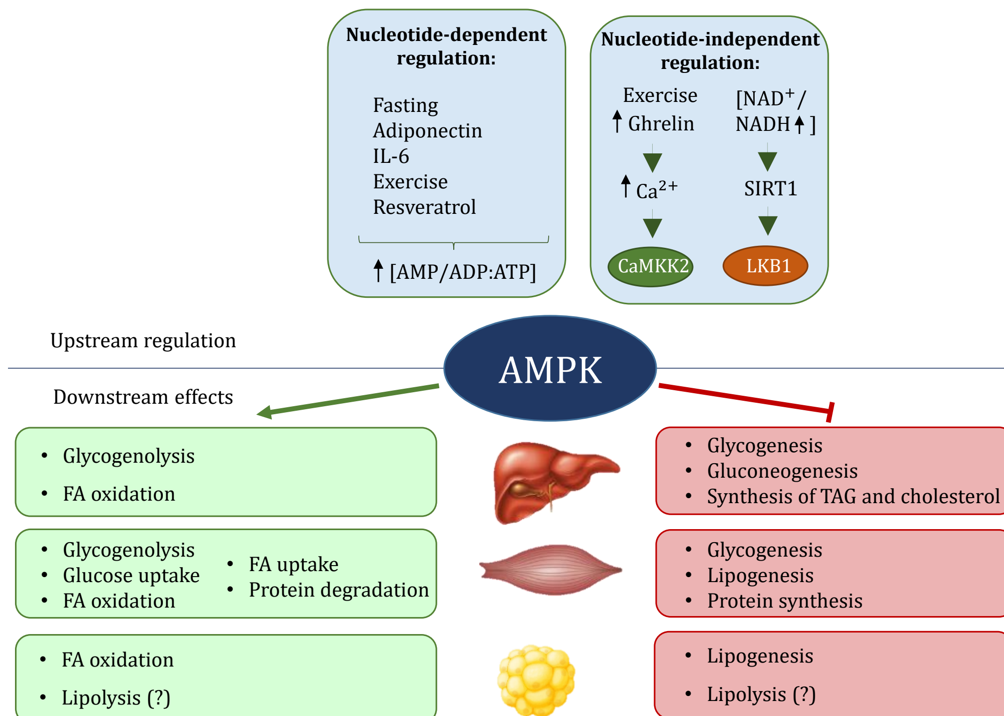

Figure 8. Summary of the metabolic actions of AMPK in different organs and tissues.

2.4. Metabolic disruption in obesity

The maintenance of all these interconnected mechanisms in order to ensure a homeostatic balance is extremely relevant on health, since its disruption can induce the development of several medical conditions, such as obesity, and metabolic diseases, such as dyslipidemia and insulin resistance. Indeed, obesity is currently considered the global epidemic of the twenty-first century, affecting more than 650 million people worldwide [152]. This increasing prevalence has also raised the appearance of several comorbidities, such as insulin resistance and dyslipidemia, contributing to a higher risk for CVD, the first cause of death globally [153]. It has been widely evidenced that the main triggering factor of these metabolic dysfunction is the overconsumption of high-energy foods, which deregulates the mechanisms involved in the mobilization of energy substrates [154].

There have been described three principal disturbances promoted by diet-induced obesity: fat accumulation in different tissues, a systemic low-grade chronic inflammation and the development of insulin resistance [155–157].

Briefly, under a constant caloric supply due to over-nutrition, the adipose tissue capacity to store this energy excess in the adipocytes through the adipogenesis process becomes unsustainable. Consequently, this tissue promotes a pro-inflammatory response through the release of adipokines, such as tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP1) and IL6 [158]. Several adipokines have been described to activate the lipolysis in the adipose tissue, triggering the release of FA to systemic circulation and other metabolic tissues that are not specialized in lipid storage, such as liver and skeletal muscle [159]. In both tissues, the accumulation of FA, diacylglycerol (DAG) and ceramides also prompts a pro-inflammatory response mediated by several serine kinases such as c-Jun N-terminal kinase 1 (JNK1), IκBα kinase beta (IKKβ) and protein kinase C (PKC), which impairs insulin-stimulated

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tyrosine phosphorylation of insulin receptor substrate 1 (IRS1) by phosphorylating the Ser residue and consequently, inhibiting IRS1 and its associated phosphatidylinositol 3-kinase (PI3K) activity [160,161]. Moreover, these adipokines induce the translocation of the nuclear factor kappa B (NF-κB) into the nucleus, which triggers a local pro-inflammatory response by stimulating the synthesis of inflammatory cytokines [162].

Insulin resistance is associated with an inhibition of insulin-mediated Akt actions, which results in the activation of the hepatic GNG process stimulated by FOXO1, the inhibition of glycogen synthesis in the liver and skeletal muscle, and the decrease of glucose uptake by the inhibition of muscular GLUT4 translocation [157,163,164]. However, in this pathologic state, insulin sensitivity is maintained in the upregulation of hepatic SREBP1c, the enhancer of the lipogenic process, stimulating TAG synthesis and its release to circulation as VLDL. These particles are exported to other tissues, such as skeletal muscle, triggering its accumulation and expanding its associated detrimental effects [165,166]. Hence, this selective insulin resistance leads to the characteristic hyperglycemia and hypertriglyceridemia reported in this pathology [166].

Relevantly, a significant decrease in AMPK activity and consequently, in the phosphorylation of several targets of this protein, such as ACC and GLUT4, have been reported in the skeletal muscle in HFD-fed rats [167]. Similarly, in humans, it has been described a clear AMPK dysfunction in insulin resistant obese patients, reporting lower AMPK activity levels in the skeletal muscle and adipose tissue [168–170]. Taking into account the key role of this protein in the regulation of the metabolic processes that are disrupted in obesity and its related disorders, AMPK has been considered a clear therapeutic target in the treatment of these conditions, as evidenced by the beneficial actions of AICAR, metformin and berberine, among other AMPK activators, in insulin resistance amelioration [171,172].

The molecular mechanisms involved in obesity-associated insulin resistance are illustrated in ***Figure 9*** [163]**.**

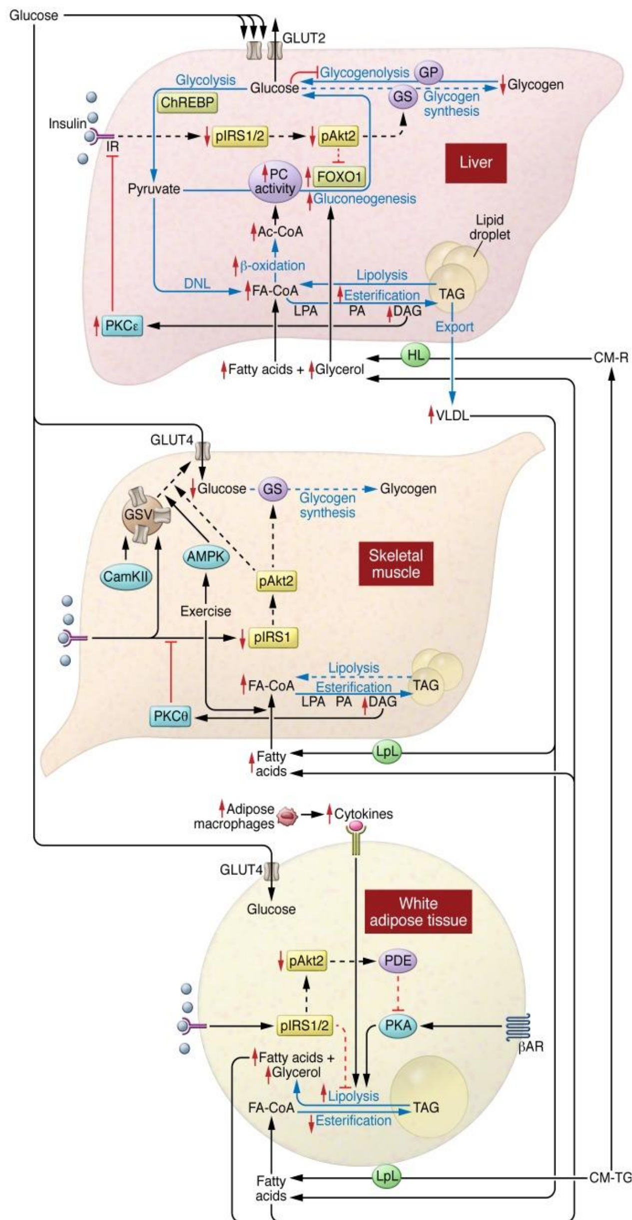

Figure 9. Signaling pathways involved in insulin resistance. Pro-inflammatory cytokines stimulate WAT lipolysis, increasing FA release, which are accumulated in the liver and skeletal muscle. The increased DAG levels stimulate the activation of PKC, which inhibits the IRS1-2/PI3K/Akt2 pathway in both liver and skeletal muscle. Consequently, insulin resistance induces a) the activation of hepatic GNG; b) the suppression of muscular GLUT4 translocation; c) the inhibition of glycogen synthesis in liver and skeletal muscle and d) the activation of lipogenesis in both tissues [163]. βAR, β-adrenergic receptor; ChERBP, CH-responsive element-binding protein; CM-R, chylomicron remnants; CM-TG, chylomicron-triglycerides; FA-CoA, fatty-acyl-CoA synthase; HL, hepatic lipase; LPA, lysophosphatidic acid; LPL, lipoprotein lipase; PA, phosphatidic acid; PC, pyruvate carboxylase; PDE, phosphodiesterase; PKA, protein kinase A.

3. PHYTOCHEMICALS: SECONDARY METABOLITES IN PLANTS

Through evolutionary history, the limited mobility of plants to escape and avoid different stresses have forced them to develop several mechanisms in order to manage with the persistent external challenges in the environment, such as climate (humidity, cold, heat and day length), biotic factors (pathogens and herbivores), harvesting conditions (pesticides), phenologic factors (maturation) and nutrient availability. Therefore, in order to enhance their chances of survival, plants have evolved secondary metabolic pathways that involve the synthesis of a wide range of bioactive non-nutritive compounds, known as phytochemicals, with different structures and functions [173]. The chemodiversity of these secondary metabolites evidences the remarkable adaptability of plants in response to several environmental stimuli that put their growth in danger, such as nutrient scarcity, extreme temperatures and pathogen or herbivore attacks [174,175].

3.1. The role of phytochemicals in plants

There are four main categories of secondary metabolites classified by their chemical structure, which have been involved in specific functions in response to different environmental cues. These compounds are: terpenes (e.g. carotenoids, sterols, glycosides and plant volatiles), phenolic compounds (e.g. flavonoids and non-flavonoids), nitrogen-containing compounds (e.g. alkaloids) and sulfur-containing compounds (e.g. glucosinolates) **(Figure 10)**.

Terpenes

Terpenes comprise the largest class of phytochemicals, containing more than 30,000 lipid-soluble compounds. They are classified according to the number of

5-carbon isoprene units that they contain and are characterized by several properties associated with plant's survival [176]. It has been reported that terpenes are synthesized in response to pathogen or herbivore attacks due to their antimicrobial and antibiotic activities and also to their defensive role, acting as toxins and feeding deterrents [177]. In addition, volatile monoterpenes can also favor plants' growth and development by attracting living organisms for pollination and seed dispersal, as well as carotenoids, which confer the colors yellow, orange and red to several fruits and vegetables [173,178].

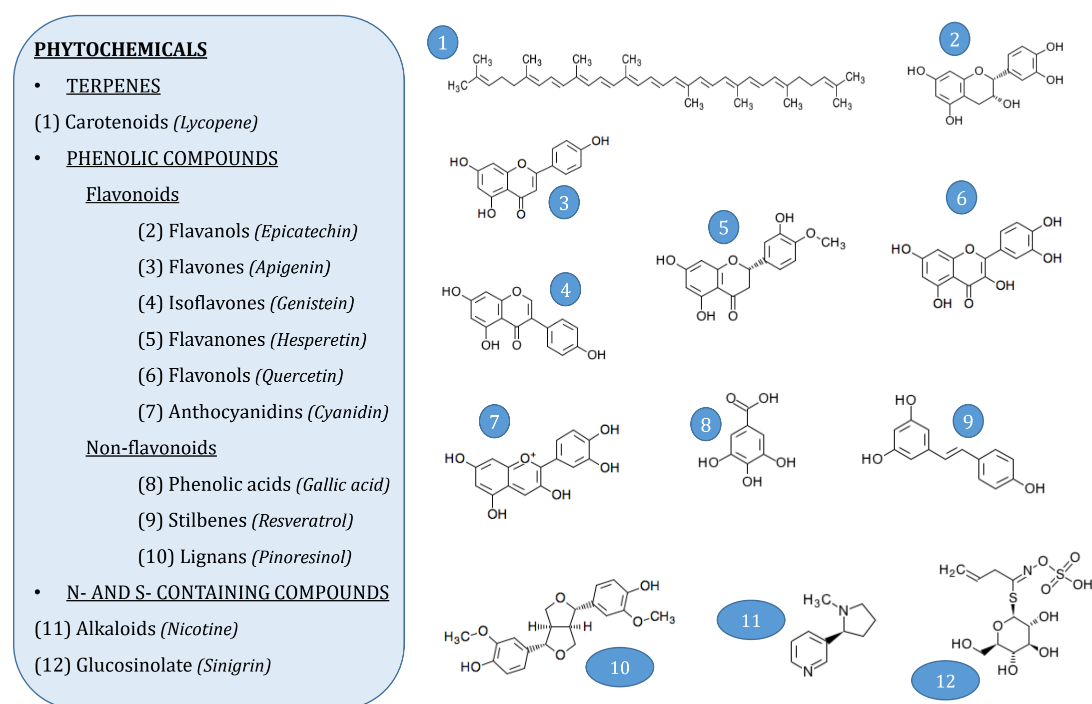

Figure 10. Classification and structural diversity of phytochemicals

Phenolic compounds

Phenolic compounds, also known as polyphenols, are characterized by having one or more aromatic rings with hydroxyl groups attached, existing more than 8,000 different structures [179]. These compounds are categorized principally into flavonoids and non-flavonoids. Flavonoids are mainly comprised by

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flavanols, flavones, isoflavones, flavanones, flavonols and anthocyanidins, while non-flavonoids contain phenolic acids, stilbenes and lignans [180]. Polyphenols play a crucial role in several functions in the plant, such as growth, reproduction and metabolism, protective mechanisms against infections and predators and also contribute to the color of plants [181]. As an example, tannins, which belong to the phenolic acids class, act as a feeding repellent for herbivores by combining with salivary proteins and producing an astringent feeling [182]. Quercetin and kaempferol, two kinds of flavonols, have been described to be crucial in pollen tube formation and in stimulating pollen germination frequency [183]. Moreover, anthocyanins, which are flavonoids found as sugar conjugates in several fruits and flowers, are colored water-soluble pigments that confer the red, purple and blue colors to fruits and vegetables, attracting pollinators and protecting them against excessive UV radiation [178,184].

Indeed, the biosynthesis of these flavonoids is markedly influenced by several annual geophysical changes, such as temperature, light quality and day length, highly differing between latitudes [185,186]. As an example, Jaakola *et al.* reported a clear effect of solar radiation on flavonoid content, illustrated by a sharp increase of hydroxycinnamic acid, anthocyanins, catechins and quercetin in the upper leaves of the bilberry fruit, which are directly exposed to sunlight, in comparison with its shaded leaves and fruits [187]. The vital role of these group of polyphenols in the protection against increased light exposure was also reported by Carvalho *et al.,* who observed that after exposing sweet potato leaves to a LD photoperiod (16 h of light/day), these plants exhibited a sharp upregulation of flavonoid synthesis-related genes and consequently, significantly higher anthocyanin, flavonols, catechins, hydroxybenzoic acid and hydroxycinnamic acid levels than those exposed to a SD photoperiod (8 h of light/day) [188]. Similarly, in a study performed with *Pinus contorta* seedlings, the exposure to a LD photoperiod (14-15.5 h of light/day) induced a vast

increase of anthocyanin levels (mainly cyanidin-3-glucoside) compared to those exposed to a shorter photoperiod [189].

Nitrogen- and Sulphur-containing compounds

Alkaloids, such as caffeine and nicotine, are the most described nitrogen-containing compounds. They are a structurally diverse group over 12,000 compounds mostly derived from non-protein amino acids, such as tyrosine, lysine, tryptophan and aspartic acid, and present in about 20% of plant species [190]. These compounds are characterized by exhibiting a strong defensive role in plants, acting as feeding deterrents and toxins to insects and animals [191].

Glucosinolates are nitrogen- and sulfur- containing compounds that structurally differ in a variable side chain, which is derived from an amino acid [190]. It has been described that these compounds are highly sensitive to several biotic and abiotic stresses, displaying sharp variations in their concentration in response to extreme temperatures, drought, nutritional deficiencies and insects attacks [192]. Indeed, there is a complex and evolutionary system that converts glucosinolates into toxic compounds (e.g. nitriles and isothiocyanates) only in case of plant damage, acting as repellents [193].

3.2. The role of phytochemicals in mammals

It has been largely described that the extensive variety of secondary metabolites found in plant-derived foods, such as fruits and vegetables, clearly define in which conditions they were harvested. Relevantly, these group of compounds have been studied and commercialized due to their bioactive properties in humans and mammals, being implicated in the prevention and treatment of several diseases, such as cancer [194], CVD [195] and obesity and its related metabolic complications [196,197]. Nevertheless, in evolutionary

terms, how these non-nutritive and exogenous compounds can be able to interact with endogenous biochemical pathways in heterotrophs have always been a mystery.

3.2.1. The xenohormesis theory

In 2003, Howitz *et al.* described that a group of small molecules extracted from plant-derived foods (resveratrol, butein and fisetin) induced a significant increase in *Saccharomyces cerevisiae* average lifespan. Focusing on resveratrol, a phenolic compound found in red wine, these authors reported that by activating SIRT1, these polyphenols inhibit the activity of the p53 tumor suppressor, promoting cell survival and suppressing apoptosis to allow cells to repair DNA damages [198]. Hence, after observing that phytochemicals produced by environmental stressed plants can interact with key proteins and promote vital processes involved in survival mechanisms not only in *S. cerevisiae*, but also in *C. elegans* and *Drosophila melanogaster*, these authors created the concept of xenohormesis [199]. The xenohormesis theory (from the Greek *xenos*, strange and *hormesis*, which defines that mild stress levels confers beneficial effects to the organism) postulates that the coevolution between plants and animals have promoted the development of a regulatory system of unpredictable variations in the environment, allowing the heterotrophs to recognize as signals the phytochemicals synthesized by plants [199]. Taking into account that each plant contains a distinctive phytochemical composition highly dependent on the environmental conditions, animals could use these chemical cues in order to be informed about the environmental status and consequently, favorably adapt their physiology and metabolism in order to increase their chances of survival [199–201]. Hence, these authors suggested that most of the beneficial effects of these compounds that are reported on health would not be associated with their antioxidant activity or responses to mild cellular damage, but rather with the direct interaction with several key

mammalian metabolic components, such as transcriptional factors, enzymes and receptors, which modulate the crucial functions in the organism [202–204].

3.2.2. How do phytochemicals interact with mammals?

Despite the wide range of phytochemicals present in the plant kingdom, most of the studies have mainly focused in the relevance of phenolic compounds on health. These bioactive compounds have been reported to exert several beneficial effects on mammals' health by interacting with key intermediates of vital processes, such as stress responses, inflammatory responses and energy utilization [205] **(Figure 11)**.

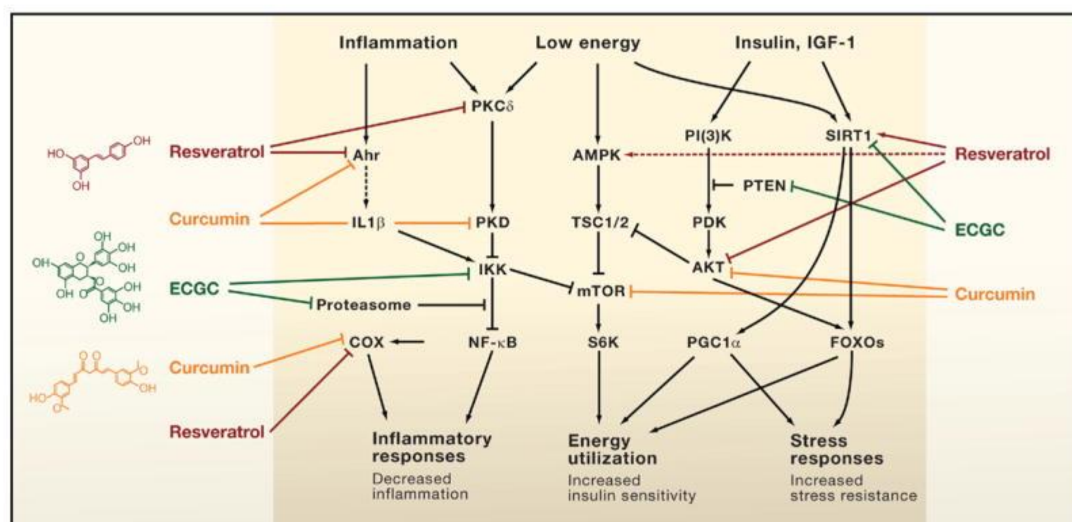

Figure 11. Modulation of key mammalian enzymes by phytochemicals present in plant-derived foods [199]**.** ECGC, epigallocatechin gallate; Ahr, aryl hydrocarbon receptor; IL1β, interleukin 1 beta; COX, cyclooxygenase; PKD, protein kinase D; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

Several epidemiological studies have associated the dietary intake of polyphenols with a lower risk of CVD, since they exert beneficial effects in the prevention and management of several CVD risk factors, such as insulin resistance, dyslipidemia, obesity and its associated inflammatory state, hypertension and atherosclerosis [206,207].

As an example, the consumption of several polyphenol-rich foods, such as coffee and green tea, which are rich in chlorogenic acids and catechins, respectively, and anthocyanin-rich fruits, such as berries, apples and pears, have been clearly associated with a lower risk of type 2 diabetes [208–211]. Regarding the mechanisms by which polyphenols could induce these beneficial effects, there have been identified several polyphenol targets in different tissue-specific functions, ranging from intestinal absorption (inhibiting disaccharidases in order to reduce the absorption of simple sugars [212]) to hepatic and muscular glucose metabolism management (enhancing muscular glucose uptake through GLUT4 upregulation [213] and inhibiting hepatic GNG via AMPK activation [214]).

In addition, it has been reported that flavonoids found in olive oil possess clear anti-inflammatory and antiatherogenic properties by improving high-density lipoprotein (HDL) function in reverse cholesterol transport and anti-inflammatory processes and decreasing LDL circulating levels [215]. Moreover, several types of polyphenols, such as procyanidins, curcumin, resveratrol and quercetin, have been reported to directly interact with several intermediates of inflammatory pathways *in vivo* and *in vitro* [204]. For example, resveratrol can induce anti-inflammatory effects by inhibiting TNF-α-induced MCP1 secretion and gene transcription in adipocytes [216]. Furthermore, the anthocyanin cyanidin-3-O-β-glucoside, found in many kinds of berries, has been described to decrease macrophage infiltration in mice through the downregulation of the receptor of MCP1 in peripheral blood monocytes [217].

The Nutrigenomics Research Group has been extensively focused in the study of the biological effects of proanthocyanidins (PA), a group of polyphenolic compounds synthesized in response to fungal infections, reporting antioxidant, anti-inflammatory, hypotensive and hypolipidemic properties [218–220]. Particularly, it has been reported that a grape seed proanthocyanidin extract

(GSPE) can exert a marked protective effect against diet-induced dyslipidemia, attenuating triglycerides and LDL cholesterol by interacting and inhibiting hepatic modulators of lipogenesis (SREBP1 and diacylglycerol O-acyltransferase 2 (DGAT2)) and VLDL assembling [221]. Moreover, it was suggested that these hypolipidemic effects were also induced by PA's interaction with key microRNAs (miR) in hepatic lipid regulation, such as miR-33 and miR-122 [222,223]. In addition, several studies performed in this group have reported that the consumption of these polyphenols in rats fed a high-palatable and energy dense cafeteria diet (CAF), attenuated the body weight, body pressure and circulating TAG levels [224] and stimulated mitochondrial functionality, oxidative capacity and fatty acid uptake in the skeletal muscle via AMPK activation [225].

Relevantly, in the search of other mechanisms of action of polyphenols, our group described an interaction between PA and several components involved in the mammalian circadian timing system. Ribas *et al.* reported that grape seed PA could significantly modulate the biological rhythms in rats, maintaining elevated melatonin levels during the light phase and regulating the gene expression of *Bmal1* in the hypothalamus [226]. In a similar study, this author described a clear modulation of *Bmal1*, *Nampt* and NAD levels in the liver by PA, evidencing that these polyphenols can regulate both central and peripheral circadian rhythms [227]. In accordance, other studies reported a stimulating effect of resveratrol, a non-flavonoid found in grapes, on SIRT1 [198,228], which in addition to be involved in anti-inflammatory and anti-aging effects, it is strongly related with the modulation of CLOCK, as it has been described in the first section.

3.3. Sweet cherry (*Prunus avium L.)*

Being within the *Rosaceae* family and *Prunus* genus, sweet cherries are considered one of the most popular seasonal fresh fruits due to its unique taste, skin color, and nutritional value [229]. They are geographically distributed worldwide, with a higher prevalence in temperate zones and typically harvested between spring and summer (June to mid-July) [230,231].

This fruit is characterized by a higher concentration of simple sugars, such as fructose, sucrose and glucose, and organic acids, such as malic, citric and lactic acids, which confer its characteristic sweetness [232]. In addition, it is an important source of vitamins and minerals, such as vitamin C, potassium, magnesium, calcium and phosphorus and phenolic compounds, being rich in phenolic acids and flavonoids [233]. Specifically, as described in the database on polyphenol content in food *Phenol-Explorer*, the phenolic composition of this cherry variant is characterized by a high content of flavonoids (1.86 mg/g), such as anthocyanins (1.71 mg/g) and flavanols (0.15 mg/g), and phenolic acids (0.88 mg/g), such as 3-caffeoylquinic (0.45 mg/g) and 3-p-coumaroylquinic acids (0.38 mg/g). Cyanidin 3-O-rutinoside (1.43 mg/g) is the major anthocyanin found in this fruit, accounting for approximately 80-90% of the total anthocyanin content, followed by cyanidin 3-O-glucoside (0.19 mg/g). In addition, the main flavanols present in this fruit are epicatechin (0.078 mg/g), catechin (0.015 mg/g), and procyanidin dimers (0.038 mg/g) and trimers (0.019 mg/g) [234]. Regarding the macronutrient composition, this fruit contains 135 mg/g of carbohydrates, 5 mg/g of lipids, 8 mg/g of proteins, 15 mg/g of dietary fiber and 837 mg/g of water [235]. This fruit is also characterized for being rich in melatonin, which has been described to protect plants from stress by acting as a reactive oxygen species (ROS) scavenger [236,237]. Relevantly, by monitoring melatonin synthesis in sweet cherries cultivars, it was described that melatonin displayed a dual peak, the first at

night and the second when the highest light intensity of the day was reported, buffering the stressed induced by heat and UV light [237].

As it has been aforementioned, not only the development but also the phytochemical content of this fruit is highly sensitive to environmental variations in several factors, such as light exposure, temperature and nutrient availability [238,239]. Different studies have evidenced that direct application of UV irradiation markedly induced a higher biosynthesis of flavonoids in this fruit as a defensive mechanism [240,241]. Indeed, it was reported that after 72 hours of UV light irradiation, sweet cherries synthesized and accumulated a higher content of anthocyanins than those exposed to a white fluorescent light (two-fold increase) [241].

The high polyphenolic content of sweet cherries and their associated antioxidant properties have been of great interest in nutrition and health areas. Indeed, several studies have reported that the consumption of this fruit can be associated with a lower risk of CVD, diabetes, cancer and other inflammatory diseases [242,243]. Wu *et al.* reported that the supplementation of purified sweet cherry anthocyanins in HFD-fed mice induced anti-obesity effects in a dose-dependent manner [244]. Specifically, 200 mg/kg of diet of sweet cherry anthocyanins induced an attenuation of serum TAG, total and LDL cholesterol, hepatic lipids and circulating IL6 levels [244]. In addition, in a study performed with 18 men and women with slightly increased CRP levels, it was reported that sweet cherry consumption for 28 days (280 g/day) decreased the circulating levels of several pro-inflammatory markers such as CRP, endothelin-1, epidermal-growth factor and IL18 [245].

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UNIVERSITAT ROVIRA I VIRGILI
PHYSIOLOGICAL AND METABOLIC ADAPTATIONS TO DIFFERENT PHOTOPERIODS: EFFECTS OF OBESITY AND SEASONAL FRUIT CONSUMPTION
Roger Mariné Casadó

II. HYPOTHESIS AND OBJECTIVES

Most mammals adapt their physiology and metabolism to external changes in order to ensure a correct homeostatic balance by anticipating the environmental variations that occur during the daily 24 hours and the annual 365 days. These cyclic physiological and metabolic adaptations are known as circadian (about a day) and circannual (about a year) rhythms.

Throughout the day, environmental conditions markedly differ during the cyclic light and darkness periods. The different features of each specie, the adaptation to the ecosystems in which they live and the advantages that light or darkness confers them in order to forage for food, have led them to exhibit a robust physiological and biochemical behavior that adapts to the day length. Therefore, the different species have evolved a strong photoperiodic response that involves a complex system of metabolic genes and proteins which oscillate 24 hours a day, enabling an enhanced homeostatic regulation of metabolism.

Similarly, environmental settings exhibit significant annual fluctuations, especially in areas far from the equator. Seasonal changes in the length of the photoperiod are reported all through the year, as well as different climatological conditions and food availability, which force animals to adapt themselves to the continuous changing environment. These processes are regulated by several mechanisms, mainly hormonal, which ensure that all the biologic functions will be performed at the optimal time and conditions.

Overall, animals and humans are highly adapted to the foreseeable changes that take place in a system mainly controlled by light. Nevertheless, these adaptations are not enough to guarantee their survival, since environment is full of unceasing unpredictable changes. Thus, each year is characterized by different rainfall intensities or temperatures, affecting every year’s food availability and consequently, changing the possibilities of accumulating body supplies or breeding.

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The Xenohormesis theory postulates that the coevolution of animals and plants have led to develop a regulatory system of unpredictable changes in the environment, in which heterotroph animals can recognize as signals the phytochemicals synthesized by plants. Especially fruits, but also other comestible parts of plants, contain a wide range of variable phytochemicals, also known as secondary metabolites. This variability of phytochemicals allows the different plants to exhibit specific colors or tastes in order to ensure their reproduction. Moreover, its concentration is highly dependent on the maturation time and on the availability of nutrients, especially water. These secondary metabolites are also crucial in plant protection against pathogens, being synthesized in a specific concentration by stressed plants. Therefore, each fruit consumed by animals is characterized by different groups of phytochemicals that depend on the variety of fruit, the kind of pathogens that it has been exposed to, the availability of nutrients and water of the soil in which it has grown and its maturation time. Relevantly, animals have evolved the ability to identify these phytochemicals as signals that allow them to favorably adapt to unpredictable environmental changes. In fact, it has been shown that these phytochemicals can directly interact with several transcriptional factors, enzymes, microRNAs or other specific molecules, being able to control the metabolism of the organisms that consume them.

Behind this evolutionary phenomenon, there is a complex biochemical base that can elucidate how these bioactive compounds can exert positive effects in the prevention of several human diseases. Our research group have been working in this area over the last years, having focused on the effects of a group of phytochemicals known as proanthocyanidins, which are polyphenolic compounds synthesized in response to fungal infections. We have described that by interacting with several mechanisms at the molecular level, these compounds can exert beneficial effects on health, reducing the systemic inflammation, preventing the dyslipidemia and ameliorating the insulin

resistance. Is it a coincidence or it has an ecological sense in the context of the adaptive responses toward unpredictable changes in the environment?

The present PhD thesis is focused in this line of research, in which **we hypothesize that the consumption of seasonal fruits out of their harvesting season induces marked changes in the regulation of physiology and metabolism, which can contribute to the appearance and exacerbation of obesity and/or related metabolic disorders, such as insulin resistance**. In other words, the intake of seasonal fruit out of season will produce an illegitimate signaling between the astronomic season and the one indicated by the bioactive compounds present in the fruit, altering the mammals' circannual rhythms and, consequently, triggering impaired physiological and metabolic responses.

We chose the sweet cherry (*Prunus avium L.)* as it is a seasonal fruit typically consumed worldwide with an interesting composition of phytochemicals. It is consumed as a fresh fruit in spring, a long day season. We have administered it lyophilized, conserving all its components, to animals submitted to different photoperiods in order to mimic the different seasonal light schedules: a short day photoperiod (6 hours of light and 18 hours of darkness), which resembles autumn/winter , and long day photoperiods (18 hours of light and 6 hours of darkness), which resembles spring/summer seasons.

In addition, we have characterized these seasonal variations in day length in normoweight and diet-induced obese animals in order to describe the molecular mechanisms involved in the metabolic adaptations to predictable changes, which is crucial to comprehend the effects of sweet cherry consumption in the different seasons. For this purpose we have included a normal day photoperiod (12 hours of light and 12 hours of darkness) as a control, which allowed us to compare the metabolic responses to long and short photoperiods with the standard conditions mainly used in preclinical studies

with animal models. We mainly focused this study in the liver and the skeletal muscle metabolisms, two central organs in the metabolic control.

Thus, the main objective of the present PhD thesis was **to evaluate whether the consumption of seasonal fruits (sweet cherry) out of season could induce detrimental effects on physiology and metabolism of normoweight and cafeteria-fed obese Fischer 344 rats, analyzing a wide variety of parameters mainly related with glucose and lipid metabolisms in blood, liver and skeletal muscles.** To achieve this purpose, the following goals were raised:

1. To evaluate, in the photoperiod-sensitive rat strain Fischer 344, the impact of the chronic exposure to different photoperiods (L12, 12h light/day; L18, 18h light/day and L6, 6h light/day; partly resembling the seasonal variations in day length) on physiology and glucose and lipid metabolisms, as well as to elucidate the underlying mechanisms (**Manuscript 1**).

2. To characterize the physiological, metabolic and molecular changes prompted by the intake of the obesogenic high-palatable and energy dense cafeteria diet in F344 rats chronically exposed to the different photoperiods (**Manuscript 2**).

3. To determine, in rats exposed to short (L6) and long (L18) photoperiods, whether the consumption of sweet cherry out of its harvesting season could produce an illegitimate signalling and consequently a) induce detrimental effects on the metabolism of normoweight animals and b) enhance the deleterious effects produced by the CAF intake (**Manuscript 3**).

III. RESULTS

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MANUSCRIPT 1

The exposure to different photoperiods strongly modulates the glucose and lipid metabolisms of normoweight Fischer 344 rats

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ABSTRACT

Seasonal variations in day length trigger clear changes in the behavior, growth, food intake and reproductive status of photoperiod-sensitive animals, such as Fischer 344 rats. However, there is little information about the effects of seasonal fluctuations in day length on glucose and lipid metabolisms and their underlying mechanisms in this model. To gain knowledge on these issues, three groups of male Fischer 344 rats were fed with a standard diet and exposed to different photoperiods for 14 weeks: normal photoperiod (L12, 12 h light/day), long photoperiod (L18, 18 h light/day), and short photoperiod (L6, 6 h light/day). A multivariate analysis carried out with 239 biometric, serum, hepatic and skeletal muscle parameters revealed a clear separation among the three groups. Compared with L12 rats, L6 animals displayed a marked alteration of glucose homeostasis and fatty acid uptake and oxidation, which were evidenced by the following observations: 1) increased circulating levels of glucose and non-esterified fatty acids; 2) a sharp downregulation of the phosphorylated Akt2 levels, a downstream post-receptor target of insulin, in both the soleus and gastrocnemius muscles; 3) decreased expression in the soleus muscle of the glucose metabolism-related microRNA-194 and lower mRNA levels of the genes involved in glucose metabolism (*Irs1*, soleus, and *Glut2*, liver), β-oxidation (*Had* and *Cpt1β*, soleus) and fatty acid transport (*Cd36*, soleus and liver). L18 animals also displayed higher blood glucose levels than L12 rats and profound changes in other glucose and lipid metabolism-related parameters in the blood, liver and skeletal muscles. However, the mechanisms that account for the observed effects were less evident than those reported in L6 animals. In conclusion, exposure to different photoperiods strongly modulated glucose and lipid metabolisms in normoweight rats. These findings emphasize the relevance of circannual rhythms in metabolic homeostasis regulation and suggest that Fischer 344 rats are a promising animal model with

which to study glucose- and lipid-related pathologies that are influenced by seasonal variations, such as obesity, cardiovascular disease and seasonal affective disorder.

Key words: photoperiod, circannual rhythms, insulin sensitivity, glucose metabolism, lipid metabolism.

1. INTRODUCTION

It has generally been established that many mammals are season- or photoperiod-sensitive and are able to change their behavior, morphology and physiology to anticipate climate and food availability changes among the seasons [1,2]. Thus, food intake, growth, energy balance and reproduction have been observed to vary during the year in some species to ensure their survival [3]. This seasonal responsiveness can be regulated by two mechanisms: the first is promoted by environmental cues that indicate the time of year, such as the day length (photoperiod), in which melatonin plays a key role [4]; the second corresponds to the endogenous circannual rhythms, which are adjusted by environmental cues and can adapt to seasonal processes as a response to variations in the photoperiod or other external signals [5]. Despite the limited impact of seasonality because of the increased use of artificial lighting, heating and air conditioning systems, humans also display seasonal changes in different anthropometric, physiologic, metabolic and behavior parameters [6–9]. Thus, the body fat significantly increases during winter in latitudes far from the equator, where greater variations in temperature, climate and daylight hours are registered, and the levels of physical activity and energy expenditure are lower in the winter than in the summer [6,7]. Since fat accretion and decreased physical activity can increase the risk of insulin resistance and cardiovascular disease (CVD), the seasonal variations in these and other parameters can place the human health at more risk in winter than in summer [7]. This fact is

illustrated by the increased number of cardiac events observed in winter both north and south of the equator, by the additional 20,000 deaths per year caused by coronary and cerebrovascular events that were reported in England and Wales during this season, and by the strong negative correlation found between CVD mortality and the hours of sunshine [9]. However, since the seasonal variation of these risk factors that trigger a peak winter mortality is influenced by changes in other exogenous factors different than day length, such as temperature and lifestyle [6,7,9], the relevance of seasonal variations in day length on health is far from being established. In this sense, the use of animal models, which can be maintained under constant temperature and social input, have emerged as a useful strategy to shed more light on how exposure to different photoperiods impacts physiology and health.

In the study of the seasonal effects, the most used animal models have been long-day breeding rodents, such as hamsters and voles, since they display quick responses to different photoperiods [10,11]. Nevertheless, one of the main drawbacks to using these models is the limited genetic information available in data repositories, in addition to the scarce molecular and biochemical tools to study these seasonal species. For this reason, in recent decades, Fischer 344 rats have become an interesting animal model with which to evaluate the effects of photoperiod exposure since they display a marked physiological and reproductive response to seasonal variations in day length [12–19]. As an example, Heideman *et al.* showed that, after exposure to photoperiods of more than 13.5 hours, young Fischer 344 rats displayed higher lean and fat mass, food intake and an increase in testis weight and size compared to those rats exposed to a shorter photoperiod, which exhibited a regressive phenotype [15,16]. Nevertheless, to the best of our knowledge, the photoperiod effects on glucose and lipid metabolisms, the impairment of which is strongly related to the appearance of risk factors for CVD, including obesity, insulin resistance and dyslipidemia, have not yet been evaluated in this animal model.

In C57BL/6J mice, Tashiro and collaborators showed that the exposure to a short photoperiod for 3 weeks produced hyperglycemia, which was partly explained by the downregulation of the glucose transporter GLUT4 in the gastrocnemius muscle [20]. These authors also demonstrated that C57BL/6J mice held under a short photoperiod displayed increased sucrose intake, body weight and fat mass and a depressive state [21,22], partly resembling subjects suffering from seasonal affective disorder (SAD), a mood disorder in which people exhibit depressive symptoms, hyperphagia, carbohydrate cravings, increased body weight gain and fat accretion, especially in winter [23]. In humans, it has been shown that a standard oral glucose tolerance test triggers a lower and slower insulin response during spring than during autumn, suggesting seasonal differences in insulin secretion and/or the different blood sugar lowering effects of this hormone at different seasons of the year [24]. In obese male subjects, it has been observed that the circulating levels of cholesterol, triglycerides and the adipocytokine leptin, which play an important role in long-term regulation of body weight and energy homeostasis, significantly increase during winter [6].

All the aforementioned findings prompted us to hypothesize that chronic exposure to different photoperiods would produce changes related with glucose and lipid metabolisms in Fischer 344 rats. Therefore, the main aim of the present study was to determine the effects of a chronic exposition to different photoperiods on glucose, lipid and energy metabolisms in normoweight Fischer 344 rats. Our goal was accomplished by analyzing different biochemical parameters and key genes and proteins involved in these metabolisms and carrying out an ^{1}H NMR metabolomic analysis of the blood, liver and skeletal muscle.

2. MATERIALS AND METHODS

2.1. Animals

The animals used were 8-week-old male Fischer 344 rats (Charles River Laboratories, Barcelona, Spain). After an adaptation period of 4 days, in which animals were housed in pairs at 22ºC under a light/dark cycle of 12 hours, they were submitted to three light schedules to emulate season's day length: short day photoperiod (n=6, L6, 6 h light—from Zeitgeber times (ZTs) 0 to 6—and 18 h darkness—from ZTs 6 to 24), normal day photoperiod (n=6, L12, 12 h light—from ZTs 0 to 12—and 12 h darkness—from ZTs 12 to 24) and long day photoperiod (n=6, L18, 18 h light—from ZTs 0 to 18—and 6 h darkness—from ZTs 18 to 24). Rats in each photoperiod were fed *ad libitum* with a standard diet (2.90 $kcal \cdot g^{-1}$; Teklad Global 14% Protein Rodent Diet 2014, ENVIGO, Sant Feliu de Codines, Barcelona, Spain). Food intake and body weight data were recorded weekly. After 14 weeks, the animals were deprived of food for one hour and were sacrificed between ZTs 1 and 2 to minimize the possible circadian variations. The blood was collected, and the serum was obtained by centrifugation and stored at -80ºC until analysis. Liver, gastrocnemius and soleus muscle were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80ºC until further analysis. The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all the procedures.

2.2. Body composition analysis

Lean and fat mass analyses were performed one week before the sacrifice using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA). The measurements were performed in duplicate. Data are expressed in absolute (g) and relative values as a percentage of body weight (%). Lean/fat mass ratio was also calculated.

2.3. Serum analysis

Enzymatic colorimetric assays were used for the analysis of glucose, total cholesterol and triglycerides (QCA, Amposta, Tarragona, Spain), phospholipids (Spinreact, St. Esteve de Bas, Girona, Spain) and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany). Serum insulin and glucagon levels were analyzed using a rat insulin ELISA kit (Millipore, Barcelona, Spain) and a rat glucagon ELISA kit (Cusabio Biotech, Wuhan, China), respectively.

2.4. Total glycogen extraction and quantification

In this method, 750 mg and 400 mg samples of liver and gastrocnemius muscle, respectively, were boiled for 20 minutes in a KOH 30% solution. Total glycogen was precipitated by adding saturated Na_2SO_4 and 95% ethanol and then centrifuged at 2560 x g for 15 min at 4ºC. Supernatants were boiled with hydrochloric acid for 2 hours to hydrolyze glycogen into glucose and neutralized with sodium chloride. Glucose levels were determined by an enzymatic colorimetric kit (QCA, Amposta, Tarragona, Spain).

2.5. Total lipid content extraction and quantification

Lipids were extracted from liver (100 mg) and gastrocnemius muscle (200 mg) using the methods described in [25] and [26], with the modifications described in [27]. The quantity of the lipids in both tissues was determined gravimetrically. Both the lipid and aqueous fractions obtained in this extraction were used to perform an NMR analysis for metabolite determination in both tissues.

2.6. Alanine Aminotransferase (ALT) and Lactate Dehydrogenase (LDH) activity

Fifty milligrams of liver and gastrocnemius muscle were homogenized in 500 μL of ALT or LDH Assay buffer and centrifuged at 10000 x g for 15 minutes at

4ºC. ALT and LDH activities were determined using an ALT Activity Assay Kit (Sigma, Madrid, Spain) and LDH Activity Assay Kit (Sigma, Madrid, Spain), respectively.

2.7. Serum extraction and ^{1}H NMR analysis for metabolite determination

Serum metabolites were extracted with methanol:water (8:1). After centrifugation (1800 x g, 10 min at 4ºC), supernatants containing soluble metabolites were placed into new vials. Pellets resulting from the aqueous extraction were washed twice with methanol:water. Supernatants were dried in an N_2 stream to remove water and stored at -80ºC.

For ^{1}H NMR analysis, the aqueous extracts obtained in the serum, liver and gastrocnemius muscle extractions were reconstituted in 700 µl of a solution containing 0.005% trisilylpropionic acid (TSP) (0.7381 mM) dissolved in D_2O phosphate buffer (0.05 M). Lipophilic extracts were subsequently dissolved in 700 µl of a solution containing 0.01% tetramethylsilane (TMS) dissolved in CD_3Cl:CD_3OD (2:1). Samples were vortexed, homogenized for 5 min and centrifuged (15 min at 14000 x g). Finally, the redissolved extractions were transferred into 5 mm NMR glass tubes.

^{1}H NMR measurements were performed following the procedure described by Vinaixa et al [28].

2.8. NMR data analysis

NMR data analysis was performed as previously described [28].

2.9. Gene expression analysis

Liver, gastrocnemius and soleus muscle total RNA and microRNA were extracted using TriPure reagent (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain) according to the manufacturer’s protocol. To isolate both the total and micro RNA species, samples were incubated overnight with 100%

isopropanol at -20ºC. The cDNA was synthetized using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) and subjected to quantitative PCR amplification using a LightCycler 480 II system with SYBR Green I Master Mix (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain). The reaction was performed according to the instructions provided by the manufacturer. The primers used for the different genes are described in **Supplementary Table 1** and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA level was calculated as a percentage of the L12 group, using the $-2^{\Delta\Delta Ct}$ method with *Ppia, β-actin, Hprt* and *Tfrc* genes as endogenous controls.

2.10. miR quantitative real-time PCR

Soleus muscle miR-194, miR-133 and miR-486 levels were measured using TaqMan Advanced miRNA Assays (Applied Biosystems, Carlsbad, CA, USA). Then, 2.5 ng of RNA was used to synthetize the cDNA using a TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA, USA), and it was subjected to quantitative PCR on the LightCycler 480 II system (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain) with SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). miR-191 was used as an endogenous miRNA.

2.11. Western Blot analysis

Total and phosphorylated (p) AMP-activated protein kinase (AMPK and (p)-AMPK) (62 kDa) and Akt serine/threonine kinase 2 (Akt2 and (p)-Akt2) (60 kDa) protein levels in the liver, soleus and gastrocnemius muscle were measured by western blot analysis as previously described [29] with some modifications. Specifically, the membranes were incubated overnight with the two primary antibodies, mouse anti-Akt2 (L79B2) and rabbit anti-(p)-Akt2 (Ser474) (Cell Signaling, Izasa SA, Barcelona, Spain), diluted 1/2500. Then, the

membranes were incubated with goat anti-mouse and goat anti-rabbit secondary antibodies (LI-COR, USA), diluted 1/10000. In the liver samples, β-actin primary antibody was used as an endogenous control (42 kDa) (Abcam, England, UK). In the soleus and gastrocnemius muscles, α-tubulin was used as a loading control (52 kDa) (Cell Signaling Technology, Barcelona, Spain).

2.12. Statistical analysis

Data are expressed as the mean ± SEM. Grubbs' test was used to detect outliers, which were discarded before subsequent analyses. Statistical analyses were performed using SPSS Statistics 22 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA followed by Duncan's *post hoc* test was used to determine significant differences among the three groups. Student's t-test was used for single statistical comparisons. The level of statistical significance was set at bilateral 5%.

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed after data normalization and autoscaling using MetaboAnalyst 3.0 software [30].

3. RESULTS

3.1. The exposure to different photoperiods altered the circulating levels of glucose and NEFAs

No significant changes among groups were found in cumulative food intake, body weight gain and body composition. Animals exposed to the L6 photoperiod showed residually lower liver weights (p=0.014, Student's t test) than the L12 group, but no changes were observed in the muscle and testes weights (**Table 1**). The analysis of serum parameters revealed that L6 and L18 animals exhibited significantly higher glucose circulating levels compared to the L12 animals (**Table 1**). Moreover, L6 rats also presented residually higher

circulating levels of NEFAS (p=0.031, Student's t test) than L12 animals (**Table 1**).

Table 1. Biometric and serum parameters in rats fed a standard diet and exposed to three different light schedules for 14 weeks.

	L6	L12	L18	
Cumulative food intake (g)	231 ± 3	230 ± 4	228 ± 5	
Biometric parameters				
Initial body weight (g)	180 ± 8	201 ± 4	195 ± 10	
Final body weight (g)	370 ± 11	381 ± 7	387 ± 13	
Body weight gain (g)	191 ± 8	180 ± 7	192 ± 8	
Liver (g)	11.86 ± 0.15	12.94 ± 0.30	12.73 ± 0.45	
Skeletal muscle (g)	2.08 ± 0.07	2.10 ± 0.04	2.12 ± 0.03	
Testes (g)	3.09 ± 0.06	3.02 ± 0.06	3.04 ± 0.04	
Fat mass (g)	45.06 ± 1.29	52.72 ± 3.65	55.64 ± 4.41	
Fat mass (%)	12.52 ± 0.34	13.93 ± 0.86	14.38 ± 0.75	
Lean mass (g)	296 ± 8	308 ± 6	310 ± 9	
Lean mass (%)	80.94 ± 1.04	81.48 ± 0.81	80.71 ± 0.67	
Lean/fat mass ratio	6.56 ± 0.22	5.98 ± 0.42	5.71 ± 0.37	
Serum parameters				
Glucose (mmol/L)	7.73 ± 0.19[a]	6.89 ± 0.09[b]	7.59 ± 0.19[a]	*P*
Insulin (ng/mL)	4.04 ± 0.66	5.41 ± 0.72	5.54 ± 0.73	
Glucagon (ng/mL)	2.66 ± 0.13	2.19 ± 0.29	2.86 ± 0.04	
Insulin:glucagon ratio	1.57 ± 0.32	2.69 ± 0.43	1.79 ± 0.24	
NEFAs (mmol/L)	0.72 ± 0.06	0.56 ± 0.03	0.62 ± 0.05	
Phospholipids (mmol/L)	3.08 ± 0.17	2.64 ± 0.15	2.83 ± 0.10	
Triglycerides (mmol/L)	1.60 ± 0.12	1.30 ± 0.12	1.39 ± 0.11	
Total cholesterol (mmol/L)	3.26 ± 0.19	2.69 ± 0.19	2.97 ± 0.09	

Male Fischer 344 rats were fed a standard diet and were exposed to three different photoperiods for 14 weeks. Data are expressed as the mean ± SEM (n=6). One-way ANOVA and Duncan's post hoc tests were performed to compare the values between the groups and

significant differences were represented with different letters (a, b). *P,* photoperiod effect. The skeletal muscle weight represents the total weight of both soleus and gastrocnemius muscles.

3.2. Rats held under different photoperiods displayed changes in the circulating levels of nitrogenate metabolites

By analysis of the serum metabolomics, which were performed using NMR, we found 8 nitrogenate metabolites with significant changes among the groups. Creatine, histamine, isoleucine, threonine and tryptophan levels were higher in the L6-photoperiod exposed animals than both the L12 and L18 animals (**Table 2**). This group exhibited higher histidine and tyrosine levels than the L18 group (**Table 2**).

The L6 group displayed lower 3-hydroxybutyrate levels than the L18 group and higher acetate levels compared to both the L12 and L18 groups (**Table 2**). All other metabolites that did not reach statistical significance are shown in **Supplementary Table 2**.

3.3. The chronic exposure to different photoperiods modified the glucose and glycogen liver content and the mRNA levels of key genes involved in hepatic glucose metabolism

To elucidate which mechanisms can be involved in the altered circulating glucose levels observed in both L6 and L18 groups, we measured different glucose metabolism-related parameters in liver, which plays an essential role in glucose homeostasis and is highly regulated by circadian rhythms [31]. L18 animals displayed residually lower levels of hepatic glucose than L6 rats (p=0.015, Student's t test) (**Figure 1A**) and less hepatic glycogen content than the L12 rats (p=0.031, Student's t test) (**Figure 1B**). The gene expression analyses carried out in the liver revealed that the L18-photoperiod exposed rats displayed a vast overexpression of glucokinase (*Gk*), a key glycolytic-related gene, compared to L6 rats. Moreover, L18 animals displayed lower mRNA levels

of the gluconeogenic gene phosphoenolpyruvate carboxykinase 1 (*Pck1*) than L12 rats and residually lower gene expression levels of fructose-1,6-biphosphatase 1 (*Fbp1*) compared to L12 and L6 animals (p=0.05 and p=0.036, Student's t test, respectively) (**Figure 2B**). L6 animals displayed decreased expression of the genes encoding the glucose transporter 2 (GLUT2) than the L12 animals (**Figure 2B**). All the metabolites obtained by NMR analysis are shown in **Supplementary Table 3.**

To further explore the mechanisms involved in the photoperiodic regulation of glucose metabolism, the phosphorylated levels of Akt2 and AMPK, two proteins involved in glucose and insulin homeostasis [32,33], were determined in the liver of the three groups of rats. Nevertheless, no changes among the groups were reported in the hepatic levels of these key proteins (**Figures 2C,E**).

3.4. The lipid content and expression of fatty acid transport-related genes changed in the liver of the photoperiod groups

L18 rats displayed significantly greater levels of glycerophosphocholine and residually higher levels of diglycerides (p=0.016, Student's t test) in this tissue than the L12 group (**Figure 1C**). These metabolic changes observed in L18 rats were accompanied by significant downregulation of the mRNA levels of the genes codifying the fatty acid transport protein 5 (*Fatp5*) and the fatty acid translocase, homologue of CD36 (*Cd36*) (**Figure 2A**) compared to that in the L12 rats. The *Cd36* mRNA levels were also significantly lower in animals exposed to the L6 photoperiod than in the L12 group animals (70.6% lower) (**Figure 2A**).

Table 2. Representative serum metabolite concentrations analyzed by nuclear magnetic resonance in response to different photoperiod exposure in animals fed a standard diet for 14 weeks.

Metabolite concentration (µmol/L)	L6	L12	L18	
3-Hydroxybutyrate	15.82 ± 1.34^{a}	20.97 ± 2.78^{ab}	27.36 ± 0.99^{b}	*P*
Acetate	45.36 ± 2.63^{a}	38.75 ± 1.11^{b}	36.84 ± 0.89^{b}	*P*
Alanine	139.29 ± 3.63^{a}	120.06 ± 5.99^{b}	133.98 ± 2.67^{a}	*P*
Creatine	81.95 ± 4.84^{a}	65.54 ± 2.09^{b}	58.55 ± 3.96^{b}	*P*
Formate	8.25 ± 1.16^{a}	6.99 ± 0.82^{a}	1.65 ± 0.64^{b}	*P*
Glutamine	140.65 ± 3.10	127.14 ± 6.28	130.03 ± 4.78	
Glycerophosphocholine	55.46 ± 0.88^{ab}	50.97 ± 1.88^{a}	58.43 ± 2.50^{b}	*P*
Histamine	5.14 ± 0.50^{a}	2.45 ± 0.33^{b}	2.06 ± 0.35^{b}	*P*
Histidine	18.86 ± 0.38^{a}	17.14 ± 0.81^{a}	14.18 ± 0.75^{b}	*P*
Isoleucine	19.56 ± 1.12^{a}	15.60 ± 0.70^{b}	14.39 ± 0.73^{b}	*P*
Lactate	1193 ± 55^{a}	1357 ± 113^{ab}	1624 ± 134^{b}	*P*
Lysine	80.10 ± 0.64	87.41 ± 3.57	77.14 ± 4.90	
Oxypurinol	4.23 ± 0.79	2.06 ± 0.17	2.69 ± 0.62	
Pyruvate	18.97 ± 0.89^{a}	15.41 ± 1.23^{b}	12.32 ± 0.62^{c}	*P*
Threonine	37.03 ± 2.22^{a}	30.01 ± 2.25^{b}	26.37 ± 2.18^{b}	*P*
Tryptophan	27.62 ± 0.73^{a}	24.50 ± 0.91^{b}	23.19 ± 0.89^{b}	*P*
Tyrosine	18.67 ± 0.55^{a}	17.62 ± 0.58^{a}	15.72 ± 0.50^{b}	*P*

Male Fischer 344 rats were fed a standard diet and were exposed to three different photoperiods for 14 weeks. Data are expressed as the mean ± SEM (n=6). All the metabolites were obtained by performing a nuclear magnetic resonance (NMR) analysis. One-way ANOVA and Duncan's post hoc tests were performed to compare the values between groups and significant differences were represented with different letters (a, b). *P*, photoperiod effect.

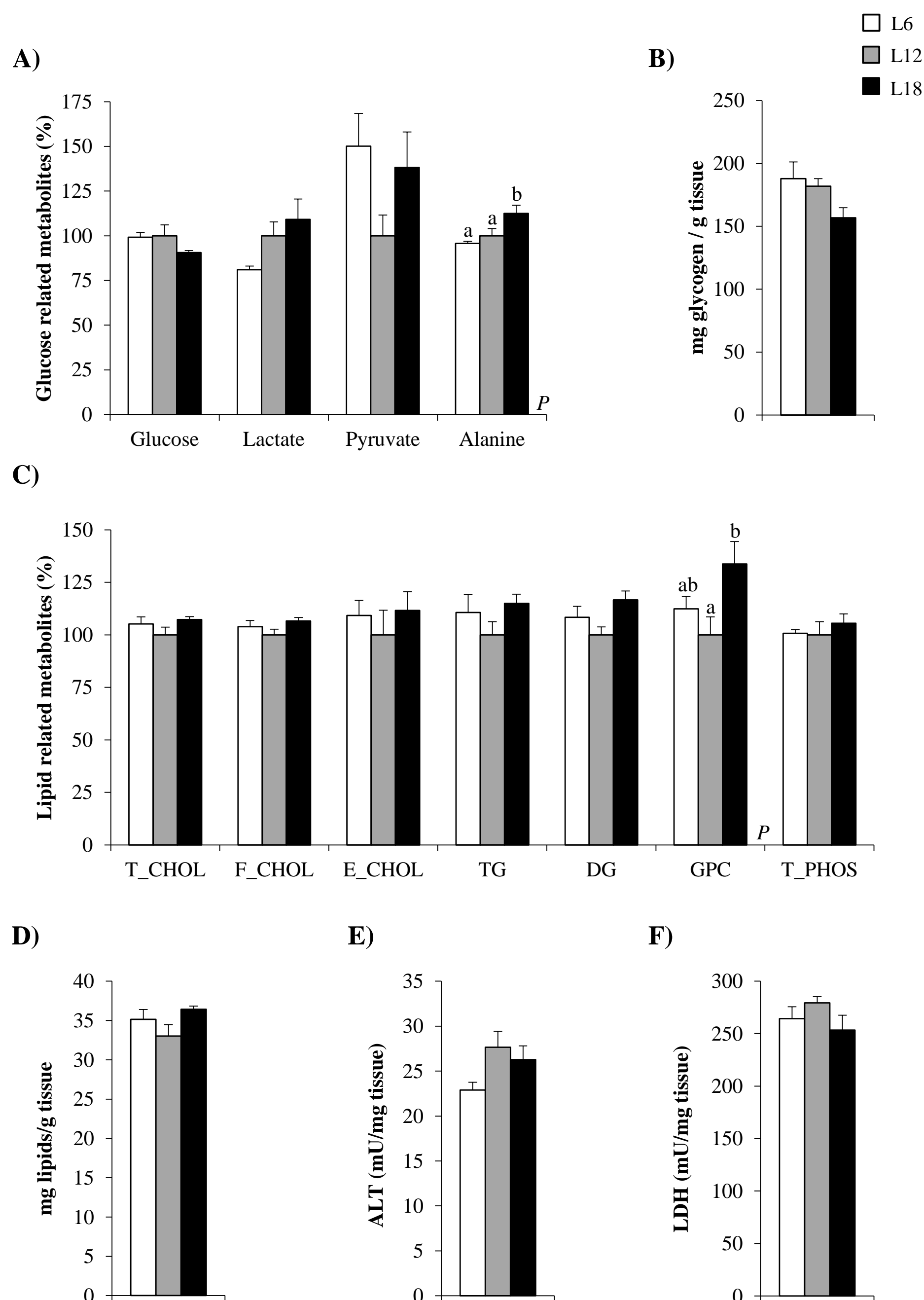
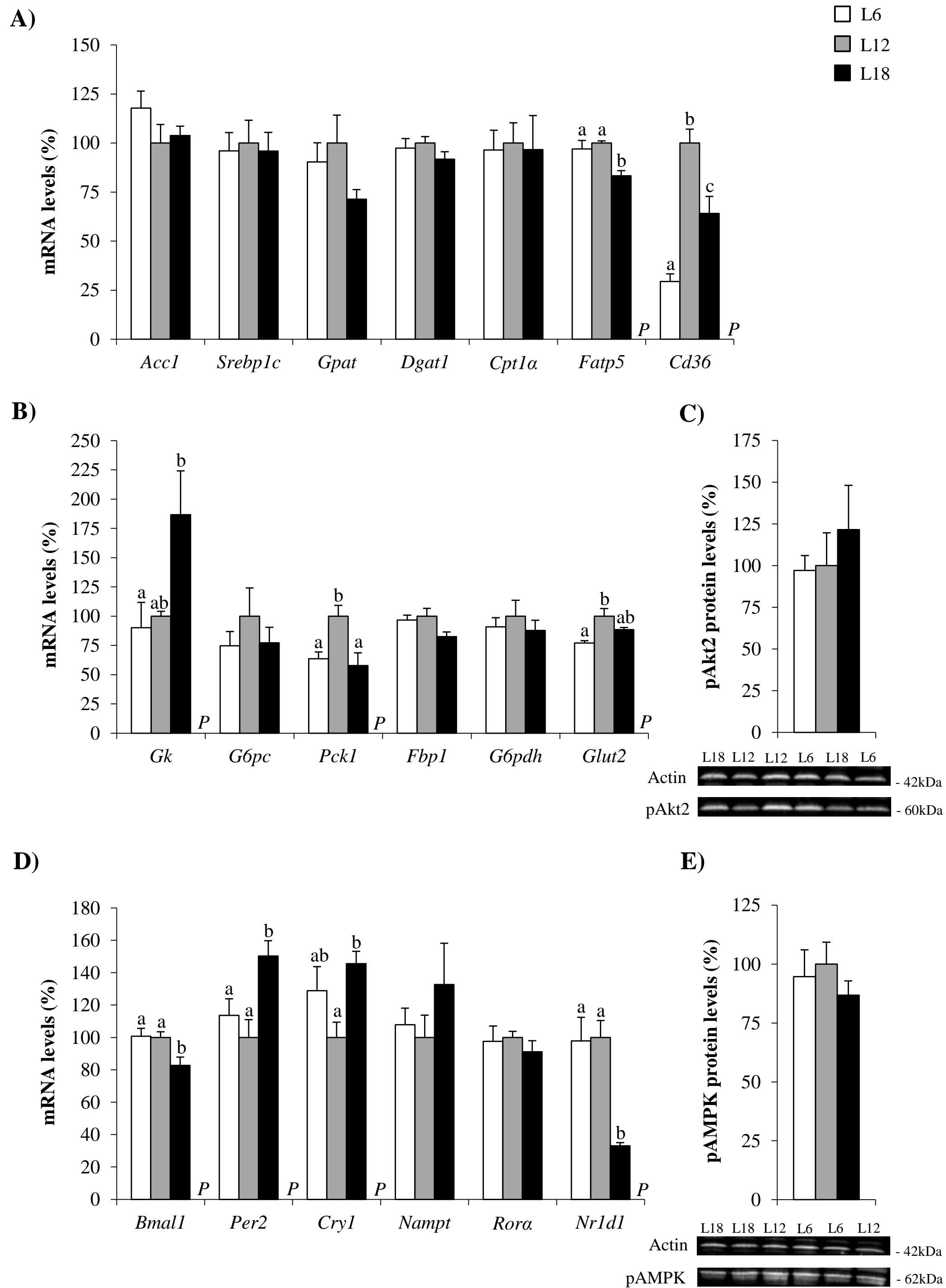


Figure 1. Hepatic glucose **(A)** and lipid-related metabolites **(C)**, glycogen **(B)** and total lipid levels **(D)**, alanine aminotransferase (ALT) **(E)** and lactate dehydrogenase (LDH) activity levels **(F)** in the liver of male Fischer 344 rats fed with a standard diet and exposed to three different photoperiods for 14 weeks. Data are represented as the mean ± SEM (n=6). Liver metabolites concentrations (expressed as µmol/g tissue) are shown in Supplementary Table 3. *P*, photoperiod effect ($p<0.05$, one-way ANOVA). [ab] Mean values with different letters are

significantly different among the groups (p<0.05, Duncan post hoc test). T_CHOL, total cholesterol; F_CHOL, free cholesterol; E_CHOL, esterified cholesterol; TG, triglycerides; DG, diglycerides; GPC, glycerophosphocholine; T_PHOS, total phospholipids.



UNIVERSITAT ROVIRA I VIRGILI
PHYSIOLOGICAL AND METABOLIC ADAPTATIONS TO DIFFERENT PHOTOPERIODS: EFFECTS OF OBESITY AND SEASONAL FRUIT CONSUMPTION
Roger Mariné Casadó

Figure 2. The mRNA levels of lipid metabolism **(A)**, glucose metabolism **(B)** and circadian rhythm-related genes **(D)** and the protein levels of pAkt2 **(C)** and pAMPK **(E)** in the liver of male Fischer 344 rats fed with a standard diet and exposed to three different photoperiods for 14 weeks. Data are represented as the mean ± SEM (n=6). *P,* photoperiod effect ($p<0.05$, one-way ANOVA). [ab] Mean values with different letters are significantly different among groups ($p<0.05$, Duncan post hoc test). *Acc1*, acetyl CoA carboxylase 1; *Bmal1*, brain and muscle Arnt-like protein-1; *Cd36*, fatty acid translocase, homologue of CD36; *Cpt1α*, carnitine palmitoyltransferase 1 alpha; *Cry1,* cryptochrome circadian clock 1; *Dgat1*, diacylglycerol acyltransferase 1; *Fatp5*, fatty acid transport protein 5; *Fbp1*, fructose-1,6-biphosphatase 1; *G6pc*, glucose-6-phosphatase, catalytic subunit; *G6pdh*, glucose-6-phosphate dehydrogenase; *Glut2*, glucose transporter 2; *Gk*, glucokinase; *Gpat*, glycerol-3-phosphate acyltransferase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Nampt*, nicotinamide phosphoribosyltransferase; *Nr1d1*, nuclear receptor subfamily 1, group D, member 1; pAkt2, phosphorylated Akt serine/threonine kinase 2; pAMPK, phosphorylated AMP-activated protein kinase; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Per2*, period circadian clock 2; *Rorα*, RAR-related orphan receptor A; *Srebp1c*, sterol regulatory element-binding protein 1c.

3.5. The exposure to different day lengths altered the mRNA levels of fatty acid transport, β-oxidation and insulin signaling-related genes and the microRNA-194 expression in the soleus muscle

To better characterize the effects of chronic exposure to different photoperiods related to the increase in the circulating levels of glucose and NEFAs, the mRNA levels of a subset of genes involved in fatty acid uptake, β-oxidation, glycolysis and insulin signaling were analyzed in both the soleus and gastrocnemius muscles of the L6, L12 and L18 rats. In the soleus muscle, animals exposed to both short and long photoperiods displayed a significant, sharp downregulation of the fatty acid transporter *Cd36* mRNA levels (56.5% and 49.8% lower, respectively) compared to L12 rats (**Figure 3C**). Both groups also exhibited lower expression of the β-oxidation-related gene carnitine palmitoyltransferase 1 beta (*Cpt1β*) but only L6 animals displayed significant lower hydroxyacyl-CoA dehydrogenase (*Had*) mRNA levels in comparison with L12 animals (**Figure 3C**). The L18-photoperiod exposed rats showed lower mRNA levels of phosphofructokinase (*Pfk*), a gene involved in the glycolytic process, than the

L12 rats (**Figure 3A**). In addition, L6 rats presented lower mRNA levels of the insulin receptor substrate 1 (*Irs1*) (p=0.007, Student's t test) (**Figure 3A**), a gene encoding a key protein involved in the insulin signaling pathway [33], and lower expression levels of the glucose metabolism-related microRNA-194 (miR-194) [34] than the L12 rats (p=0.009, Student's t test) (**Figure 3B**). No significant changes among groups were observed in the expression of these genes in the gastrocnemius muscle (**Figures 4A,C**).

3.6. The NMR metabolomic analysis revealed an effect of the photoperiod on the levels of lipid and energy intermediates in the gastrocnemius muscle

In the gastrocnemius muscle, succinate, adenosine monophosphate (AMP) and inosine monophosphate (IMP) levels were significantly higher in L6 rats compared to the other groups (**Figure 5A**), suggesting an altered energy metabolism caused by exposure to this photoperiod. This group also displayed lower levels of total cholesterol and diglycerides than the L18 group and, consequently, a lower amount of total lipids in this tissue (**Figures 5B,C**). No differences among groups were observed in the muscular glucose and glycogen levels (**Figures 5A,D**). All the metabolites obtained by the NMR analysis are shown in **Supplementary Table 4**.

3.7. Photoperiod exposure slightly modulated Cori and Cahill cycles intermediates in liver, skeletal muscle and blood

L6 animals displayed lower serum lactate levels than the L18 animals and higher circulating levels of pyruvate compared to both the L12 and L18 rats (**Table 2**). In addition, L6 animals showed higher hepatic pyruvate levels than L12 animals (p=0.042, Student's t test) (**Figure 1A**). Both L6 and L18 animals exhibited greater circulating levels of alanine compared to L12 rats (**Table 2**).

L18 animals also displayed higher hepatic alanine levels than the rats held under the other photoperiods (**Figure 1A**).

The changes in the levels of pyruvate, alanine and lactate observed among the groups prompted us to evaluate whether exposure to different photoperiods could alter the Cori and Cahill cycles by analyzing the enzymatic activity of ALT and LDH in the muscle and liver. Concerning the Cori cycle, the metabolic pathway in which lactate produced by anaerobic glycolysis in the muscles is released into the bloodstream, transported to the liver and converted to glucose, then returns to the muscles and is metabolized back to lactate [35], only a significant drop in the enzymatic activity of LDH, which catalyzes the conversion of lactate to pyruvate and back, was observed in the gastrocnemius muscle of L6 animals compared to L12 rats ($p=0.010$, Student's t test) (**Figure 5F**). Regarding the Cahill cycle, the pathway through which the muscles export pyruvate and amino groups as alanine to the liver, and receive glucose from the liver via the bloodstream [36], only a significant decrease in the ALT activity, which, in a reversible manner, converts L-glutamate and pyruvate into α-ketoglutarate and L-alanine, was observed in the liver of the L6 animals compared to L12 rats ($p=0.038$, Student's t test) (**Figure 1E**). However, no changes were observed either in the hepatic LDH (**Figure 1F**) or in the muscular ALT (**Figure 5E**) activities. Altogether, these findings would not support a photoperiod effect on the modulation of the Cahill and Cori cycles.

3.8. The phosphorylated levels of Akt2 and AMPK were photoperiodically regulated in the skeletal muscles

The profound changes triggered by the exposure to different photoperiods in the soleus mRNA levels of genes involved in fatty acid uptake, β-oxidation and insulin signaling as well as in the gastrocnemius AMP and IMP content, prompted us to analyze the phosphorylated levels of Akt2 and AMPK in both muscles to shed more light on the mechanisms that mediated the photoperiodic

effects on glucose and lipid metabolisms. In both soleus and gastrocnemius muscles, L6 photoperiod-exposed animals exhibited a sharp downregulation of the phosphorylated Akt2 levels (pAkt2) compared to L12 and L18 animals, and this decrease was greater in the soleus muscle (84.5% and 79.9% lower, respectively) than in the gastrocnemius muscle (53.6% and 60.3% lower, respectively) (**Figures 3E and 4B**). Moreover, L6 animals exhibited a vast upregulation of the phosphorylated levels of AMPK (pAMPK) compared to L12 and L18 animals in the soleus (163.8% and 167.3% higher, respectively) and gastrocnemius muscle (115% and 57.4% greater, respectively) (**Figures 3G and 4D**). L6 rats also presented a lower total protein content in both the soleus and gastrocnemius muscles than the L12 and L18 animals (**Figures 3D and 4F**).

3.9. The exposure to the long day photoperiod altered the expression of circadian rhythm-related genes in the liver and skeletal muscles

In addition to affecting seasonal rhythms, it is well known that the light-dark cycle is a key regulator of the daily rhythmicity, which is under the control of an internal circadian clock, which, in turn, plays a very important role in metabolism regulation [37,38]. Since the alteration of this body clock in both animals and humans exposed to disrupted light:dark cycles results in alterations in glucose and lipid metabolisms [39–41], we analyzed the hepatic and muscular mRNA levels of key clock genes to explore whether changes in the expression of these genes could partly explain the metabolic changes observed in rats held under different photoperiods. Animals exposed to the L18 photoperiod displayed lower mRNA levels of the brain and muscle Arnt-like protein-1 (*Bmal1*) gene and higher gene expression levels of its product, period circadian clock 2 (*Per2*), in liver and in the soleus muscle (**Figures 2D and 3F**). Furthermore, this group also showed lower mRNA levels of the nuclear receptor subfamily 1 group D member 1 gene (*Nr1d1*), the repressor of *Bmal1,*

in all three tissues (**Figures 2D, 3F and 4E**) compared to its counterparts. Hepatic cryptochrome circadian clock 1 (C*ry1*) gene expression was also greater in L18-photoperiod exposed animals than in those exposed to the L12 photoperiod (**Figure 2D**).

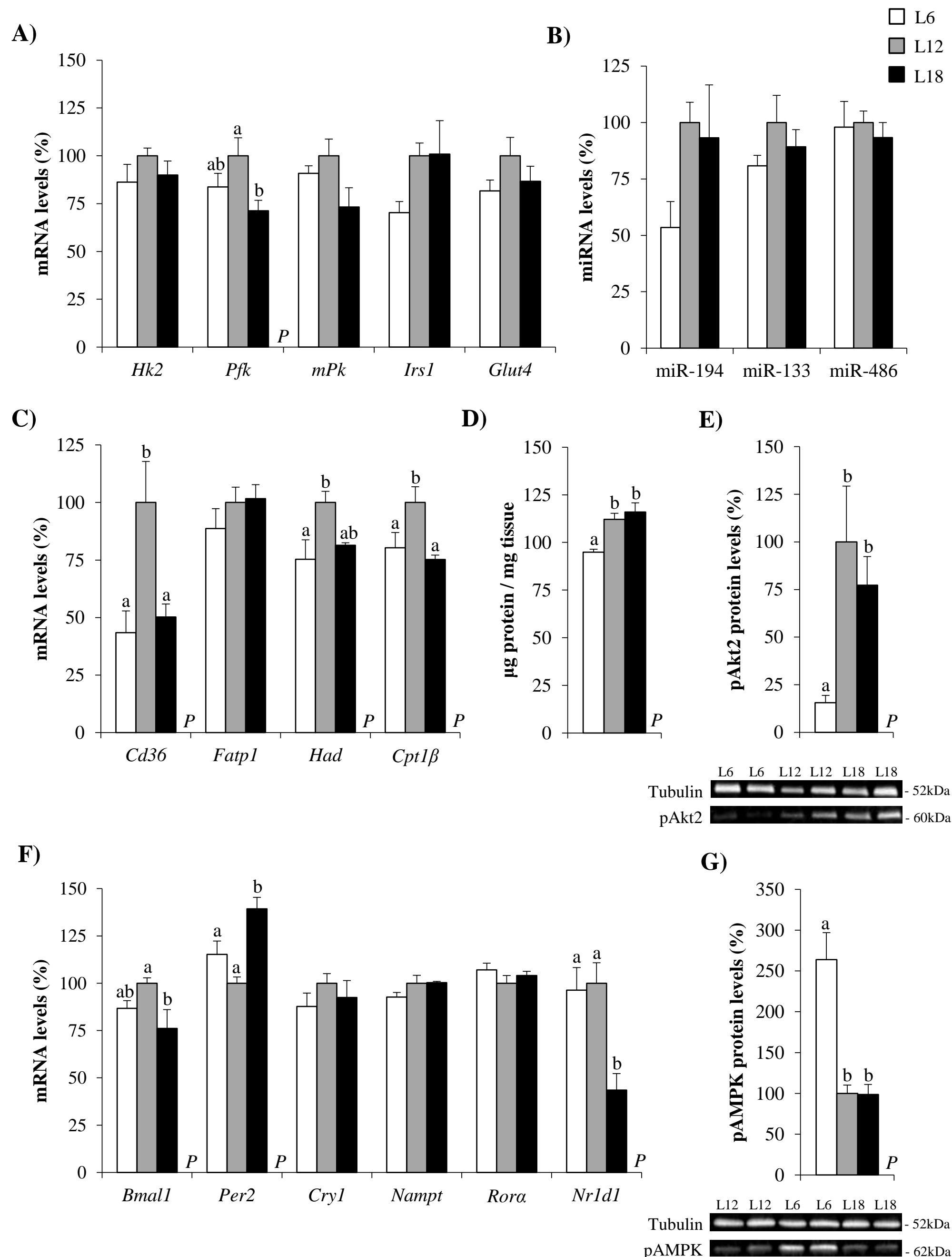


Figure 3. The mRNA levels of glucose metabolism **(A)**, lipid metabolism **(C)** and circadian

rhythm-related genes **(F)**, microRNA expression levels **(B)**, the total protein levels **(D)** and the protein levels of pAkt2 **(E)** and pAMPK **(G)** in the soleus muscle of male Fischer 344 rats fed with standard diet and exposed to three different photoperiods for 14 weeks. Data are represented as the mean ± SEM (n=6). *P*, photoperiod effect (p<0.05, one-way ANOVA). [ab] Mean values with different letters are significantly different among groups (p<0.05, Duncan post hoc test). *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Fatp1*, fatty acid transport protein 1; *Glut4*, glucose transporter 4; *Hk2*, hexokinase 2; *Had*, hydroxyacyl-CoA dehydrogenase; *Irs1*, insulin receptor substrate 1; *mPK*, pyruvate kinase type M; *Pfk*, phosphofructokinase. The rest of the genes analyzed have already been described in **Figure 2**.

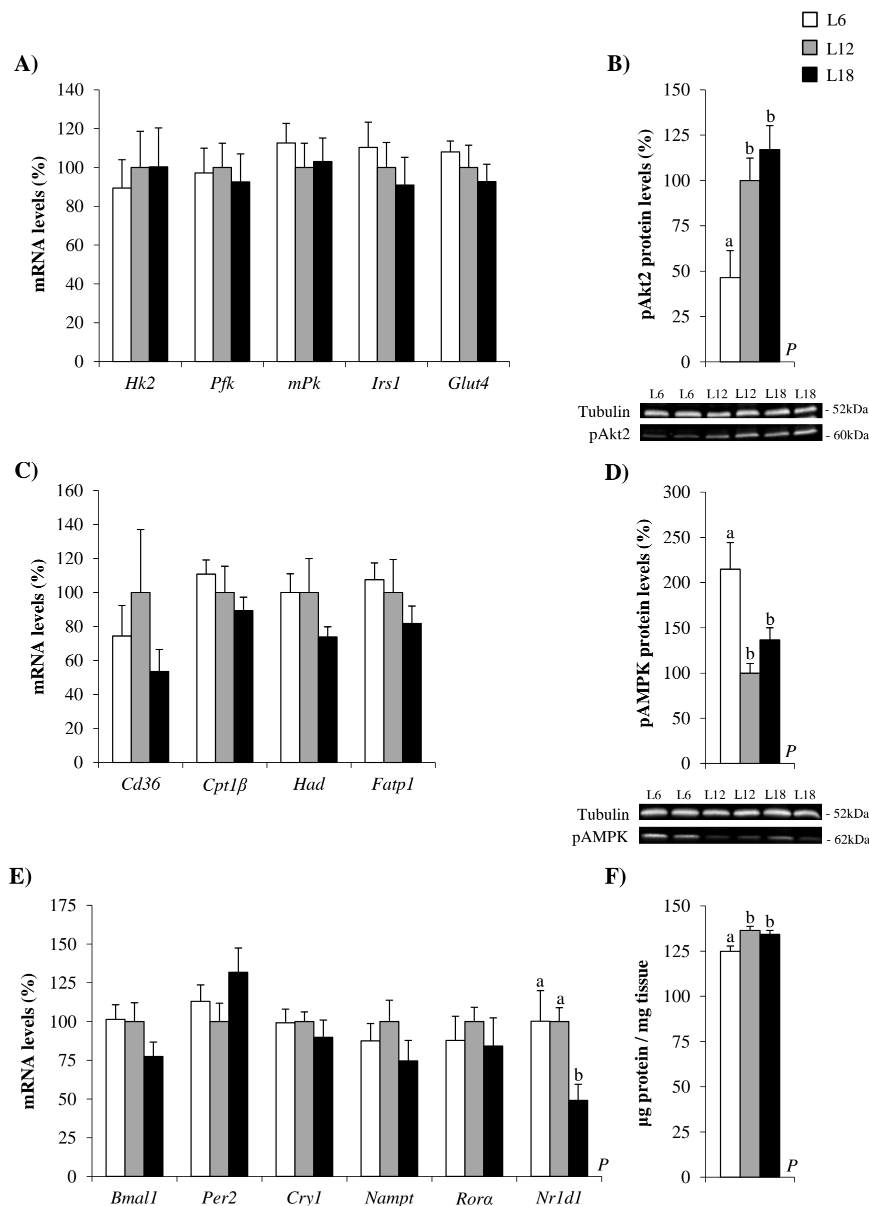


Figure 4. The mRNA levels of glucose metabolism **(A)**, lipid metabolism **(C)** and circadian rhythm-related genes **(E)**, the protein levels of pAkt2 **(B)** and pAMPK **(D)** and the total protein levels **(F)** in the gastrocnemius muscle of male Fischer 344 rats fed with a standard diet and exposed to three different photoperiods for 14 weeks. Data are represented as the mean ± SEM (n=6). *P*, photoperiod effect (p<0.05, one-way ANOVA). [ab] Mean values with different letters are significantly different among groups (p<0.05, Duncan post hoc test). The genes analyzed have been already described in **Figures 2 and 3.**

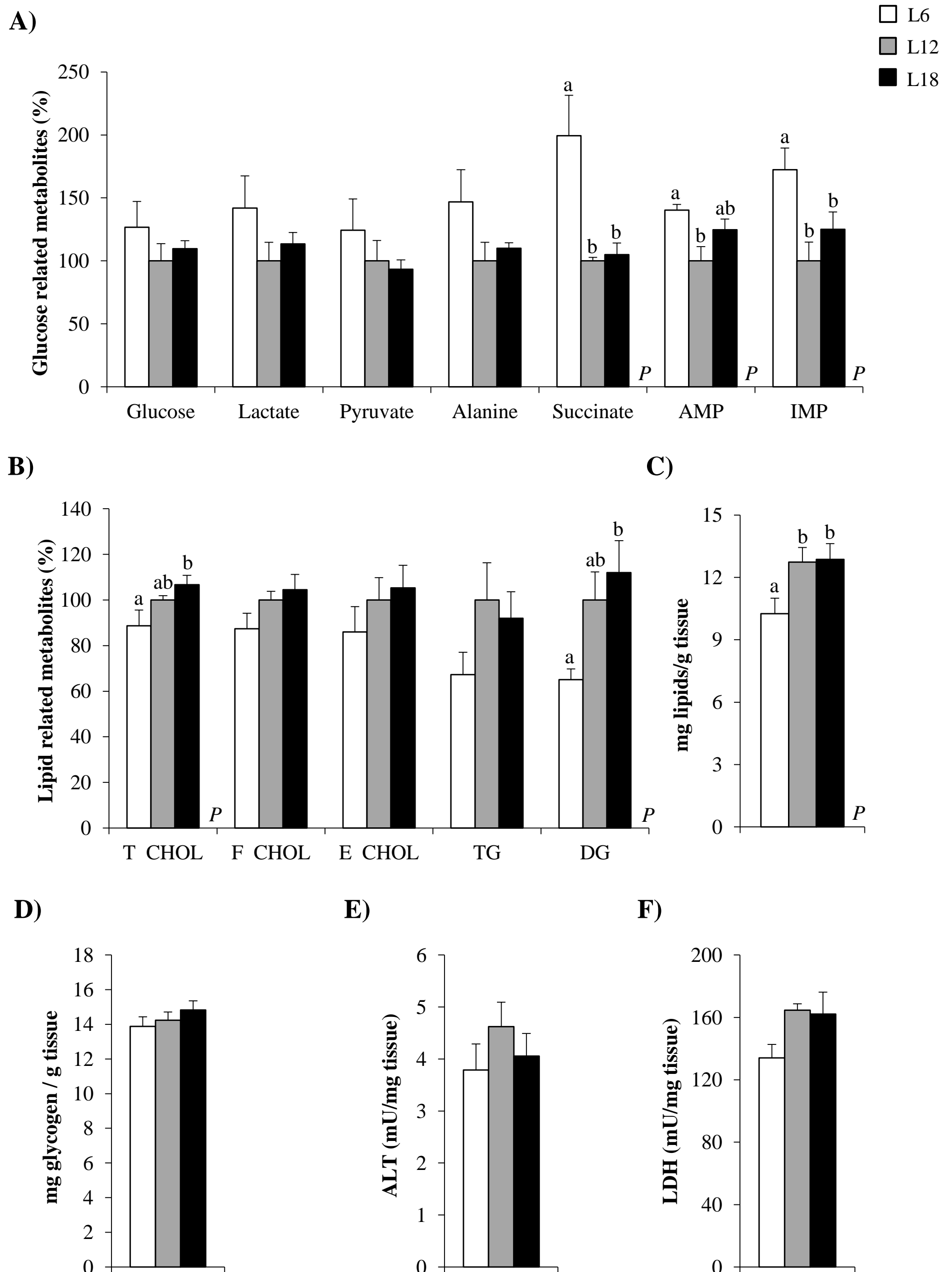


Figure 5. Glucose **(A)** and lipid-related metabolites **(B)**, total lipid **(C)** and glycogen levels **(D)**, alanine aminotransferase (ALT) **(E)** and lactate dehydrogenase (LDH) activity levels **(F)** in the gastrocnemius muscle of male Fischer 344 rats fed a standard diet and exposed to three different photoperiods for 14 weeks. Data are represented as the mean ± SEM (n=6). Gastrocnemius muscle metabolites concentrations (expressed as μmol/g tissue) are shown in the Supplementary Table 4. *P*, photoperiod effect (p<0.05, one-way ANOVA). [ab] Mean values with different letters are significantly different among groups (p<0.05, Duncan post hoc test). AMP, adenosine monophosphate; IMP, inosine monophosphate. The lipid-related metabolites analyzed have been already described in **Figure 1**.

3.10. Multivariate analysis allowed the clear differentiation of animals exposed to different photoperiods

First, all 239 parameters measured in this study were analyzed in a PLS-DA predictive model to obtain which were variables with more differences among the three groups (**Figure 6A**). The quality parameters associated with the model were acceptable. When the scores of 3 components were represented, the degree of fit of the model to the data (R^2) was 0.99, and the result of the cross validation of the model (Q^2) was 0.57, with >0.4 considered an acceptable value for a biological model [42]. Variables with a coefficient mean higher than 50 were selected for PCA multivariate analysis (**Figure 6B**).

A total of 17 variables were obtained and were used to set up a PCA analysis, in which 71% variance was explained when three components were represented. This analysis showed a clear clustering of the different animals depending on the photoperiod in which they were exposed (**Figure 6C**).

Finally, all 17 normalized variables were included in the representation of a heat map, which showed a clear hierarchical clustering among the three groups and revealed which tissues were more affected by each photoperiod (**Figure 6D**).

Among the different parameters that showed higher importance in the separation of the three photoperiod groups, we could mainly observe genes

involved in circadian rhythm regulation (*Nr1d1*, *Per2*, *Bmal1*) and in fatty acid transport and oxidation (*Cd36*, *Cpt1b*) in both liver and skeletal muscles, as well as some circulating metabolites (pyruvate, histidine, formate, creatine) and key proteins (pAkt2 and pAMPK) related to glucose and lipid metabolisms and insulin signaling in the skeletal muscle (**Figures 6B,D**).

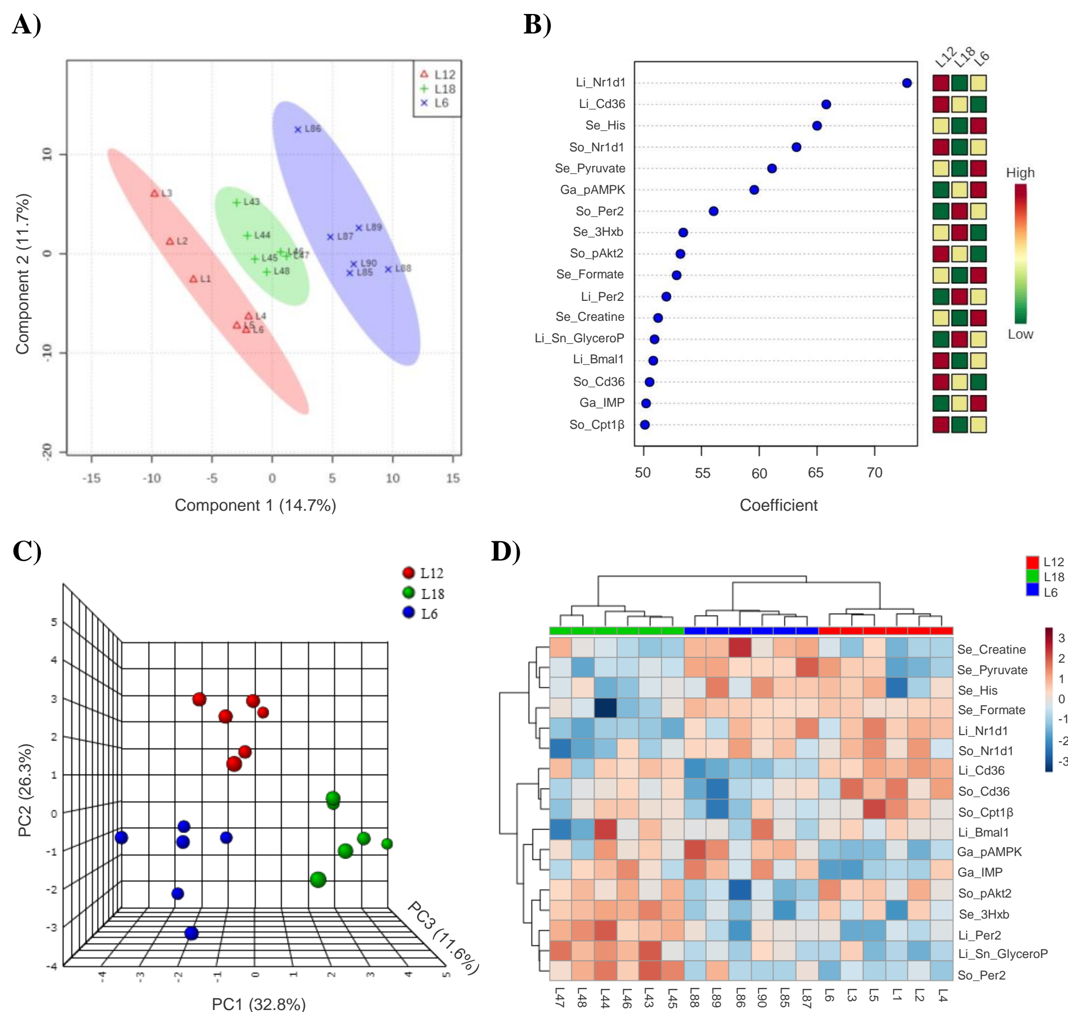


Figure 6. In this study, 239 measured parameters were used to set up a PLS-DA-predictive model **(A)**. Variables with a coefficient mean higher than 50 **(B)** were analyzed in a PCA multivariate analysis **(C)**. The 17 normalized variables were also included in the representation of a Heat Map **(D)**. 3Hxb, 3-hydroxybutyrate; Bmal1, brain and muscle Arnt-like protein-1; Cd36, fatty acid translocase; Cpt1β, carnitine palmitoyltransferase 1 beta; His, histidine; IMP, inosine monophosphate;

Nr1d1, nuclear receptor subfamily 1, group D, member 1; Per2, period circadian clock 2; Sn_GlyceroP, Sn-glycerophosphocholine; Ga, gastrocnemius muscle; Li, liver; Se, serum; So, soleus muscle.

4. DISCUSSION

In the present study, we demonstrated that male F344 rats exhibited profound changes in parameters related with glucose, lipid and energy metabolisms in the serum, liver and skeletal muscle when chronically exposed to different photoperiods. Thus, the multivariate analysis carried out with the 239 parameters analyzed in this study showed a clear clustering depending on the photoperiod in which the animals were exposed. Unexpectedly, among all these results, no significant changes were obtained in the parameters described as the most affected by exposure to different photoperiods, such as body weight gain, cumulative food intake and testes size [1,2,4]. This lack of changes in the aforementioned parameters could be explained by a possible refractoriness to short days in response to a chronic exposition to fewer hours of light. In this sense, Heideman *et al.* [15] and Shoemaker *et al*. [14] demonstrated that F344 rats could become refractory to the chronic effects of short day photoperiods on body weight [14] and testicular parameters [14,15] after 8-10 weeks of exposure. This behavior could be interpreted as an adaptive mechanism to ensure survival and avoid reproductive suppression. Therefore, it is plausible to speculate that, in our study, 14 weeks of exposure to a certain photoperiod would have reversed the photoperiodic effect on these physiological parameters. Another possible reason could rely on the fact that, in the present study, rats were constantly exposed to the same photoperiod for 14 weeks. In nature, the photoperiodic time measurement system is responsive to the direction of the change in day length, in addition to the absolute day length [1,2]. Therefore, the constant day length exposure that occurred during the experiment could have also dampened the photoperiod effects on those parameters. Interestingly, the refractory response observed in rats exposed to

both long and short photoperiods in terms of biometric parameters was not observed in a considerable amount of serum parameters, such as glucose, NEFAs, pyruvate, lactate and different amino acids (alanine, isoleucine, threonine, histidine, tryptophan and tyrosine). Remarkably, five out of seventeen parameters that showed a highest contribution to clearly differentiate the three groups in the multivariate analysis were circulating parameters (histidine, pyruvate, 3-hydroxybutyrate, creatine and formate). These results were also accompanied by clear changes in key genes and proteins involved in both glucose and lipid homeostasis, such as pAkt2, pAMPK, *Cd36* and *Irs1* levels. Altogether, our findings would indicate that the refractoriness phenomenon observed in biometric parameters were not evident at metabolic and molecular level, suggesting a mismatch in the adaptive responses between biometric and biochemical parameters to this refractory phenomenon.

In this sense, both groups of rats displayed higher circulating glucose levels compared to the L12 group. At first glance, the higher glycaemia observed in both L6 and L18 rats could be tentatively attributed to differences in the feeding state among groups at sacrifice, which could be triggered by a different feeding temporal distribution along light and darkness phases. In this regard, the food intake monitoring during the last 24 hours of the study would be useful to shed light on this issue. However, the lack of significant changes in the circulating levels of insulin and glucagon, which are markers of the post-prandial and post-absorptive situations, respectively, as well as the similar insulin:glucagon ratio observed among groups, would strongly suggest that the L6, L12 and L18 animals were at very similar feeding state and that the differences in serum glucose levels were probably explained by other mechanisms. Interestingly, in the L6 animals, the rise of blood glucose levels was accompanied by a sharp downregulation of the pAkt2 in both the soleus and gastrocnemius muscles. This protein is considered a crucial mediator of

signal transduction processes, playing a key role in apoptosis, cell proliferation and metabolism regulation [43,44] and is highly expressed in insulin-responsive tissues, such as the liver, skeletal muscle and adipose tissue [44,45]. After insulin secretion, Akt2 phosphorylation at the Ser^{474} residue promotes the redirection of GLUT4 vesicles from intracellular compartments to the plasma membrane in the skeletal muscle [32,46]. Considering that ~80% of the postprandial glucose uptake occurs in the skeletal muscle [47], it is tempting to hypothesize that the downregulation of pAkt2 observed in both skeletal muscles of L6 rats could significantly contribute to the increase in the circulating glucose levels. Furthermore, the downregulation of the soleus mRNA levels of the gene encoding IRS1, a key protein involved in the activation of Akt2 [33]; the lower levels of miR-194 observed in this tissue, which was also reported in insulin-resistant rats and in prediabetic and diabetic humans [34]; and the decreased hepatic gene expression levels of *Glut2*, the main glucose transporter in the liver [48], could also account for the elevated serum glucose levels displayed by L6 rats. Altogether, these results strongly suggest an impairment of glucose homeostasis and insulin signaling in L6 animals, which was mainly demonstrated at the molecular level in the soleus muscle. Our findings partly agree with those obtained by Tashiro *et al.* [20], who showed that mice exposed to a short photoperiod over 3 weeks displayed decreased insulin sensitivity. However, these authors described a downregulation of *Glut4* mRNA and protein levels in the gastrocnemius muscle. This result contrasts with a lack of significant regulation of this gene in our study in both soleus and gastrocnemius muscles. Since the mRNA levels of *Glut4* did not represent an accurate marker of glucose transport in the skeletal muscle [49], additional measurements focused on GLUT4 translocation could contribute to clarifying this issue. In addition, the downregulation of the hepatic gluconeogenic gene *Pck1* observed in L6 rats would neither be in agreement with the hyperglycemia displayed by these animals, since in mice exposed to disrupted

daily light-dark cycles this metabolic feature was accompanied by an increase in the mRNA levels of *Pck1* and *G6pc* in liver [39]. Further research focused on the hepatic quantification of PCK1 and/or G6PC proteins would be of value to elucidate whether there was an evident alteration of hepatic glucose homeostasis in the animals chronically exposed to the short photoperiod. As it has been fully studied, almost every tissue have molecular clocks that ensure a high robust homeostasis through the rhythmic expression of different metabolic factors [31]. In this regard, one limitation of this study is the fact that all the parameters were only analyzed at a single point. Nevertheless, Shavlakadze *et al.* have previously described that pAkt protein levels in liver and skeletal muscle did not have periodicity over 24h in *ad libitum* fed mice, differently to what was observed in 24h-fasted mice, which showed a clear rhythmicity [50]. These results, together with the fact that, in our study, the animals were only deprived of food for one hour, would suggest that the sharp downregulation of pAkt2 observed in both soleus and gastrocnemius of L6 animals would not be explained by differences in daily rhythmicity among the three groups and would reinforce our hypothesis pointing towards a decreased insulin sensitivity in these rats.

Another relevant result obtained in our study was that exposure to a short photoperiod produced a sharp upregulation of pAMPK in the soleus and gastrocnemius muscles compared to that in both the L12 and L18 groups. This finding could be explained, at least in part, by the elevated levels of AMP observed in the gastrocnemius muscle of L6 rats, since this nucleotide is a cellular stress indicator that acts as the main activator of AMPK [51]. In addition, the increase in the gastrocnemius concentration of IMP found in these animals could indirectly contribute to AMPK activation, since IMP can be converted into AMP and, therefore, may increase the AMP/ATP ratio and, consequently, the AMPK activity [52]. AMPK plays a crucial role in the maintenance of intracellular homeostasis in the skeletal muscle and it is

activated in energy-demanding conditions in order to produce some metabolic effects, such as the enhancement of glucose uptake through the stimulation of GLUT4 translocation in the skeletal muscle [53,54]. Although, at first glance, this result would not support our hypothesis and suggests an increased glucose uptake by the skeletal muscle in L6 animals, it is important to highlight that rats were sacrificed in postprandial conditions (after one hour of fasting). Thus, since the effects of the AMPK pathway on glucose metabolism are mainly produced when cells are metabolically starved, it is plausible to speculate that, in our study, the increased levels of pAMPK did not significantly contribute to enhancing the glucose uptake in the skeletal muscle. Some studies have demonstrated the presence of a cross-talk between Akt and the AMPK pathways, in which Akt can negatively regulate the AMPK activity [55–57]. Windgassen *et al.* demonstrated that Akt has a crucial role in protein synthesis activating mTOR through direct phosphorylation and inhibition of AMPK-mediated phosphorylation of TSC2, a negative regulator of mTOR [55]. In addition, the important role of Akt2 in the regulation of the skeletal muscle mass and function has also been described, although its main function is related with insulin signaling [58]. These results make it plausible to suggest that exposure to a short photoperiod could induce the activation of the proteolytic process through AMPK activation in both soleus and gastrocnemius muscles. The lower protein content observed in both skeletal muscles and the higher circulating levels of several amino acids, including the non-essential amino acid alanine—involved in the muscular ammonia detoxification [59], would reinforce this idea. Otsuka and collaborators demonstrated that exposure to a short photoperiod for three weeks significantly increased the plasma levels of many free amino acids and the marker of muscle degradation 3-methylhistidine in C57BL/6J mice, which could also support our hypothesis [21]. However, our results do not point towards an activation of the Cahill cycle, and no changes were observed in the skeletal muscle weight among the groups. Therefore,

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additional studies focused on the glutamine synthase/glutaminase system in the muscle and liver, as well as the analysis of mTOR protein or ubiquitin-ligases levels, such as MuRF-1 and MAFbx, in the gastrocnemius muscle would be needed to shed more light on this issue [60]. The higher circulating levels of amino acids found in L6 animals compared with L12 and L18 animals could also be attributed to changes in amino acid bioavailability or in the use of this molecules as an energy source for the intestinal cells in response to different chronic day length exposure [61]. Another plausible hypothesis that could contribute to explain the increased pAMPK levels observed in the soleus and gastrocnemius muscles of L6 animals would be a higher levels of activity of these rats prior to the sacrifice. Nevertheless, if this were true, and taking into account that AMPK activates lipid catabolic pathways to increase energy production [62], the upregulation of pAMPK would probably have been accompanied by an upregulation of key genes involved in β-oxidation (*Had, Cpt1β*) as well as in those involved in glucose (*Glut4*) and fatty acid uptake (*Cd36, Fatp1*) in order to deal with a higher energy demanding state. On the contrary, these animals displayed a downregulation of the mRNA levels of the fatty acid transport-related genes *Cd36* (liver and soleus muscle), which, in turn, could contribute to explain the higher circulating levels of non-esterified free fatty acids observed in L6 rats compared to L12. Jain and collaborators demonstrated that Akt2 is critically related to the stimulation of insulin-mediated fatty acid transport [63]. In agreement with these findings, the sharp downregulation of the phosphorylated Akt2 levels in the soleus muscle of L6 rats could also account for decreased fatty acid transport in the muscle, which, in turn, would produce a decrease in β-oxidation, as demonstrated by the lower mRNA levels of *Had* and *Cpt1β*. Additional research is needed to shed more light on the signaling pathways that could account for the aforementioned hepatic gene expression changes.

Although, in our study, the chronic exposure to 18 hours of light also produced clear changes in parameters related with glucose and lipid metabolisms when compared with the exposure to the 12 hours light/day photocycle, the molecular mechanisms involved in these effects were not as evident as those reported in response to the shortening of day length. Thus, the increased circulating levels of glucose observed in L18 animals compared to L12 rats cannot be explained by changes in the phosphorylated levels of Akt2 or AMPK in the liver or skeletal muscle. Since it has been described that the accumulation of hepatic lipids—mainly diglycerides—contributes to altered insulin signaling that could trigger a rise in circulating glucose levels [64], it is tempting to speculate that the increased glycerophosphocholine and diglycerides observed in L18 rats could account for this hyperglycemia. On the other hand, the decreased expression of the fatty acid transport-related genes *Fatp5* (liver) and *Cd36* (liver and soleus) and the downregulation of the soleus *Cpt1β* mRNA levels observed in L18 rats would indicate an alteration of the fatty acid metabolism. However, these molecular changes were not translated into changes in the circulating levels of NEFAs. This response could be tentatively explained by a lower release of these lipids by white adipose tissue—the major contributor of NEFAs to the bloodstream—or by an enhancement of fatty acid uptake by these fat depots or other tissues, which could be understood as a compensatory mechanism addressed to counteract the decreased uptake and utilization of these metabolites in the liver and skeletal muscle [65,66]. Another possible explanation relies on the fact that the gene expression data does not always match protein levels and, therefore, the quantification of the hepatic and muscular levels of these fatty acid metabolism-related proteins would be useful to clarify this issue.

As it has been aforementioned, one of the physiological mechanisms involved in seasonal responsiveness is the synchronization of circannual rhythms to the astronomical season using the photoperiod [67]. In spite of the interest in

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elucidating whether the clock genes can also act as circannual timers in addition to circadian timers, their complex behavior in different tissues and the influence of other environmental factors makes it difficult to fully understand how they work [68,69]. Moreover, it has been described that these clock-related nuclear receptors are involved in the regulation of lipid and glucose metabolism in liver and skeletal muscle and that their dysregulation could produce significant variations in key genes belonging to glucose and lipid metabolic pathways, such as *Gk* and *Cd36* [48,70], which were clearly affected by long photoperiod exposure in our study. In this sense, although we could only measure the circadian rhythm-related genes expression at a single point (ZT 1 to 2), we detected profound changes in *Per2, Bmal1* and *Nr1d1* in the liver and the soleus and gastrocnemius muscles of L18 animals. These results could contribute to explain the alterations in glucose and lipid metabolism observed in these rats and would suggest that L18 animals displayed a misalignment of the circadian rhythm in comparison with L12 and L6 groups. On the contrary, L6 animals showed a very similar behavior concerning the clock gene expression pattern than L12 rats, suggesting that the metabolic and biochemical changes observed between these two groups were mainly due to the chronic adaptation to different photoperiods. However, a more profound analysis carried out at different daily time points throughout a 24-h period would be needed to corroborate this hypothesis.

5. CONCLUSION

We reported that chronic exposure to short and long day lengths strongly modulates a wide range of parameters related to glucose, lipid and nitrogenate metabolism in the blood, liver and in both the soleus and gastrocnemius skeletal muscles of normoweight Fischer 344 rats. Furthermore, we have also partly elucidated the molecular mechanisms that would explain the elevated circulating levels of glucose and NEFAs observed in the animals exposed to the

short photoperiod, which were as follows: 1) a sharp downregulation of the phosphorylated Akt2 levels in both soleus and gastrocnemius muscles; and 2) decreased expression in the soleus muscle of the glucose metabolism-related microRNA-194 and lower mRNA levels of the genes involved in glucose metabolism (*Irs1*, soleus, and *Glut2*, liver), β-oxidation (*Had* and *Cpt1β*, soleus) and fatty acid transport (*Cd36*, soleus and liver). Although several studies have demonstrated the effects of circadian rhythm disruption on the development of insulin resistance [71–73], to the best of our knowledge, this is the first study that showed relevant changes in glucose and lipid metabolism produced by chronic exposition to different photoperiods in Fischer 344 rats. Despite 24-h kinetic analyses of locomotor activity, food intake and clock genes' expression would have been of great value to strengthen our findings, this study highlight the importance of circannual rhythms in the metabolic homeostasis regulation. In addition, our results pave the way for the use of Fischer 344 rats as a preclinical model to study the effect of the photoperiod on different altered conditions or diseases that occur in humans and are related to lipid and glucose metabolisms, such as obesity, CVD and SAD, which are potentially sensitive to photoperiodic changes [6,9,23]. Further studies carried out with diet-induced obese rats are planned to elucidate whether the exposure to short and long photoperiods exacerbates these metabolic responses under a situation of altered homeostasis robustness.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Cinta Bladé (CB), Lluís Arola (LA), Antoni Caimari (AC) and Josep del Bas (JdB) designed the studies; Roger Mariné (RM), Cristina Domenech (CD), AC and JdB performed the experiments and analyzed the data; RM, AC and LA wrote the manuscript. All the authors read, discussed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Nucleotide sequences of primers used for real time quantitative PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Tissue
Acc1	TGCAGGTATCCCCACTCTTC	TTCTGATTCCCTTCCCTCCT	*L*
β-actin	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	*L*
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC	*G, L, S*
Cd36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG	*G, L, S*
Cpt1α	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG	*L*
Cpt1β	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	*G, S*
Cry1	TGGAAGGTATGCGTGTCCTC	TCCAGGAGAACCTCCTCACG	*G, L, S*
Dgat1	CAGACAGCGGTTTCAGCAAT	AGGGGTCCTTCAGAAACAGAG	*L*
Fatp1	TGCTCAAGTTCTGCTCTGGA	CATGCTGTAGGAATGGTGGC	*G, S*
Fatp5	CCTGCCAAGCTTCGTGCTAAT	GCTCATGTGATAGGATGGCTGG	*L*
Fbp1	TGACCCTGCCATCAATGAGT	ATGTCTTCATTCCCCGTCGT	*L*
G6pc	ATTCCGGTGCTTGAATGTCG	TGGAGGCTGGCATTGTAGAT	*L*
G6pdh	ACCAGGCATTCAAAACGCAT	CAGTCTCAGGGAAGTGTGGT	*L*
Gk	CTGTGAAAGCGTGTCCACTC	GCCCTCCTCTGATTCGATGA	*L*
Glut2	AGTCACACCAGCACATACGA	TGGCTTTGATCCTTCCGAGT	*L*
Glut4	CCATTGCTTCTGGCTATCAC	TCCGTTTCTCATCCTTCAGC	*G, S*
Gpat	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG	*L*
Hk2	GAAGATGCTGCCCACTTACG	GCCATGCATAACCTCCTGTG	*G, S*
Had	ATCGTGAACCGTCTCTTGGT	AGGACTGGGCTGAAATAAGG	*G, S*
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG	*G, L, S*
Irs1	CTACACCCGAGACGAACACT	TAACCTGCCAGACCTCCTTG	*G, S*
mPk	AGCCTCCAGTCAATCCACAG	GCATCCTTACACAGCACAGG	*G, S*
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG	*G, L, S*
Nr1d1	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT	*G, L, S*
Per2	CGGACCTGGCTTCAGTTCAT	AGGATCCAAGAACGGCACAG	*G, L, S*
Pck1	GCAAACCAGCAAGCACAATG	CTCGAAGTGGAACCAAACCC	*L*
Pfk	GTGGATGGTGGAGAGCACAT	TCCGATGACACACAGATTGG	*G, S*
Ppia	CCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCAAAACGCT	*G, L, S*
Rorα	CCCGATGTCTTCAAATCCTTAGG	TCAGTCAGATGCATAGAACACAAACTC	*G, L, S*

Srebp1c	CCCACCCCCTTACACACC	GCCTGCGGTCTTCATTGT	*L*
Tfrc	ATCATCAAGCAGCTGAGCCAG	CTCGCCAGACTTTGCTGAATTT	*S*

The table shows the nucleotide sequences of primers used for PCR amplification. Primer pairs for PCR were designed using Primer3 software and the sequence information were obtained from Genbank. *Acc1*, acetyl CoA carboxylase 1; *β-actin*, actin beta; *Bmal1,* brain and muscle Arnt-like protein-1; *Cd36*, fatty acid translocase, homologue of CD36; *Cpt1α*, carnitine palmitoyltransferase 1 alpha; *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Cry1,* cryptochrome circadian clock 1; *Dgat1*, diacylglycerol acyltransferase 1; *Fatp1*, fatty acid transport protein 1; *Fatp5*, fatty acid transport protein 5; *Fbp1*, fructose-1,6-biphosphatase 1; *G6pc*, glucose-6-phosphatase, catalytic subunit; *G6pdh*, glucose-6-phosphate dehydrogenase; *Gk*, glucokinase; *Glut2*, glucose transporter 2; *Glut4,* glucose transporter 4; *Gpat*, glycerol-3-phosphate acyltransferase; *Hk2*, hexokinase 2; *Had*, hydroxyacyl-CoA dehydrogenase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Irs1,* insulin receptor substrate 1; *mPK*, pyruvate kinase type M; *Nampt,* nicotinamide phosphoribosyltransferase; *Nr1d1,* nuclear receptor subfamily 1, group D, member 1; *Per2,* period circadian clock 2; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Pfk*, phosphofructokinase; *Ppia*, peptidylprolyl isomerase A; *Rorα*, RAR-related orphan receptor A; *Srebp1c*, sterol regulatory element-binding protein 1c; *Tfrc*, transferrin receptor. Gene expression levels were analyzed in gastrocnemius (G) and soleus (S) muscles and liver (L).

Supplementary Table 2. Serum metabolite concentrations analyzed by Nuclear Magnetic Resonance in response to different photoperiod exposure in animals fed a standard diet for 14 weeks.

Metabolite concentration (μmol/L)	L6	L12	L18
2-Methylglutarate	1.97 ± 0.20	2.23 ± 0.08	2.40 ± 0.21
Choline	4.90 ± 0.28	4.55 ± 0.17	4.77 ± 0.16
Citrate	11.28 ± 1.18	9.40 ± 1.13	9.94 ± 0.82
Creatine Phosphate	5.15 ± 0.13	5.35 ± 0.32	5.21 ± 0.68
Cytosine	5.34 ± 0.40	4.55 ± 0.44	4.87 ± 0.46
Leucine	32.82 ± 1.32	31.66 ± 1.27	31.52 ± 1.98
Methionine	21.34 ± 0.74	19.66 ± 1.02	20.10 ± 0.88
O-acetylcarnitine	3.48 ± 0.11	3.28 ± 0.24	3.18 ± 0.19
Phenylalanine	10.20 ± 0.34	9.59 ± 0.25	9.61 ± 0.51
Serine	34.79 ± 1.43	32.70 ± 1.73	32.16 ± 1.82
Thymidine	3.92 ± 0.56	4.34 ± 0.37	4.26 ± 0.75
Valine	36.17 ± 1.51	34.33 ± 1.11	33.02 ± 1.79

Male Fischer 344 rats were fed a standard diet and were exposed to three different photoperiods for 14 weeks. Data are expressed as mean ± SEM (n=6). All the metabolites were obtained by performing a Nuclear Magnetic Resonance (NMR) analysis.

Supplementary Table 3. Liver metabolite concentrations analyzed by Nuclear Magnetic Resonance in response to different photoperiod exposure in animals fed a standard diet for 14 weeks.

	L6	L12	L18	
Aqueous fraction (μmol/g tissue)				
2-Deoxyadenosine	0.05 ± 0	0.04 ± 0.01	0.05 ± 0	
3-Hydroxybutyrate	2.41 ± 0.23	2.66 ± 0.24	2.46 ± 0.31	
Acetate	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.03	
Alanine	0.91 ± 0.01[a]	0.95 ± 0.04[a]	1.07 ± 0.04[b]	*P*
Allantoin	0.22 ± 0.02	0.22 ± 0.02	0.20 ± 0.02	
AMP	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0	
β-Alanine	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0	
Betaine	2.93 ± 0.12	3.04 ± 0.30	3.17 ± 0.09	
Carnosine	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0	
Creatine	0.03 ± 0	0.03 ± 0	0.03 ± 0	
Creatinine	0.02 ± 0	0.02 ± 0	0.02 ± 0	
Cytidine	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	
Dimethylamine	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0	
Formate	0.10 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	
Glucose	7.83 ± 0.21	7.89 ± 0.48	7.15 ± 0.09	
Glutamate	0.14 ± 0.01	0.14 ± 0.02	0.16 ± 0.02	
Glutamine	0.50 ± 0.04	0.48 ± 0.06	0.47 ± 0.01	
Glycine	0.58 ± 0.01	0.57 ± 0.03	0.56 ± 0.01	
IMP	0.06 ± 0	0.05 ± 0	0.05 ± 0.01	
Inosine	0.66 ± 0.03	0.63 ± 0.03	0.69 ± 0.03	
Isoleucine	0.10 ± 0	0.10 ± 0.01	0.10 ± 0.01	
Lactate	1.00 ± 0.02	1.24 ± 0.10	1.35 ± 0.14	
Leucine	0.28 ± 0.01	0.30 ± 0.03	0.27 ± 0.02	
Mannose	0.25 ± 0.01	0.24 ± 0.02	0.22 ± 0.01	
Methionine	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	
Niacinamide	0.38 ± 0.03	0.37 ± 0.03	0.37 ± 0.02	

N,N-dimethylglycine	0.02 ± 0	0.02 ± 0	0.02 ± 0	
O-Phosphocholine	0.07 ± 0	0.08 ± 0.02	0.06 ± 0	
Oxypurinol	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	
Phenylalanine	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	
Pyruvate	0.16 ± 0.02	0.11 ± 0.01	0.15 ± 0.02	
Sarcosine	0.04 ± 0	0.04 ± 0	0.05 ± 0.01	
Succinate	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	
Taurine	2.63 ± 0.13	2.57 ± 0.23	2.84 ± 0.20	
Trimethylamine	0.01 ± 0	0.01 ± 0	0.01 ± 0	
Tyrosine	0.09 ± 0	0.09 ± 0	0.09 ± 0	
UDP-Glucoronate	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	
Uracil	0.12 ± 0.02	0.12 ± 0.02	0.11 ± 0	
Uridine	0.61 ± 0.02	0.58 ± 0.03	0.62 ± 0.02	
Uroconate	0.19 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	
Valine	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	
Xanthine	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	
Lipid fraction (µmol/g tissue)				
ARA+EPA	0.02 ± 0	0.02 ± 0	0.02 ± 0	
DHA	0.002 ± 0	0.003 ± 0	0.003 ± 0	
Diglycerides	1.24 ± 0.06	1.15 ± 0.04	1.34 ± 0.05	
Esterified cholesterol	0.61 ± 0.04	0.55 ± 0.06	0.62 ± 0.05	
Free cholesterol	2.78 ± 0.08	2.68 ± 0.07	2.85 ± 0.04	
Glycerophosphocholine	0.04 ± 0[ab]	0.04 ± 0[a]	0.05 ± 0[b]	*P*
Linoleic acid	0.02 ± 0	0.02 ± 0	0.02 ± 0	
Lysophosphatidylcholine	2.97 ± 0.08	3.25 ± 0.33	3.02 ± 0.20	
Monoglycerides	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	
MUFA	0.03 ± 0	0.03 ± 0	0.03 ± 0	
Oleic acid	0.01 ± 0	0.01 ± 0	0.01 ± 0	
Omega-3	0.01 ± 0	0.01 ± 0	0.01 ± 0	
Phosphatidylcholine	11.23 ± 0.28	11.50 ± 0.27	12.01 ± 0.36	
Phosphoethanolamine	3.82 ± 0.08	3.55 ± 0.16	3.65 ± 0.14	

Phosphoinositol	1.21 ± 0.05	1.19 ± 0.10	1.27 ± 0.06
Plasmalogen	0.27 ± 0.01	0.24 ± 0.01	0.27 ± 0.01
PUFA	0.07 ± 0	0.07 ± 0	0.07 ± 0
Sphingomyelin	0.78 ± 0.02	0.74 ± 0.04	0.78 ± 0.02
Total cholesterol	3.37 ± 0.11	3.21 ± 0.12	3.44 ± 0.04
Total FA chain	482.15 ± 21.99	456.53 ± 23.86	505.93 ± 14.44
Total phospholipids	13.10 ± 0.23	13.01 ± 0.82	13.72 ± 0.59
Triglycerides	4.66 ± 0.36	4.21 ± 0.26	4.84 ± 0.18

Male Fischer 344 rats were fed a standard diet and were exposed to three different photoperiods for 14 weeks. Data are expressed as mean ± SEM (n=6). All the metabolites were obtained by performing a Nuclear Magnetic Resonance (NMR) analysis. One-way ANOVA and Duncan's post-hoc test were performed to compare the values between groups and significant differences were represented with different letters (a, b). *P* Photoperiod effect.

Supplementary Table 4. Gastrocnemius muscle metabolite concentrations analyzed by Nuclear Magnetic Resonance in response to different photoperiod exposure in animals fed a standard diet for 14 weeks.

	L6	L12	L18	
Aqueous fraction (μmol/g tissue)				
3-Hydroxybutyrate	0.13 ± 0.01	0.13 ± 0.03	0.14 ±0.02	
3-Hydroxyisobutyrate	0.03 ± 0	0.02 ± 0	0.02 ± 0	
3-Methyl-2-oxovalerate	0.02 ± 0	0.01 ± 0	0.02 ± 0	
Acetate	0.02 ± 0	0.02 ± 0	0.02 ± 0	
ADP	0.43 ± 0.09	0.36 ± 0.07	0.35 ± 0.07	
Alanine	1.66 ± 0.29	1.13 ± 0.17	1.24 ± 0.05	
AMP	0.08 ± 0[a]	0.06 ± 0.01[b]	0.07 ± 0.01[ab]	*P*
Anserine	4.01 ± 0.56	2.76 ± 0.35	2.91 ± 0.12	
β-Alanine	0.14 ± 0.02	0.10 ± 0.02	0.12 ± 0.01	
Carnitine	0.58 ± 0.08	0.43 ± 0.07	0.49 ± 0.02	
Carnosine	0.23 ± 0.02	0.23 ± 0.01	0.23 ± 0.01	
Choline	0.04 ± 0	0.03 ± 0	0.03 ± 0	
Creatine	14.50 ± 1.85	10.91 ± 1.35	12.27 ± 0.41	
CreatinePhosphate	0.85 ± 0.09	0.68 ± 0.13	0.97 ± 0.19	
Creatinine	0.21 ± 0.03	0.16 ± 0.02	0.17 ± 0.01	
Dimethylglycine	0.04 ± 0.01	0.03 ± 0	0.04 ± 0	
Dimethylsulfone	0.04 ± 0.01	0.03 ± 0	0.04 ± 0	
Fucose	0.15 ± 0.02	0.10 ± 0.01	0.13 ± 0.02	
Fumarate	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	
Glucose	1.72 ± 0.28	1.36 ± 0.19	1.49 ± 0.08	
Glutamate	0.36 ± 0.06	0.33 ± 0.04	0.37 ± 0.03	
Glutamine	1.44 ± 0.20	1.07 ± 0.16	1.29 ± 0.08	
Glutathione	0.13 ± 0.03	0.09 ± 0.01	0.08 ± 0.01	
Glycerol	0.32 ± 0.06	0.22 ± 0.03	0.23 ± 0.01	
Glycine	1.23 ± 0.18	0.83 ± 0.09	0.89 ± 0.06	
IMP	0.88 ± 0.09[a]	0.51 ± 0.08[b]	0.64 ± 0.07[b]	*P*

Inosine+ NAD	0.27 ± 0.05	0.21 ± 0.03	0.27 ± 0.02	
Isoleucine	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0	
Lactate	20.67 ± 3.72	14.57 ± 2.14	16.53 ± 1.31	
Leucine	0.11 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	
Lysine	0.12 ± 0.01	0.10 ± 0.01	0.10 ± 0	
Methylhistidine	0.07 ± 0.01	0.06 ± 0	0.06 ± 0.01	
NAD+	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0	
Niacinamide	0.17 ± 0.02	0.14 ± 0.02	0.16 ± 0.01	
O-Acetylcarnitine	0.19 ± 0.02	0.14 ± 0.01	0.16 ± 0.01	
Panthothenate	0.01 ± 0	0.01 ± 0	0.01 ± 0	
Phenylalanine	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0	
Proline	0.29 ± 0.04	0.23 ± 0.04	0.28 ± 0.02	
Pyruvate	0.16 ± 0.03	0.13 ± 0.02	0.12 ± 0.01	
Succinate	0.20 ± 0.03[a]	0.10 ± 0[b]	0.11 ± 0.01[b]	*P*
Taurine	6.37 ± 0.79	5.06 ± 0.61	6.12 ± 0.41	
Tyrosine	0.09 ± 0.01	0.07 ± 0.01	0.07 ± 0	
Valine	0.10 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	
Xanthine	0.05 ± 0.01	0.05 ± 0	0.05 ± 0	
Lipid fraction (µmol/g tissue)				
ARA+EPA	0.02 ± 0	0.01 ± 0	0.01 ± 0	
DHA	0.01 ± 0	0.01 ± 0	0.01 ± 0	
Diglycerides	3.82 ± 0.56	5.67 ± 0.92	5.22 ± 0.66	
Esterified cholesterol	0.17 ± 0.02	0.20 ± 0.02	0.21 ± 0.02	
Free cholesterol	0.84 ± 0.07	0.96 ± 0.04	1.00 ± 0.06	
Linoleic acid	0.03 ± 0	0.03 ± 0	0.03 ± 0	
Monoglycerides	0.07 ± 0.01	0.05 ± 0.01	0.06 ±0.01	
MUFA	0.04 ± 0	0.04 ± 0	0.04 ± 0	
Oleic acid	0.01 ± 0	0.02 ± 0	0.02 ± 0	
Omega-3	0.02 ± 0	0.01 ± 0	0.02 ± 0	
Phosphatidylcholine	4.22 ± 0.36	4.78 ± 0.14	4.94 ± 0.35	
Phosphoethanolamine	1.97 ± 0.23	2.22 ± 0.10	2.42 ± 0.22	

Phosphoinositol	0.40 ± 0.04	0.45 ± 0.02	0.48 ± 0.03	
Plasmalogen	0.63 ± 0.11	0.75 ± 0.02	0.85 ± 0.11	
PUFA	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	
Sphingomyelin	0.11 ± 0.01	0.13 ± 0	0.13 ± 0.01	
Total cholesterol	0.94 ± 0.07[a]	1.06 ± 0.02[ab]	1.13 ± 0.04[b]	*P*
Total FA chain	123.45 ± 12.73	167.15 ± 16.91	162.07 ± 14.78	
Triglycerides	3.82 ± 0.56	5.67 ± 0.92	5.22 ± 0.66	

Male Fischer 344 rats were fed a standard diet and were exposed to three different photoperiods for 14 weeks. Data are expressed as mean ± SEM (n=6). All the metabolites were obtained by performing a Nuclear Magnetic Resonance (NMR) analysis. One-way ANOVA and Duncan's post-hoc test were performed to compare the values between groups and significant differences were represented with different letters (a, b). *P* Photoperiod effect.

UNIVERSITAT ROVIRA I VIRGILI
PHYSIOLOGICAL AND METABOLIC ADAPTATIONS TO DIFFERENT PHOTOPERIODS: EFFECTS OF OBESITY AND
SEASONAL FRUIT CONSUMPTION
Roger Mariné Casadó

MANUSCRIPT 2

Intake of an obesogenic cafeteria diet affects body weight, feeding behavior and glucose and lipid metabolism in a photoperiod-dependent manner in F344 rats

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ABSTRACT

We previously demonstrated that chronic exposure to different photoperiods induced marked variations in several glucose and lipid metabolism-related parameters in normoweight Fischer 344 (F344) rats. Here, we examined the effects of the combination of an obesogenic cafeteria diet (CAF) and the chronic exposure to three different day lengths (L12, 12 h light/day; L18, 18 h light/day and L6, 6 h light/day) in this rat strain. Although no changes were observed during the first 4 weeks of adaptation to the different photoperiods in which animals were fed a standard diet, the addition of the CAF for the subsequent 7 weeks triggered profound physiologic and metabolic alterations in a photoperiod-dependent manner. Compared with L12 rats, both L6 and L18 animals displayed lower body weight gain and cumulative food intake in addition to decreased energy expenditure and locomotor activity. These changes were accompanied by differences in food preferences and by a sharp upregulation of the orexigenic genes *Npy* and *Ghsr* in the hypothalamus, which could be understood as a homeostatic mechanism for increasing food consumption to restore body weight control. L18 rats also exhibited higher glycemia than the L6 group, which could be partly attributed to the decreased pAkt2 levels in the soleus muscle and the downregulation of *Irs1* mRNA levels in the gastrocnemius muscle. Furthermore, L6 animals displayed lower whole-body lipid utilization than the L18 group, which could be related to the lower lipid intake and to the decreased mRNA levels of the fatty acid transporter gene *Fatp1* observed in the soleus muscle. Although further research is needed to elucidate the pathophysiologic relevance of these findings, our study could contribute to emphasize the impact of the consumption of highly palatable and energy dense foods regularly consumed by humans on the physiological and metabolic adaptations that occur in response to seasonal variations of day

length, especially in diseases associated with changes in food intake and preference such as obesity and seasonal affective disorder.

Key words: photoperiod, circannual rhythms, cafeteria diet, metabolic syndrome, feeding behavior, glucose homeostasis.

1. INTRODUCTION

Seasonal variations in environmental factors provide crucial information to animals, allowing them to adapt their organism through changes in many physiological and behavioral parameters [1,2]. Despite human isolation from environmental annual changes as a consequence of the appearance of heat and air-conditioning systems and artificial light in developed economies [3], several studies have proven that human patterns of birth, death or disease are season-dependent [4]. In addition, body fat mass accretion and circulating cholesterol, triglycerides, leptin, glucose and insulin levels can be significantly increased in winter, accounting for higher rates of cardiac events in this season [3,5–8]. For example, in a study performed on 1,202 Japanese male workers, Kamezaki and collaborators reported higher systolic and diastolic blood pressure and increased fasting blood glucose levels in winter than in summer, concluding marked seasonal variation in the prevalence of metabolic syndrome (MetS) [9], which is defined as a cluster of interconnected risk factors—obesity, insulin resistance, dyslipidemia and hypertension—that increase the risk of cardiovascular disease (CVD) [10,11]. These authors also described that the higher MetS incidence observed during winter was associated with a moderate increase in insulin resistance [12].

Among the different environmental conditions that vary throughout the year, some, such as temperature and food availability, are considered low predictive factors since they do not display specific timing or magnitude [13]. However, seasonal variations in day length are the main environmental cue that offers a

highly predictive signal of the correct time of year [13]. Due to the possibility of constantly controlling the photoperiod, laboratory animals have emerged as a valuable model to gain knowledge on how humans respond to seasonal variations in day length. The F344 rat strain is a clear example of an animal model that has become relevant in the study of circannual rhythms [14–18]. Our group recently described that normoweight F344 rats exposed to different photoperiods for 14 weeks displayed profound metabolic changes, highlighting the importance that the seasonal changes in day length can have on health and suggesting these rats as a promising animal model with which to study glucose- and lipid-related pathologies that are influenced by seasonal variations, such as obesity, MetS and CVD [19]. These effects were more evident in rats held under a short day (SD) photoperiod (6 h of light), which showed an insulin resistance-like phenotype, as evidenced by increased circulating glucose levels, a vast downregulation of the muscular downstream postreceptor target of insulin Akt serine/threonine kinase 2 (Akt2), and decreased gene expression of the hepatic glucose transporter *Glut2* and the muscular insulin receptor substrate 1 (*Irs1*) [19]. Our results partly agree with those reported by Tashiro *et al.*, which revealed that C57BL/6J mice exposed to SD conditions (8 h of light) for 3 weeks displayed higher circulating glucose levels, which were explained by the reduced glucose transporter 4 (GLUT4) protein levels in the gastrocnemius muscle [20]. These authors also demonstrated that this animal model exhibited increased body weight, fat mass accretion and sucrose intake and a depression-like behavior, partly mirroring the seasonal affective disorder (SAD) that occurs in humans mainly in winter [21,22].

There is little information regarding the effects of chronic exposure to different photoperiods under obesogenic conditions. In male obese Zucker rats, which display genetic obesity and type 2 diabetes due to deficiencies in leptin receptor, Larkin *et al*. described that exposure to a long day (LD) photoperiod (14 h of light) for 9 weeks increased the insulin circulating levels, lean body

mass and energy efficiency compared with exposure to an SD photoperiod (10 h of light). In addition, obese Zucker rats exhibited a more pronounced response to photoperiod exposure than their lean counterparts [23]. Nevertheless, highly caloric palatable diet-induced obese models are more representative of the etiology of obesity and MetS in modern societies [24], since genetics contributes to a lesser extent to the development of obesity and its comorbidities than sedentary lifestyles combined with excess energy intake [25]. In this sense, Togo and collaborators reported that F344 rats held under LD conditions (16 h of light) and fed a high-fat diet (HFD) for 3 weeks displayed increased body weight, epididymal adipose tissue and leptin levels compared with animals exposed to an SD (8 h of light) [26]. In another experiment performed under the same photoperiodic conditions, it was demonstrated that photoperiod regulated feeding behavior, which was evidenced by a higher preference for a low-fat, high-carbohydrate diet than for the HFD in LD F344 rats, an effect that was not observed in SD animals [26]. In contrast, Ross *et al.* described that the photoperiodic regulation of different parameters, such as the stimulation of fat mass in LD photoperiods, was dampened after HFD feeding, whereas lean mass and other photoperiod-responsive parameters were unaffected by HFD exposure [27].

Among the high caloric diets, the CAF, which contains a variety of highly palatable and energy dense foods prevalent in Western society, has become a more useful choice than a HFD to resemble metabolic and eating behavioral processes underlying diet-induced human obesity and MetS [28–30]. Nevertheless, the effects of the combination of a CAF with different photoperiod exposures on physiology and metabolic homeostasis in F344 rats have not yet been examined. In the present study, we hypothesized that physiologic- and metabolic-related parameters of CAF-fed obese F344 rats would be influenced by chronic exposure to different day lengths. Therefore, the aim of the present work was to study the photoperiodic changes in a variety of physiological and

metabolic outputs of F344 rats fed a CAF.

2. MATERIALS AND METHODS

2.1. Animals

Thirty 8-week-old male F344 rats (Charles River Laboratories, Barcelona, Spain) were housed in pairs in cages at 22ºC and submitted to three different light schedules for 11 weeks to mimic seasonal day lengths: SD photoperiod (n=10, L6, 6 h light—from Zeitgeber times (ZTs) 0 to 6—and 18 h darkness—from ZTs 6 to 24), normal day (ND) photoperiod (n=10, L12, 12 h light—from ZTs 0 to 12—and 12 h darkness—from ZTs 12 to 24) and LD photoperiod (n=10, L18, 18 h light—from ZTs 0 to 18—and 6 h darkness—from ZTs 18 to 24). The three groups were subjected to a 4-week adaptation period in which animals were fed a standard diet (STD) *ad libitum* (2.90 kcal·g^{-1}; Teklad Global 14% Protein Rodent Diet 2014, ENVIGO, Sant Feliu de Codines, Barcelona, Spain). After this period, rats were switched to a CAF for 7 weeks. The CAF contained bacon, biscuit with pâté and biscuit with cheese, carrots, muffins and milk with sugar (22 g/L). Its caloric distribution was 58.1% carbohydrate, 31.9% lipid and 10.0% protein, as previously described [31]. During the entire study, rats had free access to food and water, and body weight and food intake data were recorded once a week. After 11 weeks, animals were sacrificed by decapitation at ZT1, being deprived of food for 1 hour. Blood was collected, and serum was obtained by centrifugation and stored at -80ºC until analysis. The liver, hypothalamus and soleus and gastrocnemius muscles were rapidly weighed, frozen in liquid nitrogen and stored at -80ºC for further analysis. The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all procedures.

2.2. *Body composition analysis*

Lean and fat measurements (in grams and percentage of body weight) were performed 1 week before sacrifice using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, TX, USA).

2.3. *Indirect calorimetry*

Indirect calorimetry analyses were performed 2 weeks before sacrifice using the OxyletPro™ System (PANLAB, Cornellà, Spain). After receiving treatment at ZT0, rats were transferred to an acrylic box (Oxylet LE 1305 Physiocage, PANLAB) with free access to water and food. After an acclimation period of 3 hours, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured every 9 minutes by an O_2 and CO_2 analyzer (Oxylet LE 405 gas analyzer, PANLAB) at a constant flow rate of 600 ml/min (Oxylet LE 400 air supplier, PANLAB). At each measure, the program Metabolism 2.1.02 (PANLAB, Cornellà, Spain) calculated the respiratory quotient (RQ) as the VCO_2/VO_2 ratio and energy expenditure (EE) as $VO_2 \times 1.44 \times [3.815 + (1.232 \times RQ)]$ (kcal/day/$kg^{0.75}$) according to the Weir formula [32]. Fat and carbohydrate oxidation rates were calculated using the VCO_2 and the VO_2 measures applying the Frayn stoichiometric equations [33], which define fat oxidation rates as $1.67 \times VO_2 - 1.67 \times VCO_2 - 1.92\ n$ ($g \bullet min^{-1}$) and carbohydrate oxidation rates as $4.55 \times VCO_2 - 3.21 \times VO_2 - 2.87\ n$ ($g \bullet min^{-1}$). A nitrogen excretion rate (n) of 135 $\mu g \bullet kg^{-1} \bullet min^{-1}$ was assumed [34]. Finally, the fat and carbohydrate oxidation energy (in $kJ \bullet min^{-1}$) was obtained by using the Atwater general conversion factor. The fat and carbohydrate rates were multiplied by 37 and 16, respectively [35].

2.4. *Serum analysis*

Glucose, total cholesterol and triglycerides (QCA, Barcelona, Spain), phospholipids (Spinreact, Girona, Spain) and nonesterified free fatty acids

(NEFAs) (WAKO, Neuss, Germany) were analyzed by enzymatic colorimetric assays. Serum insulin and glucagon levels were analyzed using a rat insulin ELISA kit (Millipore, Barcelona, Spain) and a rat glucagon ELISA kit (Cusabio Biotech, Wuhan, China), respectively.

2.5. Serum extraction and ^{1}H nuclear magnetic resonance (NMR) analysis of metabolite analysis

Serum metabolites were extracted as previously described [19]. ^{1}H-NMR analysis was performed following the procedure described by Vinaixa *et al* [36].

2.6. Gene expression analysis

Total RNA extraction, cDNA synthesis and real-time quantitative PCR in the hypothalamus, liver and gastrocnemius and soleus muscles were performed as previously detailed [19]. The primers used for the different genes are described in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA level was calculated as a percentage of the L12 group using the $-2^{\Delta\Delta Ct}$ method [37] with *β-actin*, *Ppia, Hprt* and *Tfrc* genes as references.

2.7. Western Blot analysis

Total and phosphorylated (p) AMP-activated protein kinase (AMPK and (p)-AMPK) and Akt2 and (p)-Akt2 protein levels in the liver and soleus and gastrocnemius muscles were measured by Western blot analysis as previously described [19].

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) (n=8-10). The effect of photoperiod on the evolution of body weight gain, cumulative caloric intake and the cumulative intake of carbohydrate, lipids and proteins was analyzed by repeated measures (RM) analysis of variance (ANOVA) with

time as a within-subject factor and photoperiod as a between-subject factor. When the interaction between time and photoperiod (*Pxt*) was statistically significant, one-way ANOVA followed by Duncan's post hoc test was used to determine significant differences among the three groups in each point. One-way ANOVA and Duncan's post hoc test were also used to determine the photoperiod effects on biometric, serum and metabolic parameters, fiber intake and specific food items of the CAF. Student's t test was also used for single statistical comparisons. Grubbs' test was used to detect outliers, which were discarded before subsequent analyses. All statistical tests were performed with the statistical software SPSS Statistics 22 (SPSS, Inc., Chicago, IL, USA). The level of statistical significance was set at bilateral 5%.

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed after data normalization and autoscaling using MetaboAnalyst 3.0 software [38].

3. RESULTS

3.1. Exposure to both short and long photoperiods combined with CAF feeding altered food intake-related parameters and body weight

Exposure to different photoperiods during the 4-week adaptation period did not produce significant changes in body weight gain, cumulative food intake or macronutrient consumption among groups (**Figures 1A-E**). However, the shift to the CAF combined with exposure to different day lengths for 7 additional weeks triggered a photoperiod-dependent response in all parameters related with food intake ($p<0.05$, photoperiod x time interaction, RM ANOVA) (**Figures 1B-E**). Thus, L6 rats displayed significantly lower cumulative energy intake than L12 animals from week 9 onwards (**Figure 1B**), an effect that could be mainly explained by the decreased cumulative intake of carbohydrate, lipid and protein from the ninth week (**Figures 1C,D,E**). The animals chronically exposed

to the long photoperiod and fed the CAF for 7 weeks also showed significantly lower cumulative food intake than L12 rats at the end of the study (**Figure 1B**). Nevertheless, this effect was mainly attributed to the decreased cumulative carbohydrate intake observed from week 9 onwards (**Figure 1C**), since no significant differences in lipid consumption were found (**Figure 1D**), and lower cumulative protein intake in L18 rats compared with L12 animals was only observed at the end of the study (**Figure 1E**). Moreover, both L6 and L18 rats displayed a significant decrease in cumulative fiber intake than L12 rats (**Table 1**). A detailed analysis of the consumption of the different food items included in the CAF diet revealed that L6 rats ate significantly less muffins and biscuits with cheese and pâté than L12 animals, whereas L18 rats consumed less chow than L12 animals and less carrots and more bacon than the L6 and L12 groups (**Table 1**). Both L6 and L18 animals consumed numerically lower amounts of milk with sugar—the food item that was consumed more by the rats—than L12 animals and, although the difference was not statistically significant, it contributed to the observed significant decrease in the cumulative carbohydrate intake (**Table 1**).

These changes in food intake-related parameters were not associated with an overall photoperiod effect on body weight gain (**Figure 1A**). Nevertheless, L18 animals displayed residually lower body weight gain compared with the L12 group at weeks 6, 7, 9, 10 and 11 ($p<0.05$, Student's t test) and the same pattern was observed in L6 compared to L12 rats at weeks 6, 7, 8 and 9 ($p<0.05$, Student's t test). In addition, at the end of the study, both L6 and L18 groups displayed lower body weight compared with L12 animals ($p=0.055$ and $p=0.042$, respectively, Student's t test) (**Table 2**).

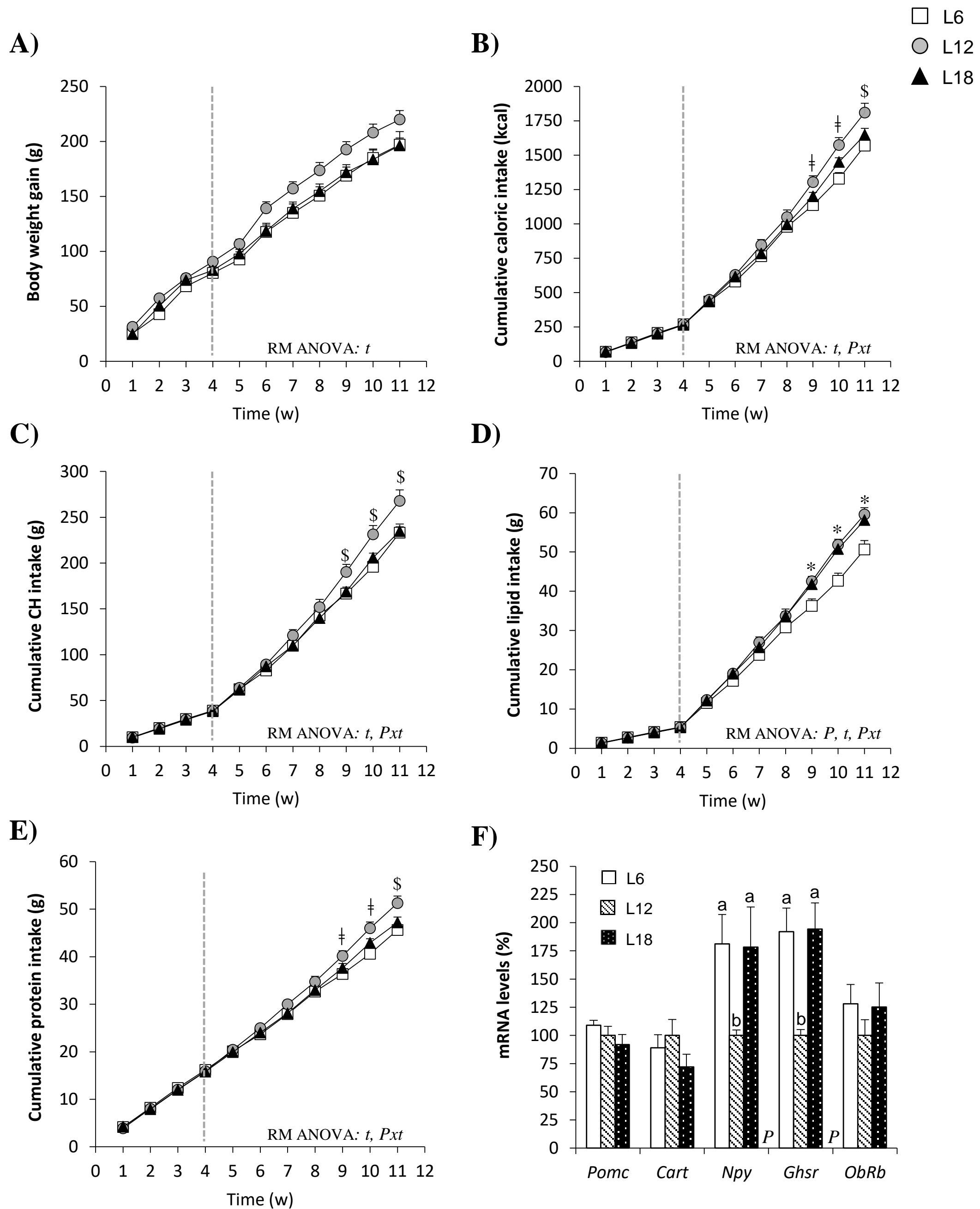

Figure 1. Body weight gain (**A**), cumulative caloric intake (**B**), CH intake (**C**), lipid intake (**D**), protein intake (**E**) and hypothalamic mRNA levels of genes related to food intake control (**F**) in male F344 rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. The end of the 4-week adaptation period is represented by a vertical dotted line. Data are expressed as the mean ± SEM (n=8-10). *P*, photoperiod effect; *t*, time effect; *Pxt*, photoperiod x time interaction effect (p<0.05, RM ANOVA). $^{\$}$p<0.05 L12 versus L18 and L6

groups; $^{\Delta}$ p<0.05 L12 versus L18 group; ‡ p<0.05 L12 versus L6 group; * p<0.05 L6 versus L12 and L18 groups; ^{ab} Mean values with unlike letters were significantly different among groups (one-way ANOVA and Duncan's post hoc test). CH, carbohydrate; *Cart,* cocaine and amphetamine-regulated transcript; *Ghsr,* ghrelin receptor; *Npy*, neuropeptide Y; *ObRb*, long-form leptin receptor; *Pomc*, proopiomelanocortin.

3.2. Hypothalamic mRNA levels of genes related with food intake control were vastly regulated by chronic exposure to both L6 and L18 photoperiods

To shed light on the described photoperiod effects on caloric intake, we analyzed the mRNA levels of different genes related with the regulation of food intake in the hypothalamus of diet-induced obese rats. Intriguingly, we noted a sharp upregulation of the orexigenic neuropeptide Y (*Npy)* gene in both L6 and L18 groups compared with those exposed to the L12 photoperiod (81.2% and 78.3% higher, respectively), whereas no changes in the anorexigenic neuropeptides proopiomelanocortin (*Pomc*) and cocaine and amphetamine-regulated transcript (*Cart*) were observed (**Figure 1F**). In addition, the ghrelin receptor (*Ghsr*) mRNA levels were significantly increased in L6 and L18 animals compared to the L12 group (92% and 94.2% higher, respectively) (**Figure 1F**). No changes in the leptin receptor (*ObRb*) gene in response to different photoperiod exposures were noted (**Figure 1F**).

3.3. CAF-fed obese rats exposed to different photoperiods displayed significant differences in body composition

Compared to L12 animals, both L18 and L6 groups displayed lower absolute lean mass (**Table 2**). L18 animals also showed decreased skeletal muscle weight, significantly lower relative lean mass and, consequently, a lesser lean/fat mass ratio than their L12 counterparts (p<0.05, Student's t test) (**Table 2**).

Table 1. Cumulative intake of different cafeteria diet food items and fiber at the end of the 7-week dietary study.

Cumulative intake	L6	L12	L18	
Chow (g)	31.5 ± 3.5	42.7 ± 5.6	29.5 ± 2.0	
Cheese and pâté biscuits (g)	50.4 ± 2.8	62.0 ± 4.4	55.4 ± 2.5	
Bacon (g)	21.7 ± 2.6[a]	23.7 ± 2.7[a]	34.1 ± 2.4[b]	*P*
Carrots (g)	48.3 ± 4.7[a]	53.3 ± 1.1[a]	35.0 ± 2.9[b]	*P*
Muffins (g)	38.2 ± 4.5[a]	49.3 ± 1.1[b]	50.7 ± 1.5[b]	*P*
Milk with sugar (ml)	399 ± 7	446 ± 34	371 ± 33	
Fiber (g)	4.33 ± 0.14[a]	5.31 ± 0.22[b]	4.14 ± 0.16[a]	*P*

Male Fischer 344 rats were exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). One-way ANOVA and Duncan's post hoc tests were performed to compare differences between groups. Significant differences are represented by different letters (a, b). *P,* photoperiod effect.

3.4. Chronic exposure to different photoperiods modified circulating serum glucose levels and some serum metabolites analyzed by NMR

The analysis of serum showed that L18 photoperiod-exposed animals exhibited higher circulating glucose levels compared to the L6 group (p=0.013, Student's t test), whereas no difference in either circulating insulin or glucagon levels was observed between groups (**Table 2**). In addition, only 5 circulating metabolites obtained by NMR analysis were significantly different between the photoperiod groups. Glutamate, glycine and taurine were higher in L12 animals than the L6 and L18 groups (**Supplementary Table 2**). Moreover, L18 animals displayed higher circulating levels of proline compared to the L6 group and lower choline levels than their counterparts (**Supplementary Table 2**). This group also exhibited residually lower levels of 3-hydroxybutyrate (p=0.05 versus L12 rats, Student's t test) and lactate (p=0.052 and p=0.048, compared to L6 and L12 animals, respectively, Student's t test) (**Supplementary Table 2**).

Table 2. Biometric and serum parameters in F344 rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks.

	L6	L12	L18	
Biometric parameters				
Initial body weight (g)	210 ± 4	221 ± 4	215 ± 4	
Final body weight (g)	407 ± 12	441 ± 11	411 ± 8	
Liver (g)	14.2 ± 0.5	14.8 ± 0.4	14.7 ± 0.4	
Skeletal muscle (g)	2.11 ± 0.05	2.23 ± 0.04	2.09 ± 0.03	
Testes (g)	3.00 ± 0.04	3.03 ± 0.07	2.92 ± 0.02	
Fat mass (g)	85.8 ± 3.3	84.4 ± 4.2	89.5 ± 3.8	
Fat mass (%)	21.5 ± 0.8	19.5 ± 0.9	22.0 ± 0.6	
Lean mass (g)	294 ± 8[a]	317 ± 6[b]	292 ± 5[a]	*P*
Lean mass (%)	73.7 ± 0.8[ab]	75.0 ± 0.8[b]	72.1 ± 0.5[a]	*P*
Lean/fat mass ratio	3.48 ± 0.18	3.92 ± 0.25	3.30 ± 0.12	
Serum parameters				
Glucose (mmol/L)	9.11 ± 0.27	9.73 ± 0.52	10.15 ± 0.26	
Insulin (ng/mL)	5.82 ± 0.24	6.52 ± 0.82	6.59 ± 0.47	
Glucagon (ng/mL)	2.53 ± 0.12	2.64 ± 0.06	2.63 ± 0.10	
Insulin:glucagon ratio	2.21 ± 0.13	2.53 ± 0.34	2.51 ± 0.21	
NEFAs (mmol/L)	1.52 ± 0.24	1.37 ± 0.13	1.57 ± 0.18	
Phospholipids (mmol/L)	3.87 ± 0.33	3.78 ± 0.22	4.18 ± 0.24	
Triglycerides (mmol/L)	5.30 ± 0.58	5.18 ± 0.39	5.28 ± 0.32	
Total cholesterol (mmol/L)	3.30 ± 0.41	3.33 ± 0.40	3.85 ± 0.41	

Male Fischer 344 rats were exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). One-way ANOVA and Duncan's post hoc tests were performed to compare differences between groups. Significant differences are represented by different letters (a, b). *P*, photoperiod effect. The skeletal muscle weight represents the total weight of both the soleus and gastrocnemius muscles.

3.5. Exposure to different day lengths significantly modulated whole-body substrate oxidation, EE and locomotor activity

L6 animals displayed a significant increase in the RQ compared with their L12 and L18 counterparts (**Figure 2A**). Consequently, this group exhibited a residual increase in carbohydrate oxidation rates compared to L18 rats (p=0.027, Student's t test) (**Figure 2B**) and a significant decrease in the fat oxidation levels compared with the L12 and L18 groups (29.8% and 21.5% lower, respectively) (**Figure 2C**). Thus, these findings revealed that exposure to a short photoperiod highly boosted the use of carbohydrate instead of lipids as an energy source. Both L6 and L18 animals showed lower EE and locomotor activity than L12 rats (**Figures 2D,E**).

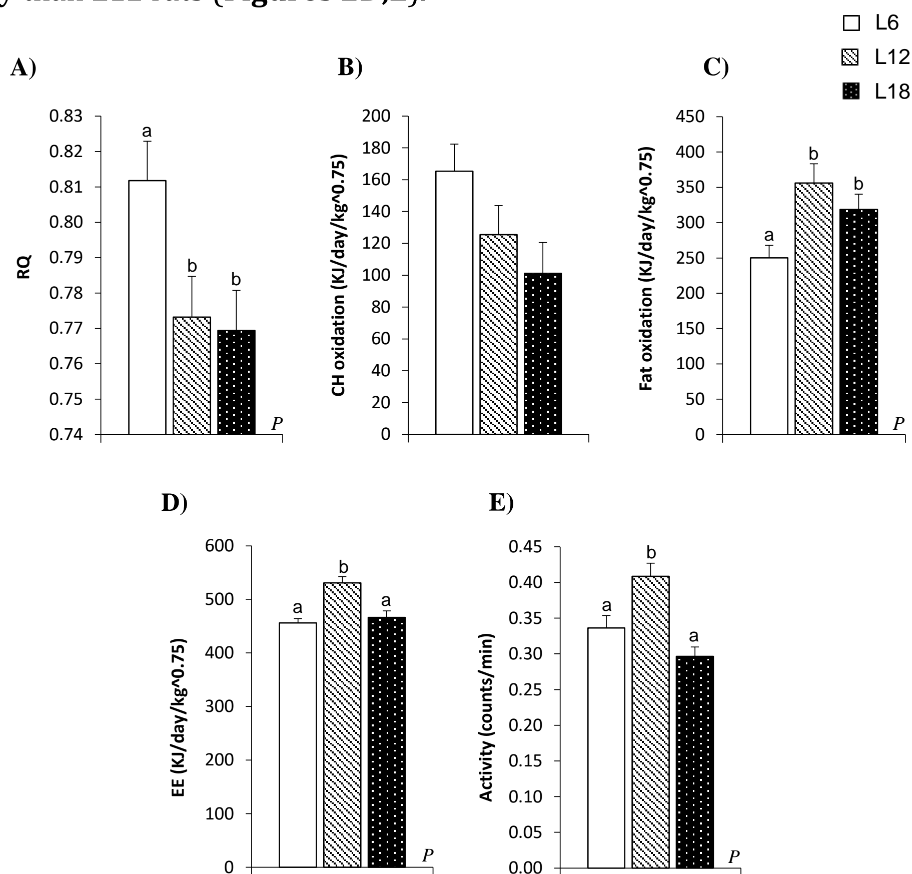

Figure 2. Respiratory quotient (RQ) (**A**), carbohydrate (CH) oxidation (**B**), fat oxidation (**C**), energy expenditure (EE) (**D**) and locomotor activity (**E**) in male F344 rats exposed to three

different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). *P*, photoperiod effect. [ab] Mean values with unlike letters were significantly different among groups (one-way ANOVA and Duncan's post hoc test).

3.6. Skeletal muscle fatty acid uptake- and β-oxidation-related genes were modulated by exposure to different day length schedules

To elucidate the mechanisms involved in the photoperiodic modulation of whole-body substrate oxidation, we analyzed different lipid metabolism-related parameters in the skeletal muscle of CAF-fed obese rats. L6 photoperiod-exposed animals exhibited significant downregulation of the fatty acid transport protein 1 (*Fatp1*) mRNA levels compared to the L12 and L18 groups in the soleus muscle (30.3% and 32.6% lower, respectively) (**Figure 3A**). The expression of this gene showed very similar behavior in the gastrocnemius muscle (33.9% and 36.6% lower in L6 animals compared with L12 and L18 rats, respectively), although the differences were not statistically significant (p=0.053 versus L18 rats, Student's t test) (**Figure 4A**). Moreover, L6 animals exhibited a clear trend towards decreased expression of the β-oxidation-related gene, hydroxyacyl-CoA dehydrogenase (*Had*), in the gastrocnemius muscle compared with the L18 group (p=0.056, Student's t test) (**Figure 4A**) and numerically lower carnitine palmitoyltransferase 1 beta (*Cpt1β*) gene expression in the soleus muscle compared with L12 animals (p=0.068, Student's t test) (**Figure 3A**). No differences in the phosphorylated levels of AMPK were found in either the soleus or gastrocnemius muscle among groups (**Figures 3C and 4C**).

3.7. Exposure to different day lengths altered the phosphorylated levels of Akt2 and other glucose metabolism-related genes in both the soleus and gastrocnemius muscles

To better understand the higher glycemia observed in L18 photoperiod-exposed animals, some parameters related with glucose homeostasis were

analyzed in the skeletal muscle, which is considered the main contributor of postprandial glucose uptake in the organism [39]. L18 animals exhibited a sharp downregulation of the pAkt2 protein levels in the soleus muscle compared with the L6 group (49.2% lower) (**Figure 3D**). Furthermore, residual downregulation of the *Irs1* mRNA levels was observed in the gastrocnemius muscle of L18 animals compared to L6 rats (p=0.030, Student's t test) (**Figure 4B**). Intriguingly, in the soleus muscle, L18 animals displayed higher *Irs1* mRNA levels compared with L12 rats, and L6 animals exhibited a downregulation of *Glut4* (p<0.05, Student's t test) (**Figure 3B**).

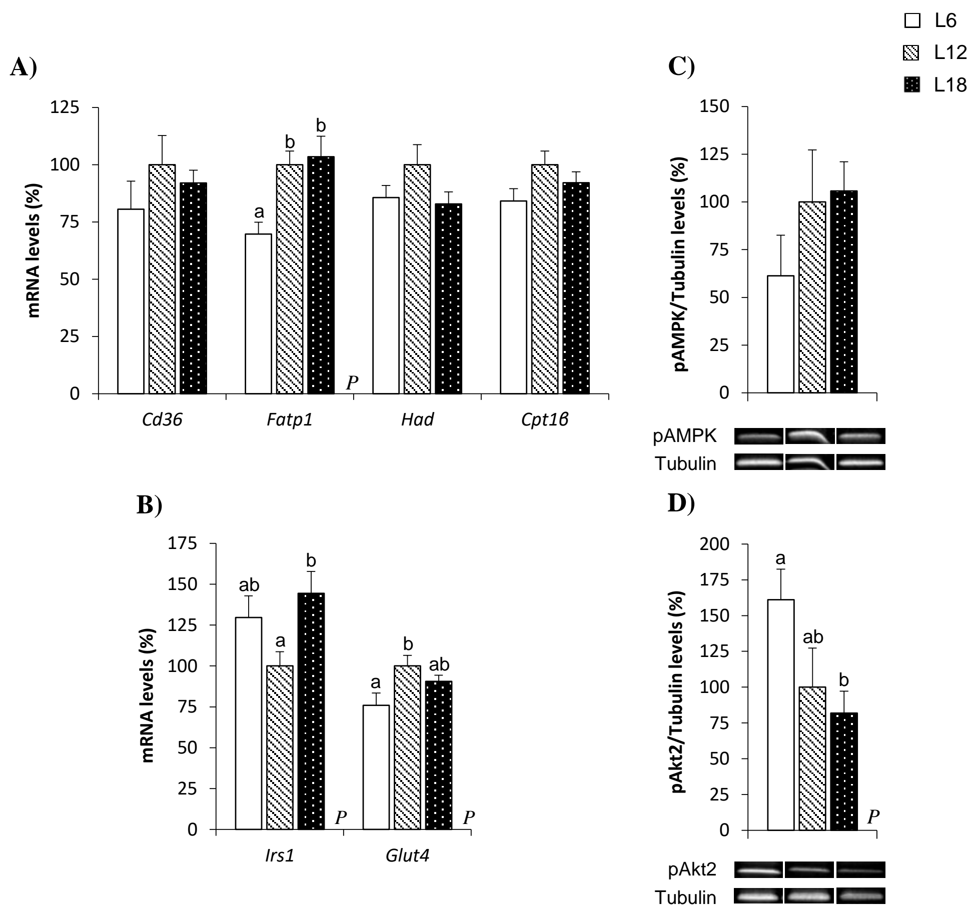

Figure 3. The mRNA expression of genes involved in lipid (**A**) and glucose (**B**) metabolism and pAMPK (**C**) and pAkt2 (**D**) protein levels in the soleus muscle of male F344 rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are

expressed as the mean ± SEM (n=8). *P*, photoperiod effect. [ab] Mean values with unlike letters were significantly different among groups (one-way ANOVA and Duncan's post hoc test). *Cd36*, fatty acid translocase, homologue of CD36; *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Fatp1*, fatty acid transport protein 1; *Glut4*, glucose transporter 4; *Had*, hydroxyacyl-CoA dehydrogenase; *Irs1*, insulin receptor substrate 1; pAkt2, phosphorylated Akt serine/threonine kinase 2; pAMPK, phosphorylated AMP-activated protein kinase.

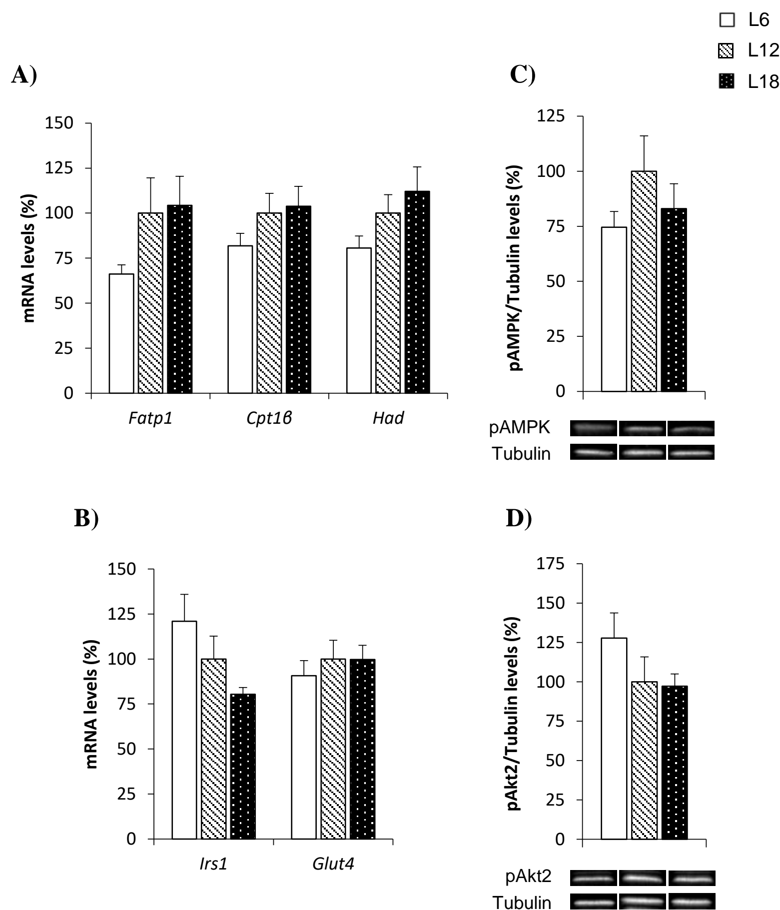

Figure 4. The mRNA expression of genes involved in lipid (**A**) and glucose (**B**) metabolism and pAMPK (**C**) and pAkt2 (**D**) protein levels in the gastrocnemius muscle of male F344 rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=8). *P*, photoperiod effect. [ab] Mean values with unlike letters were significantly different among groups (one-way ANOVA and Duncan's post hoc test). The genes and proteins analyzed have already been described in **Figure 3.**

3.8. Chronic exposure to different photoperiods induced pronounced changes in key genes involved in glucose and lipid homeostasis in the liver

In contrast to what was observed in the skeletal muscle, L18 photoperiod-exposed animals displayed a significant drop in the gene expression of several fatty acid uptake- and β-oxidation-related genes in the liver. Compared with the L6 and L12 groups, L18 animals exhibited residually lower fatty acid transport protein 5 (*Fatp5*) mRNA levels (p=0.025 and p=0.041, respectively, Student's t test) and a significant reduction in carnitine palmitoyltransferase 1 alpha (*Cpt1α)* gene expression (31.7% and 35.3% lower, respectively) (**Figure 5A**). This group also displayed lower fatty acid translocase, homolog of CD36 (*Cd36*) and *Had* mRNA levels than the L6 group (p=0.045 and p=0.027, respectively, Student's t test) (**Figure 5A**). The analysis of glucose metabolism-related genes revealed a residual increase in the glucose transporter 2 (*Glut2*) mRNA levels in L18 animals compared to those exposed to the L6 photoperiod (p=0.032, Student's t test) (**Figure 5B**). No significant changes in either pAMPK or pAkt2 protein expression were observed among groups (**Figures 5C,D**).

3.9. Hepatic and muscular core clock genes were significantly modulated by chronic exposure to different photoperiods

A very similar expression pattern of genes related to circadian rhythms was observed in the liver and the soleus and gastrocnemius muscles among the three photoperiod-exposed groups. L18 animals displayed lower brain and muscle Arnt-like protein 1 (*Bmal1*) mRNA levels in the soleus muscle and the liver than the L6 group (**Figures 6A,C**) and exhibited an upregulation of the period circadian clock 2 (*Per2*) mRNA levels in both the soleus and gastrocnemius muscles and the liver compared to L6 and L12 photoperiod-exposed animals (**Figures 6A,B,C**). Similarly, cryptochrome circadian clock 1 (*Cry1*) gene expression levels were greater in L18 animals than in L6 and L12

rats, but these changes were only significant in the liver (p=0.039 and p=0.031, respectively, Student's t test) (**Figure 6C**). Finally, the gene that encodes the *Bmal1* transcription inhibitor nuclear receptor subfamily 1 group D member 1 (NR1D1) protein was sharply downregulated in the three analyzed tissues of L18 animals compared to the L12 group and to a greater extent compared with L6 animals (**Figures 6A,B,C**).

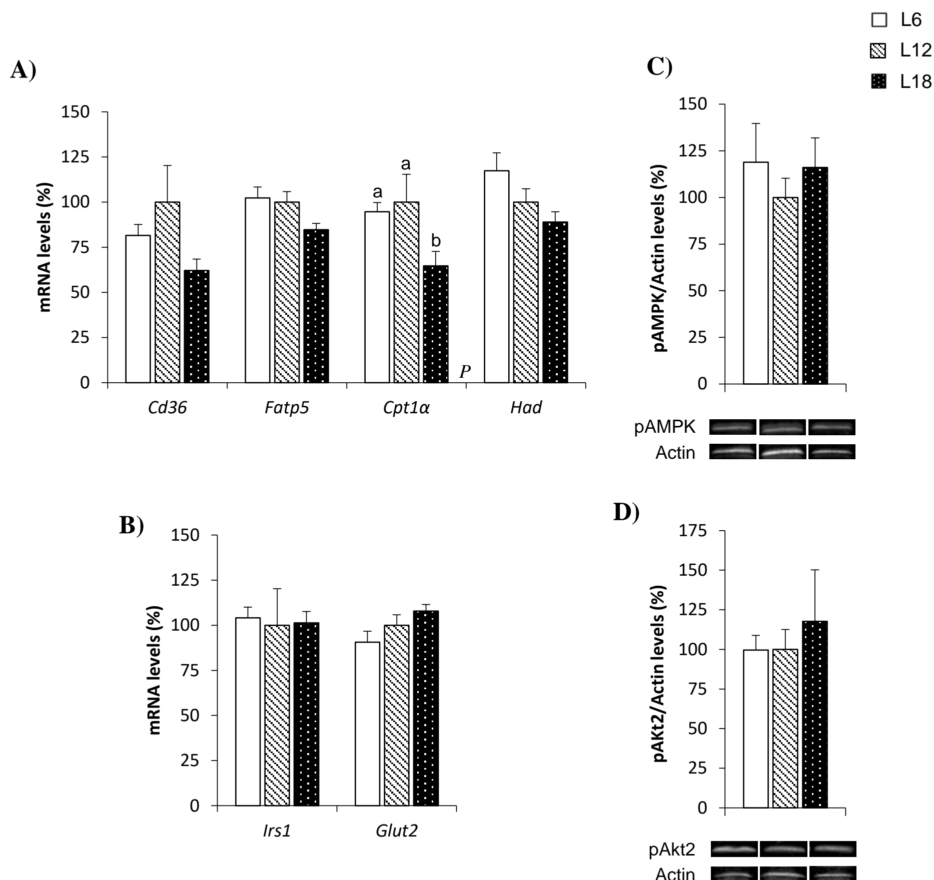

Figure 5. The mRNA expression of genes involved in lipid (**A**) and glucose (**B**) metabolism and pAMPK (**C**) and pAkt2 (**D**) protein levels in the liver of male F344 rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=8). *P*, photoperiod effect. [ab] Mean values with unlike letters were significantly different among groups (one-way ANOVA and Duncan's post hoc test). *Cpt1α*, carnitine palmitoyltransferase 1 alpha; *Fatp5*, fatty acid transport protein 5; *Glut2*, glucose

transporter 2. The rest of the genes and proteins analyzed have already been described in **Figure 3**.

3.10. The multivariate analysis revealed a marked clustering among the different photoperiod-exposed CAF groups

The 112 biometric, biochemical, physiological and molecular parameters evaluated in the present study were used to set up a PLS-DA predictive model to detect marked clustering among the photoperiod groups (**Figure 7A**). After representing the three components' scores, the quality parameters associated with this model were satisfactory. In this sense, the degree of fit of the model to the data, which is represented by R^2, was 0.98. Furthermore, the cross-validation of this model (Q^2) was 0.59; with a threshold of >0.4, this biological model is considered acceptable [40]. Taking into account the good quality of this predictive model, we selected those variables with a coefficient mean higher than 30 to set up a PCA (**Figure 7B**).

After representing the 28 selected variables that exhibited a higher relevance in the separation of the three groups in the PCA, the 56.8% variance was explained. As shown in ***Figure 7C***, clear clustering revealed a strong differential response of each group towards chronic exposure to different photoperiods. Among these variables, we mainly observed circadian rhythm-related genes (*Bmal1*, *Per2, Cry1*, *Rorα* and *Nr1d1*) in the three analyzed tissues, hypothalamic orexigenic genes (*Npy* and *Ghsr*), glucose metabolism-related parameters (pAkt2 protein and *Glut4* and *Irs1* gene expression) in the skeletal muscle and physiologic parameters measured by indirect calorimetry analysis (RQ, EE and fat oxidation).

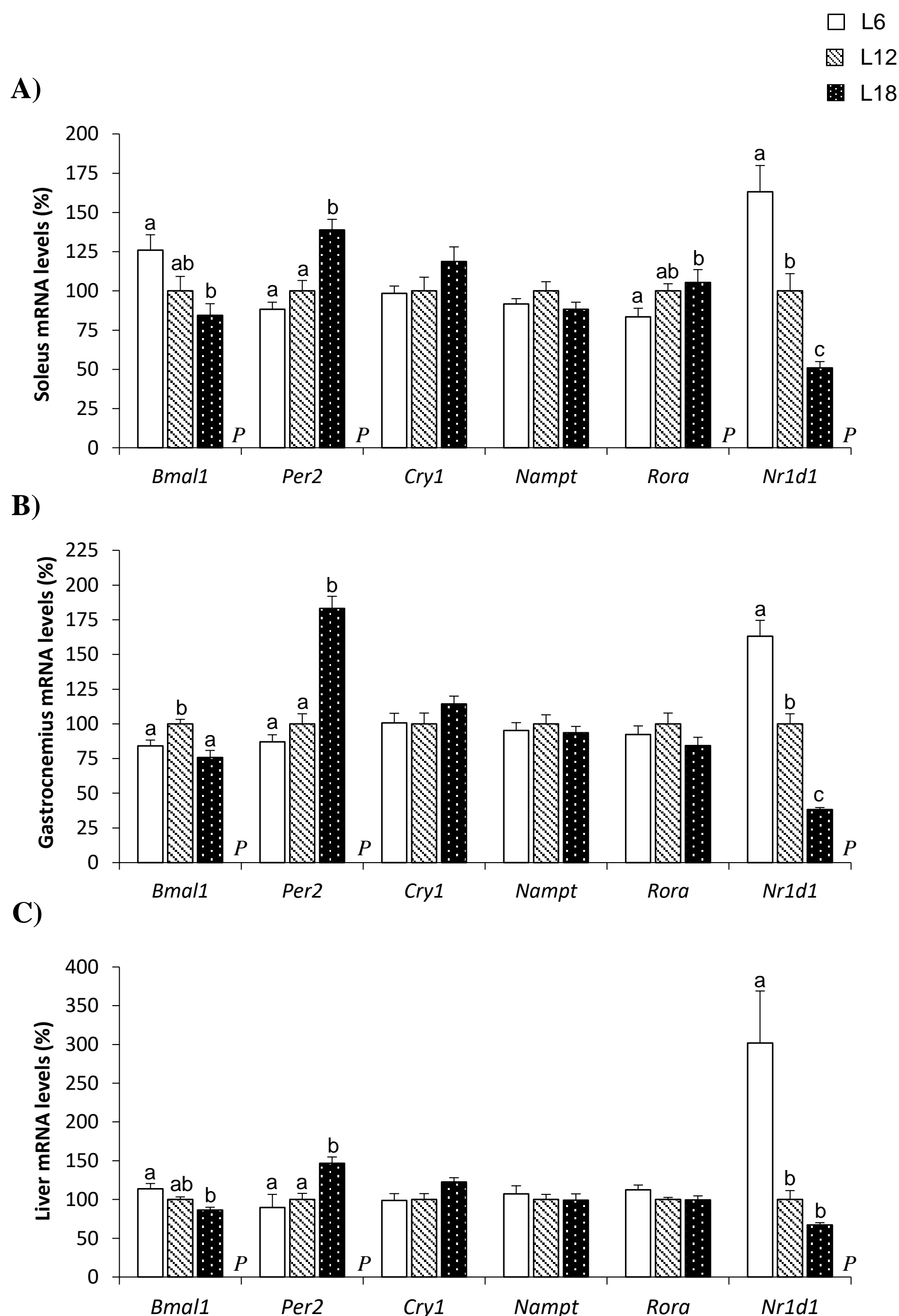

Figure 6. The mRNA expression of circadian core-clock genes in the soleus (**A**) and gastrocnemius (**B**) muscles and the liver (**C**) in male F344 rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=8). *P*, photoperiod effect. [abc] Mean values with unlike letters were significantly different among groups (one-way ANOVA and Duncan's post hoc test). *Bmal1*,

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brain and muscle Arnt-like protein-1; *Cry1,* cryptochrome circadian clock 1; *Nampt,* nicotinamide phosphoribosyltransferase; *Nr1d1,* nuclear receptor subfamily 1, group D, member 1; *Per2*, period circadian clock 2; *Rorα*, RAR-related orphan receptor alpha.

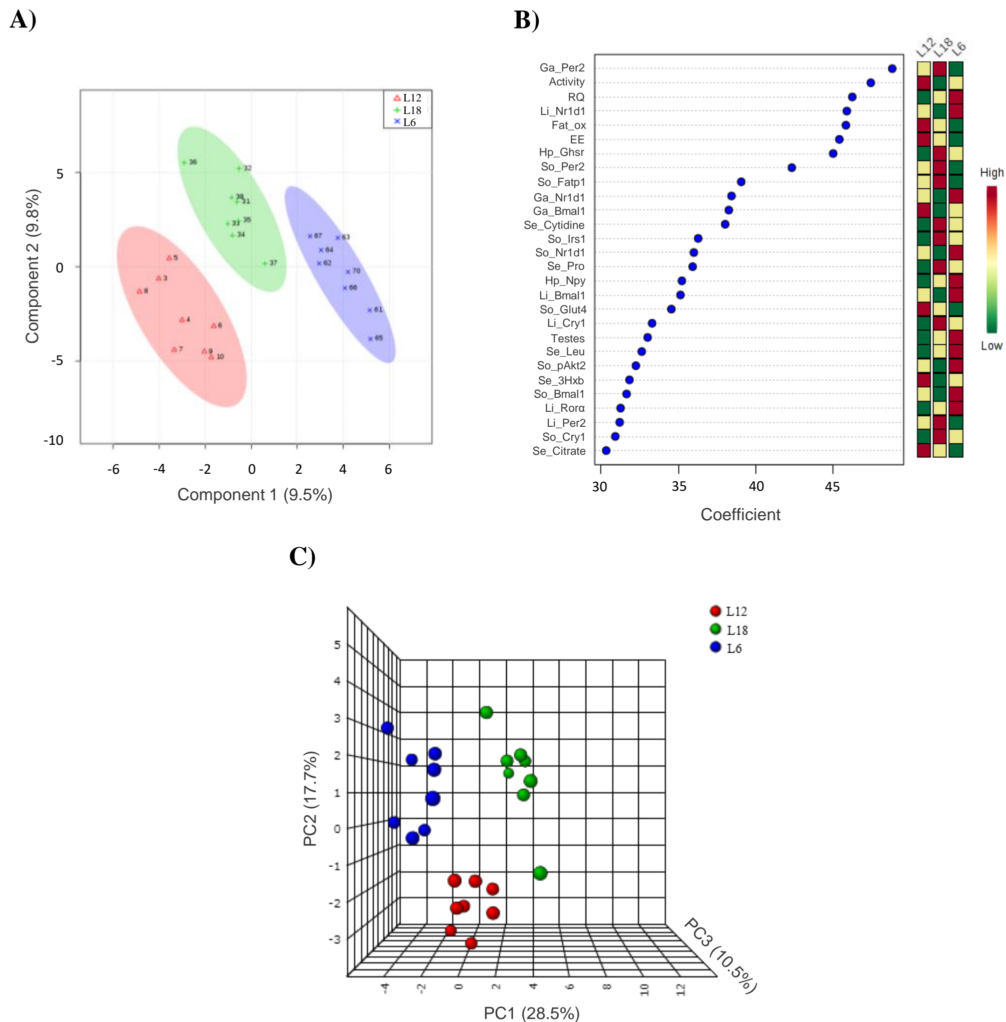

Figure 7. The 112 analyzed parameters were used to set up a PLS-DA predictive model (**A**). The 28 variables with a coefficient mean higher than 30 (**B**) were analyzed by PCA (**C**). 3Hxb, 3-hydroxybutyrate; *Bmal1*, brain and muscle Arnt-like protein-1; *Cry1*, cryptochrome circadian clock 1; EE, energy expenditure; Fat_ox, fat oxidation; *Fatp1*, fatty acid transport protein 1; Ga, gastrocnemius muscle; *Ghsr*, ghrelin receptor; *Glut4*, glucose transporter 4; Hp, hypothalamus; *Irs1*, insulin receptor substrate 1; Leu, leucine; Li, liver; *Npy*, neuropeptide Y; *Nr1d1*, nuclear receptor subfamily 1, group D, member 1; pAkt2, phosphorylated Akt serine/threonine kinase 2; *Per2*, period circadian clock 2; Pro, proline; *Rorα*, RAR-related orphan receptor alpha; RQ, respiratory quotient; Se, serum; So, soleus muscle.

4. DISCUSSION

In the present study, we demonstrated that chronic exposure to 3 different photoperiods (L6, L12 and L18) combined with the intake of the obesogenic diet CAF induced profound changes in a wide range of physiological and metabolic parameters in F344 rats. Briefly, we demonstrated: 1) decreased final body weight, cumulative caloric intake and EE and increased hypothalamic mRNA levels of the orexigenic genes *Npy* and *Ghsr* in both L6 and L18 animals; 2) significant changes in the preferences for different food items included in the CAF among the three photoperiod groups; 3) decreased EE and locomotor activity in both L6 and L18 rats; 4) higher circulating glucose levels in L18 animals compared with L6 rats, which were accompanied by a downregulation of the phosphorylated levels of Akt2 in the soleus muscle and lower *Irs1* mRNA levels in the gastrocnemius muscle; and 5) reduced whole-body lipid utilization in L6 animals, which was supported by the downregulation of fatty acid transporters and β-oxidation-related genes in both skeletal muscles. In addition, the multivariate analysis carried out with 112 parameters, including biometric- and food intake-related parameters as well as biochemical and molecular variables in the blood, liver, skeletal muscles and hypothalamus, revealed a clear clustering among the three photoperiod groups, reinforcing the relevance of changes in seasonal day length in physiology and metabolism in the obese state.

Numerous studies have already shown a marked photoperiod effect on several physiologic, behavioral and reproductive parameters in healthy Fischer 344 rats exposed to different day length schedules [14–18]. In this sense, our group previously reported that, under normoweight conditions, chronic exposure to different photoperiods induced relevant changes in a variety of glucose and lipid metabolism-related parameters in this model [19]. However, in terms of biometric and reproductive parameters, normoweight F344 rats held under an

SD photoperiod exhibited a significant photorefractory response after 14 weeks, not displaying the widely described short photoperiod-like regressive phenotype in body weight, body composition and testes size [14,17] compared to animals exposed to an LD photoperiod [19]. In the present study, surprisingly, an adaptation period of 4 weeks to the different photoperiods did not manifest into changes in body weight or food intake in F344 rats fed an STD, which clearly differs from other studies in which marked variations in cumulative food intake (15 days) and body weight gain (20 days) were rapidly reported after exposure to different day lengths [17]. However, the inclusion of the CAF for the subsequent 7 weeks provoked slight but significant decreases of body weight gain and final body weight in both L6 and L18 animals, which could be mostly attributed to the decreased cumulative energy intake displayed by both groups of rats over the course of the CAF intervention. Intriguingly, this lower caloric intake was accompanied by a sharp upregulation of the hypothalamic expression of the gene encoding the orexigenic neuropeptide NPY, which is one of the main enhancers of appetite [41], and by increased mRNA levels of the receptor of ghrelin, a gastric orexigenic hormone that acts through the activation of NPY neurons [42]. One possible explanation for these contradictory results could be a differential feeding state among groups at sacrifice, which could markedly regulate leptin and ghrelin systems [43]. However, the lack of variation in the circulating insulin and glucagon levels among groups strongly suggests that all animals were sacrificed under a very similar feeding status and, therefore, it seems very unlikely that differences in this parameter could account for the observed findings. Body weight is mainly determined by the balance between EE and energy intake [41]. Thus, at first glance, the lower energy intake observed in both L6 and L18 rats could be understood as an adaptive response to fit with the decreased EE observed in these groups in an attempt to maintain an optimal weight. Nevertheless, these decreases in both caloric intake and EE were translated into a progressive loss

of weight gain, suggesting an impairment of the mechanisms involved in body weight control in animals held under the SD and LD photoperiods. In this context, it is plausible to hypothesize that the activation of the orexigenic pathways could be a compensatory mechanism used to promote food intake in an attempt to counteract the lower body weight increase and, therefore, to recover the energy balance in L6 and L18 rats. One limitation of the obtained data is the fact that they only represent the endpoint of the study. Additional studies in which the animals are sacrificed at a later time point would be of great value to shed more light on this issue.

In addition to changes in energy intake, photoperiod clearly altered food preferences and macronutrient intake. Thus, the higher preference for some fat-rich solid food items included in the CAF (bacon and muffins) observed in L18 animals compared with L6 rats may explain the higher lipid consumption of animals exposed to a long-duration day than those held under the SD photoperiod, despite the fact that both groups attained a very similar overall caloric intake. Furthermore, the increased consumption of bacon reported in L18 rats compared with L12 animals and the similar muffin intake reported in both groups over the course of the study may account for the lack of significant differences in cumulative lipid intake between ND and LD photoperiod-exposed animals. The increased fat intake and higher preference for fat-rich foodstuffs observed in L18 rats compared with L6 animals is not consistent with the results obtained by Togo and collaborators, which reported that F344 rats exposed to a long photoperiod (16 h of light/day) during 3 weeks displayed a significant preference for a low-fat, high-carbohydrate diet than for a high-fat, low-carbohydrate diet, although no differences in protein and fat intake were reported compared to animals exposed to a short photoperiod (8 h of light /day) [26]. One possible explanation for these discrepancies could rely on the different experimental designs in terms of study length and/or diet compositions. Further analyses related with the hedonic regulation of food

intake, such as hippocampal expression of genes and proteins involved in dopaminergic pathways (dopamine receptor D5 (DRD5) and dopamine transporter (DAT)) [44] and more specific tests concerning feeding behavior, such as the two-bottle preference test [45], would be of high relevance to gain further insight into the relevance of photoperiod exposure on lipid and carbohydrate preferences in CAF-fed rats.

The lower cumulative protein intake observed in both L6 and L18 animals could explain, at least in part, the decrease in absolute lean mass observed in both groups [46]. Since quantitative magnetic resonance analysis of lean mass provides a precise measurement of muscle mass [47] and L18 animals showed lower skeletal muscle weight than L12 rats, it seems clear that chronic exposure to the LD photoperiod slowed down muscle mass accretion. Although skeletal muscle accounts for ≃80% of the postprandial circulating glucose uptake [39], this finding was not translated into significant changes in the circulating levels of glucose between animals held under ND and LD photoperiods. Nevertheless, we reported a significant increase in blood glucose of L18 animals compared with L6 rats, which could be partly explained by the significant downregulation of the phosphorylated levels of the downstream postreceptor target of insulin Akt2 [48] and lower expression of the insulin signaling-related gene *Irs1*—involved in the activation of Akt2 [49]—observed in the soleus and gastrocnemius muscle, respectively, of the group exposed to the long-duration day. Although we recently reported that chronic exposure to 18 hours of light induced an increase in circulating glucose levels compared with L12 photoperiod-exposed normoweight F344 rats, these results are not in agreement with the idea that short-duration days are more associated with impairments in glucose metabolism and insulin signaling than long photoperiods, as was previously described by our group in F344 rats [19] and by Tashiro *et al*. in C57BL/6J mice [20]. However, in the present study, the lack of significant changes in *Irs1* and *Glut4* gene expression in the soleus muscle

between L18 and L6 animals and the increased mRNA levels of the hepatic glucose transporter *Glut2* in the L18 group do not fully support the hypothesis of a lower insulin sensitivity phenotype in LD-exposed CAF-fed animals. Taking into account that gene expression data do not always match the protein levels, further analyses concerning GLUT4 translocation and the levels of other proteins involved in glucose and insulin signaling pathways are needed. Nevertheless, to the best of our knowledge, this is the first study to report a clear interaction between an obesogenic diet and day length seasonal variations in the regulation of glucose homeostasis, although the mechanisms involved in these diet-dependent photoperiod effects deserve further research.

We previously reported that normoweight L6 photoperiod-exposed animals displayed increased circulating NEFAs levels, which were accompanied by a downregulation of the mRNA levels of the gene encoding the fatty acid transporter CD36 in the soleus muscle and liver and by decreased expression of the β-oxidation-related genes, *Cpt1β* and *Had*, in the soleus muscle [19]. In the present study, exposure to the L6 photoperiod combined with CAF intervention also produced profound changes in lipid metabolism, as illustrated by the reduced whole-body fat oxidation rates in L6 animals compared with L12 and L18 rats. The lower lipid substrate utilization observed in this group could be mainly attributed to its significantly lower lipid intake, decreased mRNA levels of the fatty acid transporter gene, *Fatp1*, observed in both the soleus and gastrocnemius muscles, and the clear trend towards lower mRNA levels of the β-oxidation-related genes, *Cpt1β* and *Had*, observed in the soleus and gastrocnemius muscle, respectively. However, in contrast to what was observed in normoweight rats, the molecular changes related with fatty acid metabolism observed in the skeletal muscles of L6 CAF-fed animals were not translated into elevated circulating NEFAs. One possible explanation for these results could be the enhancement of fatty acid transport and β-oxidation pathways observed in the liver, which is illustrated by the upregulation of *Cd36, Fatp5, Cpt1α* and *Had*

mRNA levels observed in L6 animals compared to L18 rats. This liver-specific response could be explained as a compensatory action addressed to increase the hepatic fatty acid supply to maintain fatty acid homeostasis and, therefore, to avoid the rise of blood NEFAs. Nevertheless, further analyses of genes and proteins involved in fatty acid metabolism in the liver and peripheral tissues that significantly contribute to fatty acid uptake and β-oxidation, such as white adipose tissue [50], would be useful to corroborate this hypothesis.

We previously described that chronic exposure to different photoperiods in normoweight rats modulated the circadian core-clock transcriptional machinery, inducing profound changes in the gene expression levels of the major circadian clock transcriptional activator *Bmal1* [51], its product, *Per2*, and its inhibitor, *Nr1d1*, in the liver and both the soleus and gastrocnemius muscles in L18 animals [19]. Taking into account that several studies have proven that these genes are markedly implicated in the regulation of feeding behavior [41] and different metabolic pathways, such as gluconeogenesis or fatty acid β-oxidation [52], we speculate that the circadian rhythm-related gene expression changes observed in normoweight L18 rats could account, at least in part, to the physiologic and metabolic changes observed in this group, despite the limitation of only performing a single-point measurement of each gene (ZT1-2) [19]. In the present study, CAF-fed rats chronically exposed to different day lengths displayed a very similar gene expression pattern of core-clock genes than those reported in our previous study [19], suggesting that the CAF did not alter the circadian transcriptional machinery regulation. Remarkably, despite the fact that the hepatic and muscular gene expression profiles of circadian rhythm-related genes were very similar, we observed a very different response in genes related to fatty acid uptake and oxidation between the liver and skeletal muscles. Thus, although the marked variations in the mRNA levels of *Nr1d1* and *Per2* in the liver and skeletal muscles may partly explain the differences in physiologic and metabolic parameters observed among the

different photoperiod groups, the abovementioned results would make it difficult to establish the relevance of these gene expression changes on the observed effects. Therefore, further analyses performed at different time points throughout a 24-hour period are needed to shed more light on this issue.

5. CONCLUSION

In conclusion, we demonstrated that the consumption of a CAF triggered marked variations in outputs related with body weight regulation, feeding behavior and metabolism in F344 rats chronically exposed to different photoperiods. Relevantly, we observed a very similar behavior concerning caloric intake and biometric parameters in obese rats exposed to both short and long photoperiods, describing a decrease in body weight gain, lean mass, energy intake and EE compared with animals exposed to 12 hours of light. These changes were accompanied by a significant increase in the hypothalamic expression of the orexigenic genes *Npy* and *Ghsr*, in both groups of animals, which could be interpreted as a mechanism for increasing food intake to restore body weight homeostasis. Nevertheless, these common outputs between L6 and L18 animals were associated with different metabolic adaptations, as was illustrated by 1) the higher circulating glucose levels observed in animals held under the long photoperiod than L6 rats, which would be partly attributed to decreased pAkt2 protein levels and *Irs1* mRNA levels in the soleus and gastrocnemius muscles, respectively; and 2) the lower whole-body fat utilization in L6 animals, an effect that could be partly associated with decreased fat intake and the downregulation of fatty acid uptake- and β-oxidation-related genes in skeletal muscles. Since a seasonal cycle of food consumption in humans has been described [53,54], our study could contribute to highlight the relevance of the intake of highly palatable and energy dense foods prevalent in Western societies in the physiological and metabolic adaptations that occur in response to seasonal variations of day length,

especially in diseases associated with changes in food intake frequency and preference, such as obesity [41] and SAD [53]. The impact of these findings on human physiology and health deserves further research.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Cinta Bladé (CB), Lluís Arola (LA), Antoni Caimari (AC) and Josep del Bas (JdB) designed the studies; Roger Mariné (RM), Cristina Domenech (CD), AC and JdB performed the experiments and analyzed the data; RM, AC and LA wrote the manuscript. All the authors read, discussed and approved the final version of the manuscript.

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System and Brain Serotonin Precursor Availability. J Clin Endocrinol Metab 2001;86:5848–53.

SUPPLEMENTARY MATERIAL

Supplementary table 1. Nucleotide sequences of primers used for real time quantitative PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Tissue
β-actin	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	*L*
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC	*G, L, S*
Cart	AGAAGAAGTACGGCCAAGTCC	CACACAGCTTCCCGATCC	*H*
Cd36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG	*L, S*
Cpt1α	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG	*L*
Cpt1β	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	*G, S*
Cry1	TGGAAGGTATGCGTGTCCTC	TCCAGGAGAACCTCCTCACG	*G, L, S*
Fatp1	TGCTCAAGTTCTGCTCTGGA	CATGCTGTAGGAATGGTGGC	*G, S*
Fatp5	CCTGCCAAGCTTCGTGCTAAT	GCTCATGTGATAGGATGGCTGG	*L*
Ghsr	TCAGCCAGTACTGCAACCTG	GGAGAGATGGGATGTGCTGT	*H*
Glut2	AGTCACACCAGCACATACGA	TGGCTTTGATCCTTCCGAGT	*L*
Glut4	CCATTGCTTCTGGCTATCAC	TCCGTTTCTCATCCTTCAGC	*G, S*
Had	ATCGTGAACCGTCTCTTGGT	AGGACTGGGCTGAAATAAGG	*G, L, S*
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG	*G, L, S*
Irs1	CTACACCCGAGACGAACACT	TAACCTGCCAGACCTCCTTG	*G, L, S*
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG	*G, L, S*
Npy	TGGACTGACCCTCGCTCTAT	GTGTCTCAGGGCTGGATCTC	*H*
Nr1d1	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT	*G, L, S*
ObRb	AGCCAAACAAAAGCACCATT	TCCTGAGCCATCCAGTCTCT	*H*
Per2	CGGACCTGGCTTCAGTTCAT	AGGATCCAAGAACGGCACAG	*G, L, S*
Pomc	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGTTC	*H*
Ppia	CCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCAAAACGCT	*G, S*
Rorα	CCCGATGTCTTCAAATCCTTAGG	TCAGTCAGATGCATAGAACACAAACTC	*G, L, S*
Tfrc	ATCATCAAGCAGCTGAGCCAG	CTCGCCAGACTTTGCTGAATTT	*S*

The table shows the nucleotide sequences of primers used for PCR amplification. Primer pairs for PCR were designed using Primer3 software and the sequence information were obtained from Genbank. *β-actin*, actin beta; *Bmal1,* brain and muscle Arnt-like protein-1;

Cart, cocaine and amphetamine-regulated transcript; *Cd36*, fatty acid translocase, homologue of CD36; *Cpt1α*, carnitine palmitoyltransferase 1 alpha; *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Cry1,* cryptochrome circadian clock 1; *Fatp1*, fatty acid transport protein 1; *Fatp5*, fatty acid transport protein 5; *Ghsr*, ghrelin receptor; *Glut2*, glucose transporter 2; *Glut4,* glucose transporter 4; *Had*, hydroxyacyl-CoA dehydrogenase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Irs1,* insulin receptor substrate 1; *Nampt,* nicotinamide phosphoribosyltransferase; *Npy,* neuropeptide Y; *Nr1d1,* nuclear receptor subfamily 1, group D, member 1; *ObRb,* long-form leptin receptor; *Per2,* period circadian clock 2; *Pomc*, proopiomelanocortin; *Ppia*, peptidylprolyl isomerase A; *Rorα*, RAR-related orphan receptor A; *Tfrc*, transferrin receptor. Gene expression levels were analyzed in the hypothalamus (H), liver (L) and the gastrocnemius (G) and soleus (S) muscles.

Supplementary table 2. Concentration of representative serum metabolites analyzed by Nuclear Magnetic Resonance in diet-induced obese rats exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks.

Metabolite concentration (μmol/L)	L6	L12	L18	
3-Hydroxybutyrate	61.7 ± 5.8	65.4 ± 4.7	53.2 ± 3.2	
Acetate	64.3 ± 2.5	67.8 ± 3.1	67.6 ± 3.2	
Alanine	166.6 ± 8.8	170.5 ± 5.6	177.5 ± 7.8	
Creatine	103.2 ± 5.7	116.6 ± 4.4	109.5 ± 6.7	
Choline	6.3 ± 0.3 [a]	5.9 ± 0.1 [a]	5.4 ± 0.1 [b]	*P*
Formate	23.9 ± 1.1	25.0 ± 0.5	25.1 ± 1.3	
Glutamine	171.4 ± 4.6	177.2 ± 4.5	176.1 ± 5.7	
Glutamate	39.2 ± 1.3 [a]	44.7 ± 2.2 [b]	39.4 ± 1.5 [a]	*P*
Glycerophosphocholine	34.9 ± 5.2	37.0 ± 5.5	27.8 ± 2.9	
Glycine	64.4 ± 2.7 [a]	71.3 ± 1.1 [b]	65.3 ± 1.8 [a]	*P*
Histidine	22.5 ± 0.7	23.4 ± 0.8	22.2 ± 0.9	
Isoleucine	34.8 ± 1.9	32.3 ± 0.9	36.5 ± 3.1	
Lactate	1259 ± 77	1314 ± 107	1048 ± 63	
Leucine	31.6 ± 1.8	27.9 ± 0.6	32.7 ± 2.9	
Lysine	124.8 ± 4.8	119.6 ± 3.9	127.1 ± 8.7	
Phenylalanine	29.2 ± 0.7	31.1 ± 0.8	29.0 ± 0.7	
Proline	52.6 ± 3.3 [a]	55.3 ± 2.5 [ab]	62.2 ± 1.9 [b]	*P*
Pyruvate	22.9 ± 0.9	26.4 ± 2.2	23.2 ± 1.8	
Serine	85.5 ± 2.2	85.5 ± 3.0	85.4 ± 3.1	
Taurine	353 ± 10 [a]	391 ± 17 [b]	340 ± 10 [a]	*P*
Threonine	52.8 ± 2.9	52.3 ± 2.7	52.6 ± 3.9	
Tryptophan	44.1 ± 1.5	45.0 ± 1.3	43.5 ± 1.8	
Tyrosine	33.1 ± 0.8	34.1 ± 1.3	30.6 ± 1.2	
Valine	49.0 ± 2.2	46.7 ± 1.9	51.1 ± 4.3	

Male Fischer 344 rats were exposed to three different photoperiods for 11 weeks and fed a cafeteria diet for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). All the metabolites were obtained by performing a nuclear magnetic resonance (NMR) analysis. One-

way ANOVA and Duncan's post hoc tests were performed to compare the values between groups and significant differences were represented with different letters (a, b). *P*, photoperiod effect.

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MANUSCRIPT 3

Cherry consumption out of season alters lipid and glucose homeostasis in normoweight and cafeteria-fed obese Fischer 344 rats

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ABSTRACT

The xenohormesis theory postulates that animals, through the consumption of chemical cues, mainly polyphenols, synthetized by plants, are able to favorably adapt to changing environmental conditions. We hypothesized that the intake of fruits with a seasonally distinctive phenotype (in terms of bioactive compounds) produced a metabolic response that depends on mammals' circannual rhythms and that fruit intake out of season can lead to a disruption in characteristic seasonal metabolism. Fischer 344 rats were chronically exposed to short (L6, 6h light/day) and long (L18, 18h light/day) photoperiods in order to simulate autumn and spring seasons, respectively, and were fed either a standard diet (STD) or an obesogenic cafeteria diet (CAF) and orally treated with either vehicle or 100 mg.kg-1.day-1 of lyophilized sweet cherry (*Prunus avium L.*), a fruit consumed during long-day seasons. Cherry consumption exerted a marked photoperiod-dependent effect, inducing more changes when it was consumed out of season, which was apparent in the following observations: 1) in L6 STD-fed rats, a downregulation of the phosphorylated (p) levels of the downstream postreceptor target of insulin Akt2 in the soleus muscle and an enhancement of fatty acid transport and β-oxidation-related pathways, which was evidenced by increased *Had* gene expression (soleus) and pAMPK levels (soleus and gastrocnemius); 2) an increase in whole-body fat oxidation and circulating levels of glucose and insulin in L6-CAF-fed obese rats. Although the pathophysiological significance of these results requires further research, our findings could contribute to highlighting the importance of the consumption of seasonal fruits to maintain optimal health.

Key words. Seasonal fruits, circannual rhythms, cherry consumption, polyphenols, metabolic homeostasis, xenohormesis theory.

1. INTRODUCTION

Obesity has become a global epidemic as more than 1.9 billion adults were overweight and of these, over 650 million were obese in 2016 [1]. Taking into account that the main factor contributing to the development of obesity and its related disorders is an imbalance between food intake and energy expenditure [2,3], national policies have been focused on recommending healthy habits, such as the adherence to lower caloric diets, higher fruit and vegetable intake and increased physical activity. However, this strategy has shown a limited effectiveness, since the incidence and prevalence of obesity are still increasing every year. Thus, there is an urgent need to find new factors that can contribute to the development of obesity and related pathologies in order to improve lifestyle-based strategies to successfully counteract this worldwide epidemic.

It is widely accepted that mammals are capable of changing their physiology, behavior and metabolism as an adaptive mechanism to address seasonal variations in environmental factors that can compromise their food availability and survival, such as day length and climate [4,5]. In humans, especially those living in latitudes far from the equator, this seasonal responsiveness is illustrated by higher fat mass accretion and fasting circulating levels of cholesterol, triglycerides, glucose, insulin and leptin during winter and by increased energy expenditure and physical activity during summer [6–8]. The seasonal variations in these parameters seem to have a more negative impact on human health during the cold dark part of the year, since a clear negative correlation was found between cardiovascular disease mortality and day length [8]. Nevertheless, despite the evidence, the impact of factors other than day length, such as physical activity and temperature together with the augmented use of heating and artificial lighting systems [6,7], makes it difficult to determine the importance of seasonal day length variations on human health. To minimize these limitations and to more accurately elucidate how day length

affects physiology and metabolism, the use of animal models held under constant and controlled temperature conditions and social input has gained interest over recent decades. Thus, in C57BL/6J mice, the exposure to a short photoperiod (8:16 h light:darkness) for 3 weeks induced depression-like behavior accompanied by an increase in sucrose intake, body weight, fat mass and hyperglycemia [9,10], which represent the main symptoms of seasonal affective disorder (SAD), a mood disorder with a high incidence in winter [11]. Using the photosensitive Fischer 344 rat strain [12–15], we recently demonstrated the relevance of circannual rhythms in the regulation of lipid and glucose metabolism, suggesting that this model could be a useful tool to study glucose- and lipid-related pathologies that are influenced by seasonal variation, such as obesity, cardiovascular disease and SAD. Thus, by carrying out a multivariate analysis including 239 biometric, serum, hepatic and skeletal muscle parameters, we showed a clear clustering that was dependent on the photoperiod to which the animals were exposed. Furthermore, we demonstrated that the exposure to a short photoperiod (6:18 h light:darkness) for 14 weeks induced a marked increase in circulating glucose and free fatty acid levels that was accompanied by changes in key glucose, insulin and lipid metabolism-related proteins and genes in the skeletal muscles and liver [16].

In addition to animals and humans, plants exhibit a high sensitivity toward seasonal changes in the environment and produce some chemical signals, known as phytochemicals, whose composition and levels can be influenced by different environmental factors, such as the temperature, light quality, stress and day length [17]. An extensive variety of these signals, such as polyphenols, can be found in fruits and vegetables in our diet, and these signals are characterized by their antioxidant [18–21] and anti-inflammatory [19,22] properties, among others. Moreover, it has been shown that these bioactive compounds can directly interact with specific enzymes or receptors from signaling pathways in the organism that consumes them [23,24]. Based on

these concepts, Howitz and Sinclair developed a theory called xenohormesis (from *xeno,* stranger and *hormesis*, the term for the protective response provided by mild biological stress) [25]. This theory postulates that heterotrophic organisms acquired the ability to recognize stress signaling compounds synthetized by other species, being informed about the status of the environment or food availability. Therefore, these signals allow them to adapt to these conditions in a beneficial way, leading to a higher chance of survival [25–27].

Currently, life's globalization guarantees the commercialization of fruits and vegetables from across the world, which makes possible the consumption of seasonal fruits throughout the year. Here, we hypothesize that the intake of fruits with a seasonal distinctive phenotype (in terms of bioactive compounds) produces a metabolic response that depends on mammals' circannual rhythms. Therefore, fruit consumption out of its harvesting season could trigger erroneous signaling, leading to a disruption in characteristic seasonal metabolism, which could contribute to the development of obesity and related disorders.

To shed light on this issue, we conducted a proof-of-concept study in which Fischer 344 rats were exposed to short and long photoperiods in order to mimic autumn and spring light schedules, respectively, and were treated with lyophilized sweet cherry (*Prunus avium* L.), a fruit typically consumed during spring. The main aim of the study was to evaluate whether cherry consumption out of season could induce erroneous signaling, which could consequently a) negatively affect the metabolism of normoweight rats and b) enhance the detrimental effects caused by the obesogenic cafeteria diet.

2. MATERIALS AND METHODS

2.1. Fruit preparation and characterization

Royal Dawn sweet cherries (*Prunus avium L.*) were cultivated in Argentina and acquired in Mercabarna (Barcelona, Spain). After discarding the pits, cherries were frozen in liquid nitrogen, crushed and lyophilized by using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Barcelona, Spain) at -55ºC. Then, cherries were crushed again in order to ensure their easy dilution in water, aliquoted and protected from light and humidity. The phenolic composition of this cherry variant is characterized by a high content of flavonoids (1.86 mg/g), such as anthocyanins (1.71 mg/g) and flavanols (0.15 mg/g), and phenolic acids (0.88 mg/g), such as 3-caffeoylquinic (0.45 mg/g) and 3-p-coumaroylquinic acids (0.38 mg/g). Cyanidin 3-O-rutinoside (1.43 mg/g) is the main anthocyanin present in this fruit, accounting for approximately 80-90% of the total anthocyanin content, followed by cyanidin 3-O-glucoside (0.19 mg/g). In addition, the main flavanols present in this fruit are epicatechin (0.078 mg/g), catechin (0.015 mg/g), and procyanidin dimers (0.038 mg/g) and trimers (0.019 mg/g) [28]. This fruit contains 135 mg/g of carbohydrate, 5 mg/g of lipids, 8 mg/g of proteins, 15 mg/g of dietary fiber and 837 mg/g of water [29].

2.2. Experimental design

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures. ***Experiment one.*** Twenty-four 8-week-old male Fischer 344 (F344) rats (Charles River Laboratories, Barcelona, Spain) were housed in pairs in cages at 22ºC and exposed to 2 different photoperiods to mimic the day length of different seasons: the autumn season (short day photoperiod, L6, 6 h light and 18 h darkness) and the spring season (long day photoperiod, L18, 18 h light and 6 h darkness). Both groups (n=12 per group)

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were subjected to a 4-week adaptation period during which they were fed *ad libitum* with a STD (2.90 kcal·g^{-1}; Teklad Global 14% Protein Rodent Diet 2014, ENVIGO, Sant Feliu de Codines, Barcelona, Spain). After this period, rats of each photoperiod were divided into 2 groups (n=6 per group) according to the treatment received over 10 weeks: vehicle (VE) or lyophilized cherry (CH) at a dose of 100 mg per kg of body weight (diluted in water). The vehicle group was supplemented with the same volume of a sugar mixture solution (glucose:fructose 1:1, 20 mg per kg of body weight) in order to administer the same amount of sugars as those given to the cherry-supplemented rats. Both treatments were administered orally using a 1-ml syringe in a volume of 0.3-0.4 ml. Considering the rat's average weight as 350 g and that 100 g of fresh cherries approximately yields 33 g of lyophilized fruit, the dose of lyophilized cherry used in this study was equivalent to a daily consumption of 4.26 g of fresh cherry without pits per day for a 60-kg human [30]. Body weight and food intake were recorded weekly, and after 10 weeks, animals were sacrificed by decapitation at Zeitgeber time (ZT) 1, after being deprived of food for 1 h. ***Experiment two.*** Forty 8-week-old male F344 rats were housed in the same conditions described in the first experiment. After a 4-week adaptation period during which the animals were fed a STD, rats were switched to a CAF and distributed into 2 different groups according to the treatment received (VE or CH) for 7 weeks, which is a period of time that allows the development of obesity and metabolic syndrome (MetS)-related alterations in CAF-fed rats [31]. The cafeteria diet contained bacon, biscuit with pâté and biscuit with cheese, carrots, muffins and milk with sugar (220 g/L), and its caloric distribution was 58.1% carbohydrate, 31.9% lipid and 10.0% protein. Sacrifice was performed as described above. In both experiments, blood was collected, and serum was obtained by centrifugation and stored at -80ºC until analysis. The liver and the soleus and gastrocnemius muscles were rapidly weighed, frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.3. Body composition analysis

Lean and fat mass measurements were performed without anesthesia 1 week before sacrifice using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA). Data are expressed in absolute (g) and relative values as a percentage of body weight (%). The lean/fat mass ratio was also calculated.

2.4. Indirect calorimetry

Indirect calorimetry analyses were performed 2 weeks before sacrifice in the CAF-fed rats study using an Oxylet ProTM System (PANLAB, Cornellà, Spain). After receiving the treatment at ZT0, rats were transferred to an acrylic box (Oxylet LE 1305 Physiocage, PANLAB, Cornellà, Spain) with free access to water and food. After an acclimation period of 3 h (from ZT0 to ZT3), the indirect calorimetry analyses were performed for 21 h (from ZT3 to ZT24). The respiratory quotient (RQ), energy expenditure (EE) and oxidation levels of carbohydrate and lipids were calculated as previously defined [32].

2.5. Serum analysis

Circulating levels of glucose, total cholesterol, triglycerides (QCA, Barcelona, Spain), phospholipids (Spinreact, Girona, Spain) and nonesterified free fatty acids (NEFAs) (WAKO, Neuss, Germany) were analyzed by enzymatic colorimetric assays. Serum insulin and glucagon levels were analyzed using a rat insulin ELISA kit (Millipore, Barcelona, Spain) and a rat glucagon ELISA kit (Cusabio Biotech, Wuhan, China), respectively.

2.6. Gene expression analysis.

Total RNA extraction from the liver and gastrocnemius and soleus muscles, cDNA synthesis and real-time quantitative-PCR were performed as previously described [16]. The primers used to amplify the different genes are described in *Supplementary Table 1* and were obtained from Biomers.net (Ulm, Germany).

The relative expression levels of each mRNA were calculated as a percentage of the nontreated L6 group, using the $-2^{\Delta\Delta Ct}$ method with the *β-actin*, *Ppia, Hprt* and *Tfrc* genes as reference genes.

2.7. Western blot analysis

Total and phosphorylated (p) AMP-activated protein kinase (AMPK and (p)-AMPK) and Akt serine/threonine kinase 2 (Akt2 and (p)-Akt2) protein levels in the liver and the soleus and gastrocnemius muscles were measured in both experiments by western blot analysis as previously described [16].

2.8. Statistical analysis

Data are expressed as the mean ± SEM (n=6 and n=10 in experiments one and two, respectively). Grubbs' test was used to detect outliers, which were discarded for subsequent analyses. Fruit (*F*), photoperiod (*P*) and fruit x photoperiod interaction (*FxP*) effects within groups were determined by performing two-way ANOVA (2 x 2 factorial designs: fruit (VE or CH) x photoperiod (L6 or L18)). When the interaction between fruit and photoperiod was statistically significant according to two-way ANOVA, Student's t test was performed to compute pairwise comparisons between groups (i.e., the photoperiod effect within fruit groups and the fruit effect within the L6 and L18 groups). Student's t test was also used for single statistical comparisons. All the statistical tests were performed with the statistical software SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). The level of significance was set at bilateral 5%.

3. RESULTS

3.1. *The exposure to different photoperiods altered the body composition and serum parameters in STD-fed animals*

Regardless of the treatment received, L18 photoperiod-exposed rats displayed higher absolute (grams) and relative (%) fat mass and a lower lean/fat mass ratio than those exposed to the short photoperiod (*P* effect, $p<0.05$, two-way ANOVA) (**Table 1**). Overall, circulating levels of glucose, NEFAs and triglycerides were significantly higher and insulin levels were significantly lower in L6 rats compared to L18 animals (*P* effect, $p<0.05$, two-way ANOVA), although these photoperiod effects were numerically more evident between the cherry-supplemented groups (**Table 1**).

3.2. *Gene and protein expression analyses revealed clear photoperiodic effects on glucose and lipid metabolisms and circadian rhythm regulation in the skeletal muscles and liver of normoweight rats*

Both groups of STD-fed rats exposed to the short photoperiod displayed a significant downregulation of *Irs1* mRNA levels in the soleus muscle (*P* effect, $p=0.031$, two-way ANOVA), and this effect was more evident in the non-supplemented animals (32.3% lower in STD-VE-L6 than in STD-VE-L18 animals) (**Figure 1B**).

Regardless of the treatment received, L6 animals displayed a vast downregulation of the levels of phosphorylated Akt2 in both the soleus and gastrocnemius skeletal muscles in comparison with L18 animals (*P* effect, $p<0.001$, two-way ANOVA) (**Figure 1D and 2D**).

Similar to what was observed for the pAkt2 levels, a clear photoperiod effect was discerned on the phosphorylated AMPK levels, which were significantly increased in both skeletal muscles of L6-photoperiod exposed animals (*P* effect, $p<0.001$, two-way ANOVA) (**Figures 1E and 2E**).

The chronic exposure to the long day photoperiod significantly downregulated the hepatic expression of the genes encoding the fatty acid transport protein 5 (FATP5) and two key enzymes involved in β-oxidation, carnitine palmitoyltransferase 1 alpha (CPT1α) and HAD (*P* effect, p<0.05, two-way ANOVA) (**Figure 3A**).

The expression analyses of circadian rhythm-related genes revealed that, regardless of the treatment received, the normoweight rats exposed to the long photoperiod displayed higher period circadian clock 2 (*Per2*) and lower nuclear receptor subfamily 1 group D member 1 gene (*Nr1d1*) mRNA levels than both L6 groups in the soleus and gastrocnemius muscles as well as in the liver (*P* effect, p<0.05, two-way ANOVA) (**Figures 1C, 2C, 3B**).

3.3. Cherry consumption scarcely affected biometric and serum parameters in normoweight rats

No changes were observed in cumulative food intake, body weight gain, tissue weights and body composition after the administration of lyophilized cherry either in the STD-CH-L6 or STD-CH-L18 groups (**Table 1**). Both cherry-supplemented groups displayed lower serum cholesterol levels than their respective nontreated counterparts (*F* effect, p=0.043, two-way ANOVA) (**Table 1**). Only glucagon levels were differently modulated depending on which photoperiod cherry was consumed (*FxP* effect, p=0.035, two-way ANOVA), being slightly increased in STD-CH-L6 animals and decreased in STD-CH-L18 rats compared to the STD-VE-L6 and STD-VE-L18 groups, respectively, although these pairwise comparisons did not reach statistical significance (**Table 1**).

Table 1. Food intake, biometric and serum parameters in normoweight rats exposed to two different photoperiods for 14 weeks and supplemented with vehicle or lyophilized cherry for the last 10 weeks.

	STD-VE-L6	STD-CH-L6	STD-VE-L18	STD-CH-L18	2wA
CFI (kcal)	541 ± 9	534 ± 13	540 ± 12	527 ± 12	
Feed efficiency ratio	15.54 ± 0.74	16.79 ± 1.09	16.62 ± 1.05	15.96 ± 1.16	
Biometric parameters					
Initial body weight (g)	286 ± 8	287 ± 8	297 ± 14	304 ± 4	
Final body weight (g)	370 ± 11	377 ± 14	387 ± 13	388 ± 5	
Body weight gain (g)	84 ± 5	90 ± 7	89 ± 4	84 ± 5	
Liver (g)	11.86 ± 0.15	12.66 ± 0.57	12.73 ± 0.45	12.83 ± 0.26	
Skeletal muscle (g)	2.08 ± 0.07	2.04 ± 0.12	2.12 ± 0.03	2.13 ± 0.03	
Testes (g)	3.09 ± 0.06	3.08 ± 0.05	3.04 ± 0.04	3.16 ± 0.10	
Fat mass (g)	45.06 ± 1.29	50.41 ± 2.82	55.64 ± 4.41	58.38 ± 3.11	*P*
Fat mass (%)	12.52 ± 0.34	13.43 ± 0.57	14.38 ± 0.75	15.17 ± 0.70	*P*
Lean mass (g)	296 ± 8	299 ± 12	310 ± 9	307 ± 5	
Lean mass (%)	80.94 ± 1.04	79.69 ± 0.62	80.71 ± 0.67	79.95 ± 0.76	
Lean/fat mass ratio	6.56 ± 0.22	5.99 ± 0.29	5.71 ± 0.37	5.33 ± 0.32	*P*
Serum parameters					
Glucose (mmol/L)	7.73 ± 0.19	8.08 ± 0.15	7.59 ± 0.19	7.42 ± 0.19	*P*
Insulin (ng/mL)	4.04 ± 0.66	4.03 ± 0.79	5.54 ± 0.73	6.15 ± 0.96	*P*
Glucagon (ng/mL)	2.66 ± 0.13	2.87 ± 0.05	2.86 ± 0.04	2.04 ± 0.40	*FxP*
Insulin:glucagon ratio	1.57 ± 0.32	1.54 ± 0.30	1.79 ± 0.24	4.01 ± 1.29	
NEFAs (mmol/L)	0.72 ± 0.06	0.72 ± 0.08	0.62 ± 0.05	0.49 ± 0.04	*P*
Phospholipids (mmol/L)	3.08 ± 0.17	2.80 ± 0.15	2.83 ± 0.10	2.71 ± 0.09	
Triglycerides (mmol/L)	1.60 ± 0.12	1.58 ± 0.13	1.39 ± 0.11	1.18 ± 0.09	*P*
TChol (mmol/L)	3.26 ± 0.19	2.90 ± 0.13	2.97 ± 0.09	2.73 ± 0.12	*F*

Standard diet-fed male Fischer 344 rats were exposed to two different photoperiods for 14 weeks and supplemented with vehicle or lyophilized cherry for the last 10 weeks. Data are expressed as mean ± SEM (n=6). Two-way ANOVA analysis (2 × 2 factorial designs: fruit (VE or CH) × photoperiod effect (L6 or L18) was used to evaluate differences among groups. *F*, fruit effect; *P*, photoperiod effect; *FxP*, interaction fruit x photoperiod effect. The skeletal muscle

weight represents the total weight of both soleus and gastrocnemius muscles. CFI, cumulative food intake; Tchol, total cholesterol.

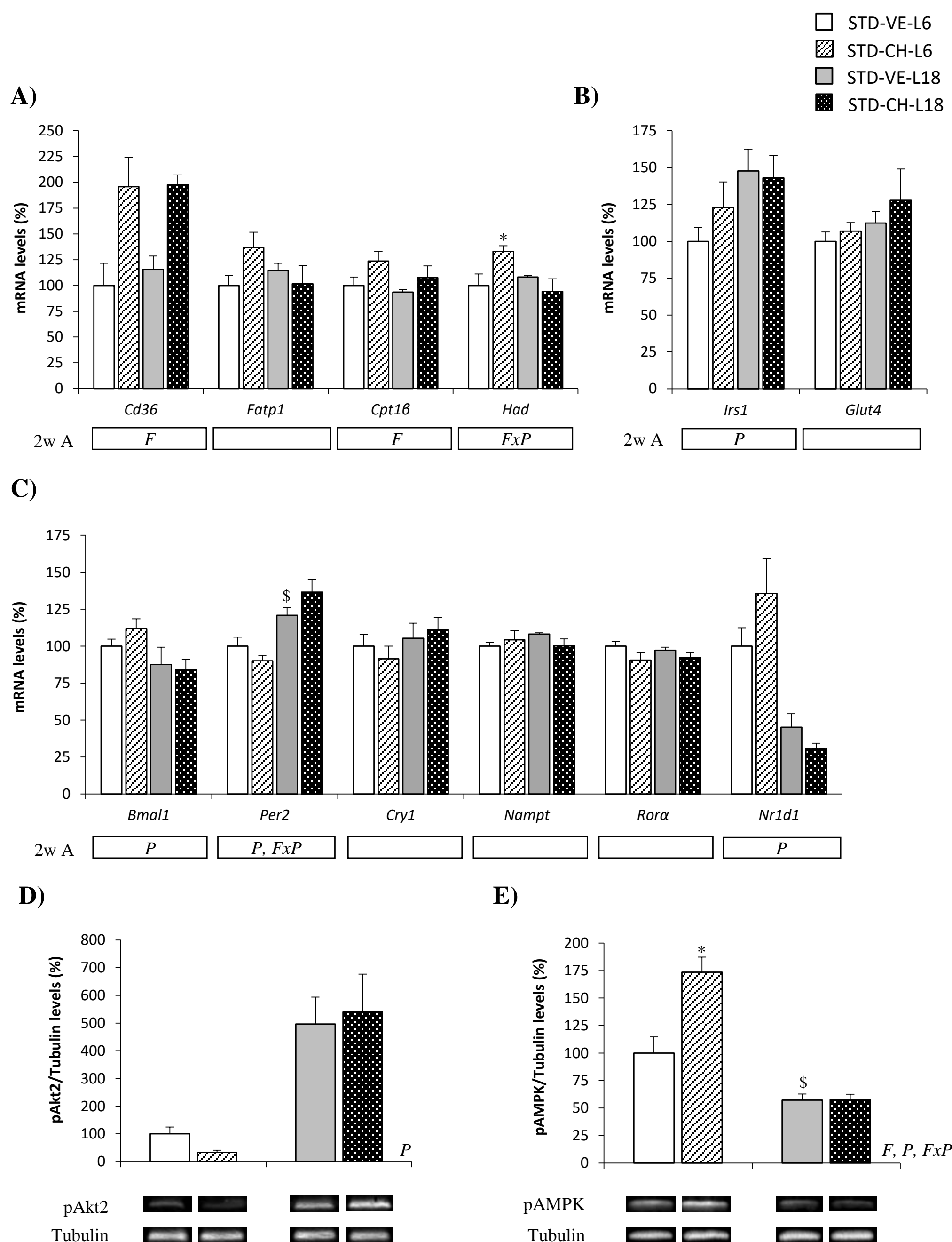

Figure 1. The mRNA levels of lipid metabolism (**A**), glucose metabolism (**B**) and circadian rhythm-related genes (**C**), and pAkt2 (**D**) and pAMPK protein levels (**E**) in the soleus muscle of standard diet-fed male Fischer 344 rats exposed to two different photoperiods for 14 weeks

and supplemented with vehicle or lyophilized cherry for the last 10 weeks. Data are represented as the mean ± SEM (n=6). *F*, fruit effect; *P*, photoperiod effect; *FxP*, fruit x photoperiod interaction effect (two-way ANOVA analysis, p<0.05). * The effect of cherry consumption within photoperiod groups (Student's t test, p<0.05); $ The effect of photoperiod within vehicle groups (Student's t test, p<0.05). *Bmal1,* brain and muscle Arnt-like protein-1; *Cd36*, fatty acid translocase, homologue of CD36; *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Cry1,* cryptochrome circadian clock 1; *Fatp1*, fatty acid transport protein 1; *Glut4,* glucose transporter 4; *Had*, hydroxyacyl-CoA dehydrogenase; *Irs1,* insulin receptor substrate 1; *Nampt,* nicotinamide phosphoribosyltransferase; *Nr1d1,* nuclear receptor subfamily 1, group D, member 1; pAkt2, phosphorylated Akt serine/threonine kinase 2; pAMPK, phosphorylated AMP-activated protein kinase; *Per2,* period circadian clock 2; *Rorα*, RAR-related orphan receptor A.

3.4. Cherry supplementation modulated the expression of genes involved in fatty acid transport and β-oxidation in the soleus muscle of normoweight rats

In the soleus muscle of both the STD-CH-L6 and STD-CH-L18 groups, cherry consumption produced a significant increase in the mRNA levels of the fatty acid transporter-related gene *Cd36* and β-oxidation-related gene *Cpt1β* (**Figure 1A**). Interestingly, in this tissue, the effects of this fruit on lipid metabolism-related genes were enhanced when it was consumed out of its season. Thus, the expression of the key gene involved in β-oxidation, *Had*, showed a different pattern of expression depending on the photoperiod in which cherry was consumed (*FxP* effect, p=0.022, two-way ANOVA), being only significantly upregulated when this fruit was given to the L6-photoperiod-exposed rats (p=0.026, Student's t test) (**Figure 1A**). A very similar pattern was observed for the fatty acid transport protein 1 (*Fatp1*) gene expression levels, although the differences were not statistically significant (*FxP* effect, p=0.066, two-way ANOVA) (**Figure 1A**).

Cherry supplementation produced minimal effects on the expression of lipid metabolism-related genes in the gastrocnemius muscle, only increasing the mRNA levels of *Had* in both the STD-CH-L6 and STD-CH-L18 groups (*F* effect,

p=0.024, two-way ANOVA), although this effect was much more pronounced in the STD-CH-L18 animals (50.4% higher than the STD-VE-L18 rats) (**Figure 2A**).

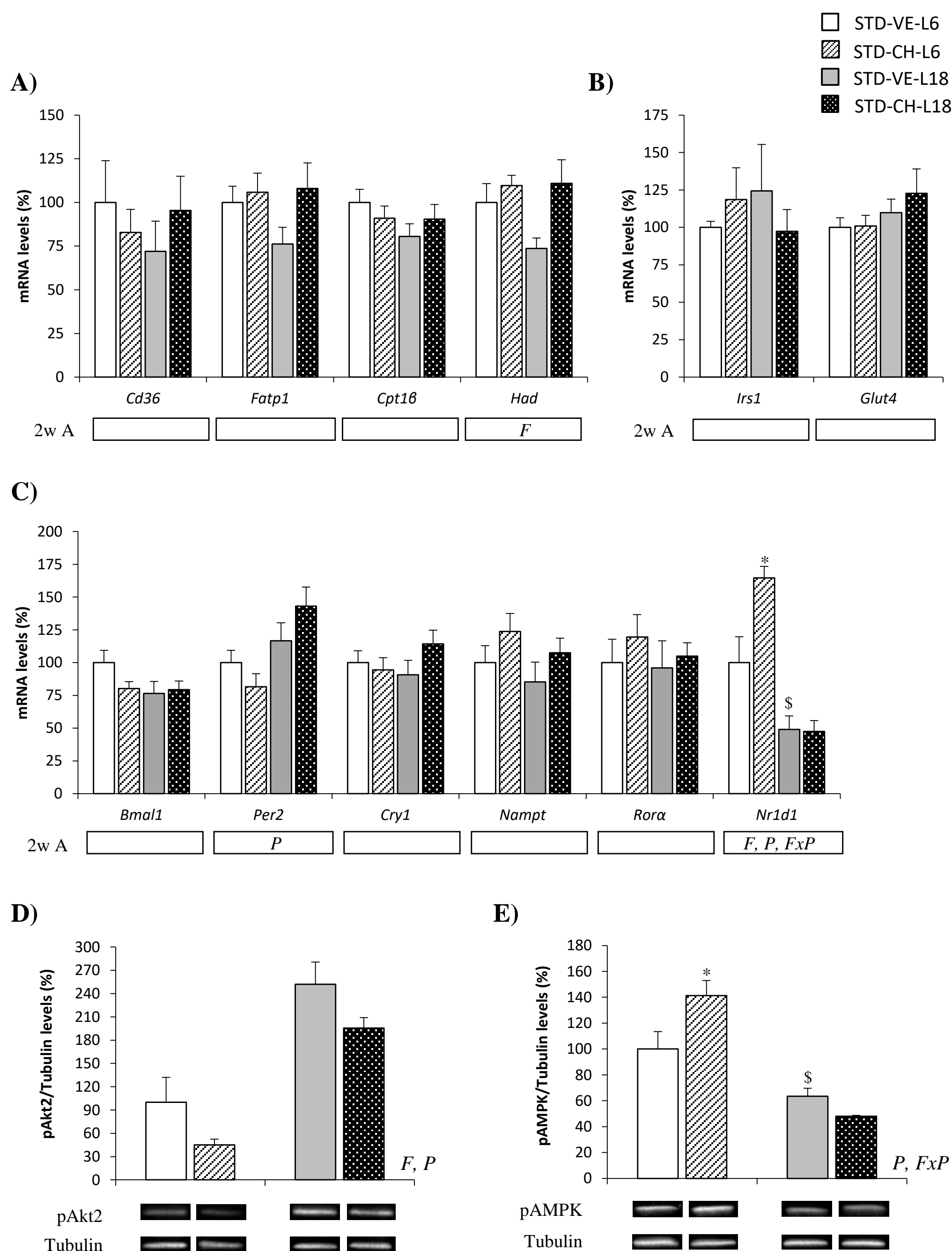

Figure 2. The mRNA levels of lipid metabolism (**A**), glucose metabolism (**B**) and circadian rhythm-related genes (**C**), and pAkt2 (**D**) and pAMPK protein levels (**E**) in the gastrocnemius

muscle of standard diet-fed male Fischer 344 rats exposed to two different photoperiods for 14 weeks and supplemented with vehicle or lyophilized cherry for the last 10 weeks. Data are represented as the mean ± SEM (n=6). *F*, fruit effect; *P*, photoperiod effect; *FxP*, fruit x photoperiod interaction effect (two-way ANOVA analysis, p<0.05). * The effect of cherry consumption within photoperiod groups (Student's t test, p<0.05); $ The effect of photoperiod within vehicle groups (Student's t test, p<0.05). The genes and proteins analyzed have already been described in Figure 1.

3.5. Chronic cherry intake out of season profoundly altered phosphorylated Akt2 and AMPK levels in both the soleus and gastrocnemius muscles of normoweight rats

Despite the fact that, overall, cherry intake did not significantly modulate pAkt2 levels, a pairwise comparison carried out between the L6 groups revealed a clear decrease in the active form of this protein in the soleus muscle in the STD-CH-L6 animals compared with their nontreated counterparts (67.3% lower, p=0.029, Student's t test) (**Figure 1D**). In addition, cherry consumption also decreased pAkt2 protein levels in the gastrocnemius muscle of both L6 and L18 groups (*F* effect, p=0.030, two-way ANOVA) (**Figure 2D**).

Relevantly, a marked interaction effect was reported in pAMPK levels in both the soleus (*FxP* effect, p=0.026, two-way ANOVA) and gastrocnemius (*FxP* effect, p=0.008, two-way ANOVA) muscles. Thus, STD-CH-L6 animals exhibited a sharp increase in pAMPK levels compared to those treated with vehicle in both the soleus and gastrocnemius muscles (73.5% and 41.3% higher, respectively), whereas these effects were not observed in any of the skeletal muscles when the animals subjected to the long photoperiod were chronically supplemented with this fruit (**Figure 1E and 2E**).

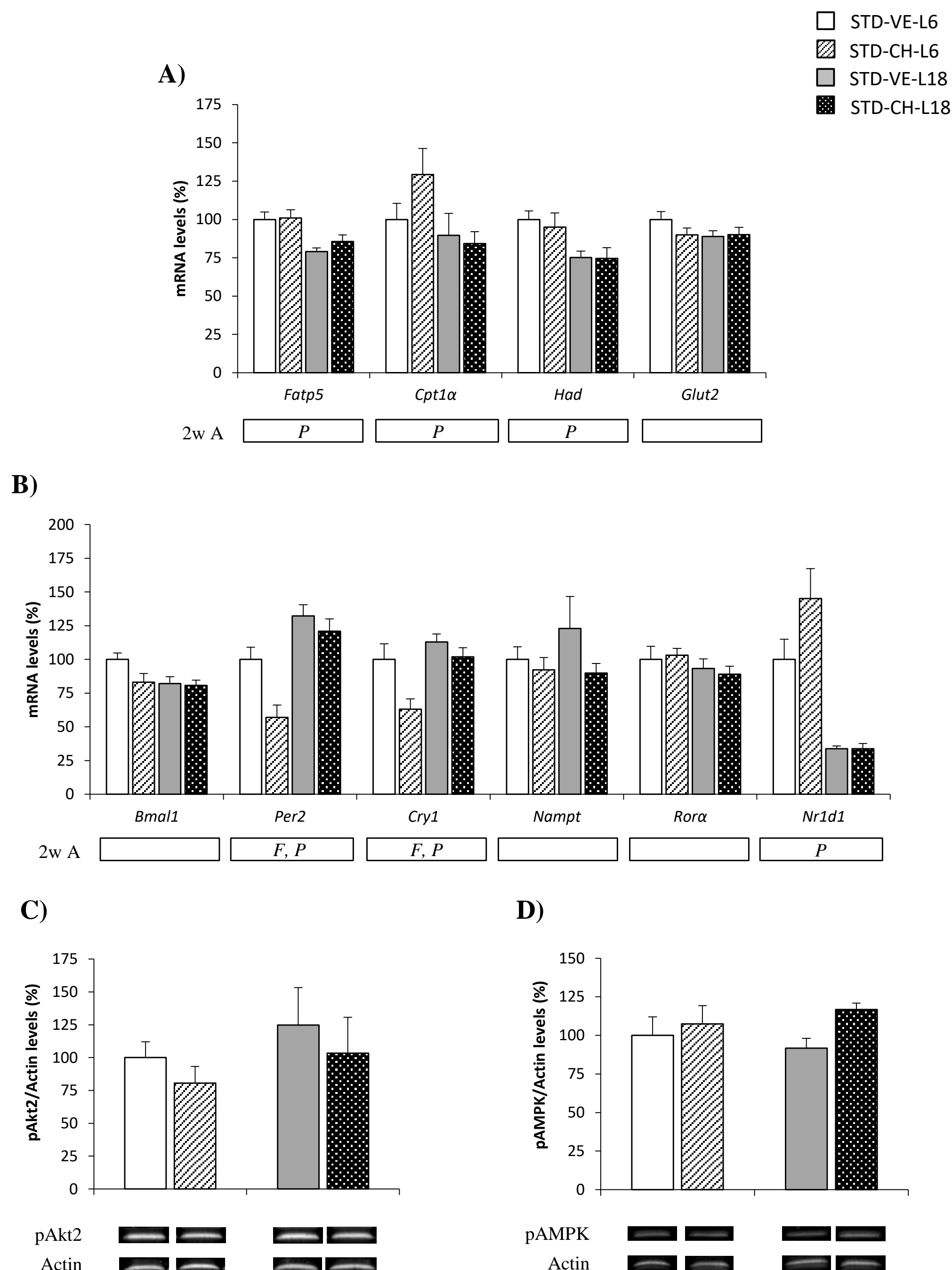

Figure 3. The mRNA levels of lipid and glucose metabolism (**A**), and circadian rhythm-related genes (**B**), and pAkt2 (**C**) and pAMPK protein levels (**D**) in the liver of standard diet-fed male Fischer 344 rats exposed to two different photoperiods for 14 weeks and supplemented with vehicle or lyophilized cherry for the last 10 weeks. Data are represented as the mean ± SEM (n=6). *F*, fruit effect; *P*, photoperiod effect (two-way ANOVA analysis, $p<0.05$). *Cpt1α*, carnitine

palmitoyltransferase 1 alpha; *Fatp5*, fatty acid transport protein 5; *Glut2*, glucose transporter 2. The rest of the genes and proteins analyzed have already been described in Figure 1.

3.6. Cherry consumption slightly modulated the expression of circadian rhythm-related genes in normoweight rats exposed to the short photoperiod

Cherry intake out of its season was able to modulate the expression of different circadian rhythm-related genes in the liver and skeletal muscles. Specifically, STD-CH-L6 rats exhibited higher *Nr1d1* mRNA levels in the gastrocnemius muscle than their nontreated controls (p=0.014, Student's t test), an effect that was not observed in L18 animals (*FxP* effect, p=0.021, two-way ANOVA) (**Figure 2C**). In addition, although both groups supplemented with cherry displayed a significant decrease in the hepatic expression of the genes encoding PER2 (*F* effect, p=0.007, two-way ANOVA) and cryptochrome circadian clock 1 (CRY1) (*F* effect, p=0.001, two-way ANOVA), this overall effect was mainly attributed to the decrease in the mRNA levels of these genes in STD-CH-L6 animals (43.1% and 36.9% lower, respectively, compared with the STD-VE-L6 rats) (**Figure 3B**).

3.7. CAF feeding induced obesity and other MetS-like alterations in male Fischer 344 rats

Although the experiments performed with STD-fed and CAF-fed animals were carried out independently and had different lengths, the numerical comparisons between the data included in ***Table 1*** and ***Table 2*** revealed that rats that were fed a CAF showed an evident increase in cumulative energy intake, body weight gain and fat mass as well as in circulating levels of glucose, insulin, triglycerides, total cholesterol, phospholipids and NEFAs. Therefore, these results strongly suggest that, as expected [32,33], CAF-fed rats developed obesity and other MetS-like alterations, such as hyperglycemia and dyslipidemia.

Table 2. Food intake, biometric and serum parameters in diet-induced obese rats exposed to two different photoperiods for 11 weeks and supplemented with vehicle or lyophilized cherry for the last 7 weeks.

	CAF-VE-L6	CAF-CH-L6	CAF-VE-L18	CAF-CH-L18	2wA
CFI (kcal)	1299 ± 48	1198 ± 30	1384 ± 39	1215 ± 58	*F*
Feed efficiency ratio	9.06 ± 0.52	10.10 ± 0.65	8.25 ± 0.45	10.18 ± 0.61	*F*
Biometric parameters					
Initial body weight (g)	290 ± 7	285 ± 5	298 ± 5	302 ± 7	
Final body weight (g)	407 ± 12	404 ± 10	411 ± 8	423 ± 7	
Body weight gain (g)	117 ± 7	120 ± 6	113 ± 5	121 ± 3	
Liver (g)	14.21 ± 0.54	13.83 ± 0.45	14.71 ± 0.35	15.10 ± 0.45	
Skeletal muscle (g)	2.11 ± 0.05	2.07 ± 0.04	2.09 ± 0.03	2.08 ± 0.04	
Testes (g)	3.00 ± 0.04	2.98 ± 0.04	2.92 ± 0.02	3.04 ± 0.02*	*FxP*
Fat mass (g)	85.84 ± 3.27	88.29 ±5.31	89.51 ± 3.80	89.68 ± 3.91	
Fat mass (%)	21.53 ± 0.79	21.82 ± 1.04	22.03 ± 0.63	21.70 ± 0.66	
Lean mass (g)	294 ± 8	296 ± 4	292 ± 5	299 ± 5	
Lean mass (%)	73.68 ± 0.76	72.50 ± 1.18	72.07 ± 0.54	72.50 ± 0.62	
Lean/fat mass ratio	3.48 ± 0.18	3.46 ± 0.25	3.30 ± 0.12	3.37 ± 0.12	
Serum parameters					
Glucose (mmol/L)	9.11 ± 0.27	11.23 ± 0.86*	10.15 ± 0.26$	10.02 ± 0.51	*FxP*
Insulin (ng/mL)	5.82 ± 0.24	7.31 ± 0.59	6.59 ± 0.47	7.42 ± 0.49	*F*
Glucagon (ng/mL)	2.53 ± 0.12	2.74 ± 0.09	2.63 ± 0.10	2.56 ± 0.07	
Insulin:glucagon ratio	2.21 ± 0.13	2.71 ± 0.23	2.51 ± 0.21	2.94 ± 0.25	*F*
NEFAs (mmol/L)	1.52 ± 0.24	1.92 ± 0.27	1.57 ± 0.18	1.56 ± 0.16	
Phospholipids (mmol/L)	3.87 ± 0.33	4.22 ± 0.42	4.18 ± 0.24	4.03 ± 0.11	
Triglycerides (mmol/L)	5.30 ± 0.58	5.76 ± 0.66	5.28 ± 0.32	5.35 ± 0.14	
TChol (mmol/L)	3.30 ± 0.41	4.10 ± 0.64	3.85 ± 0.41	3.81 ± 0.31	

Cafeteria diet-fed male Fischer 344 rats were exposed to two different photoperiods for 11 weeks and supplemented with vehicle or lyophilized cherry for the last 7 weeks. Data are expressed as mean ± SEM (n=10). Two-way ANOVA analysis (2 × 2 factorial designs: fruit (VE or CH) × photoperiod effect (L6 or L18) was used to evaluate differences among groups. *F*, fruit effect; *FxP*, interaction fruit x photoperiod effect. * The effect of cherry consumption within

photoperiod groups (Student's t test, p<0.05); $ the effect of photoperiod within vehicle groups (Student's t test, p<0.05). The skeletal muscle weight represents the total weight of both soleus and gastrocnemius muscles. CFI, cumulative food intake; Tchol, total cholesterol.

3.8. CAF consumption dampened the photoperiod effects on biometric and serum parameters observed in standard diet-fed rats

Unlike what was observed in normoweight rats, the exposure to different photoperiods produced slight alterations in biometric and serum parameters in diet-induced obese rats. Thus, only the circulating glucose levels were significantly higher in the CAF-VE-L18 animals in comparison with the CAF-VE-L6 rats (p=0.013, Student's t test) (**Table 2**).

3.9. The exposure to different day lengths altered the whole-body substrate utilization and modulated different lipid metabolism-related genes in the skeletal muscles and liver of diet-induced obese rats

Overall, L18 CAF-fed animals displayed significantly lower RQ than L6 obese rats (*P* effect, p=0.030, two-way ANOVA), and this effect was much more evident when the comparison was carried out between the noncherry-supplemented groups (**Figure 4A**). Consequently, the CAF-VE-L18 rats showed increased lipid utilization (p=0.028, Student's t test) (**Figure 4B**) and decreased carbohydrate oxidation (p=0.027, Student's t test) (**Figure 4C**) compared with their L6 nontreated counterparts.

The enhancement of fat oxidation observed in CAF-VE-L18 animals was accompanied by a significant upregulation of the fatty acid transporter gene *Fatp1* in the soleus muscle (p=0.006, Student's t test) (**Figure 5A**) and higher mRNA levels of the *Fatp1, Cpt1β* and *Had* genes in the gastrocnemius muscle (*P* effect, p<0.05, two-way ANOVA) (**Figure 6A**). Intriguingly, hepatic *Cd36, Fatp5, Cpt1α* and *Had* mRNA levels were significantly decreased in L18-photoperiod

exposed animals in comparison with their L6 counterparts (*P* effect, p<0.05, two-way ANOVA) (**Figure 7A**).

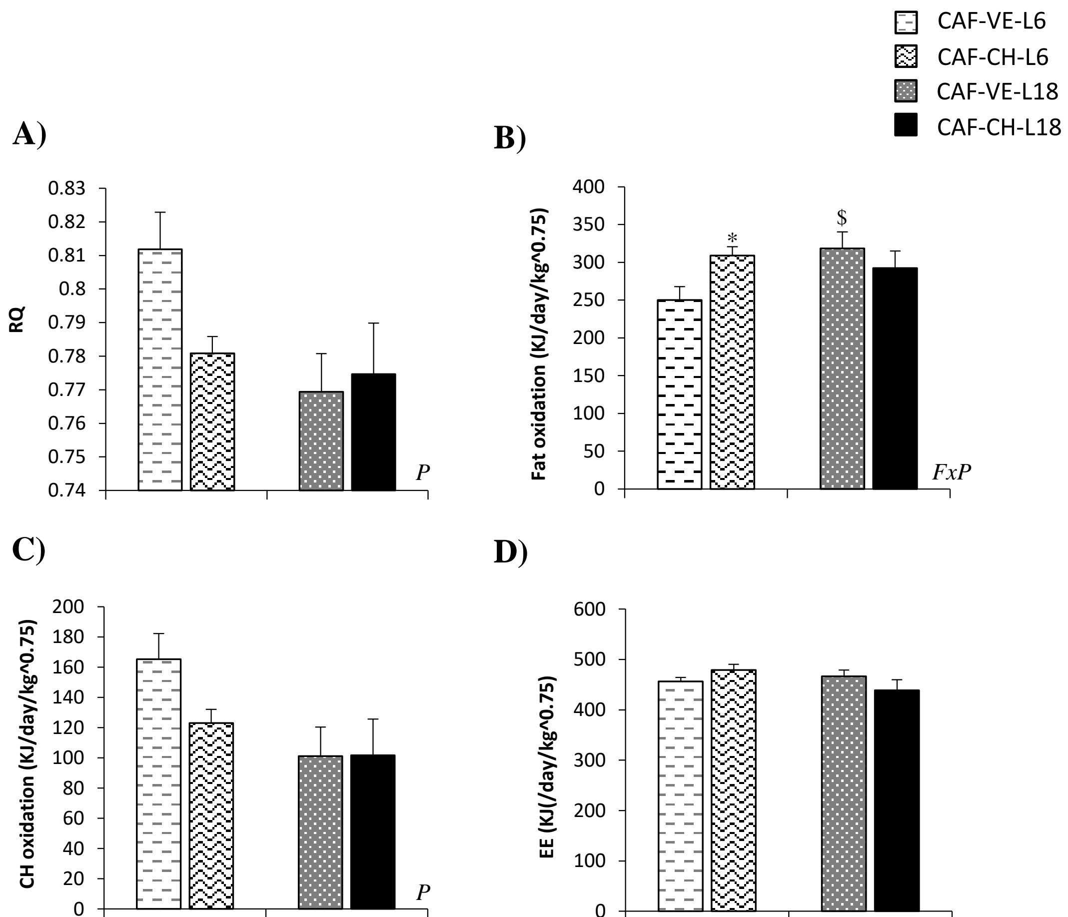

Figure 4. Respiratory quotient (RQ) (**A**), fat oxidation (**B**), carbohydrate (CH) oxidation (**C**) and energy expenditure (EE) (**D**) of cafeteria diet-fed male Fischer 344 rats exposed to two different photoperiods for 11 weeks and supplemented with vehicle or lyophilized cherry for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). *P*, photoperiod effect; *FxP*, fruit x photoperiod interaction effect (two-way ANOVA analysis, p<0.05). * The effect of cherry consumption within photoperiod groups (Student's t test, p<0.05); $ The effect of photoperiod within vehicle groups (Student's t test, p<0.05).

3.10. The expression of glucose metabolism and circadian rhythm-related genes and the phosphorylated levels of Akt2 were photoperiodically regulated in the skeletal muscles and liver of obese rats

Both groups of CAF-fed rats exposed to the L18 photoperiod displayed a slight, but significant, increase in *Glut4* and *Glut2* mRNA levels in both muscles and the liver, respectively (*P* effect, p<0.05, two-way ANOVA), and exhibited lower hepatic *Irs1* mRNA levels (*P* effect, p=0.037, two-way ANOVA) than their L6 counterparts (**Figures 5B, 6B and 7B**). CAF-VE-L18 animals also showed a residual decrease in *Irs1* gene expression levels in the gastrocnemius muscle when compared with the CAF-VE-L6 group (p=0.029, Student's t test) (**Figure 6B**). In addition, the protein expression analysis revealed an overall significant downregulation of pAkt2 levels in the soleus muscle of CAF rats exposed to the L18 photoperiod (*P* effect, p=0.002, two-way ANOVA) (**Figure 5D**).

Diet-induced obese rats also exhibited a strong photoperiod effect in the mRNA levels of the analyzed circadian rhythm-related genes in the liver and both skeletal muscles. In these tissues, *Per2* and *Cry1* mRNA levels were significantly greater in CAF rats exposed to the L18 photoperiod (*P effect*, p<0.05, two-way ANOVA), whereas in these groups, *Nr1d1* gene expression was lower in comparison with their L6 counterparts (*P effect*, p<0.001, two-way ANOVA) (**Figures 5C, 6C and 7C**). In addition, *Bmal1* mRNA levels decreased in both the soleus muscle and liver of L18 CAF-fed animals, whereas the expression of the *Rorα* gene increased in the soleus muscles and decreased in the liver in these groups of rats (*P* effect, p<0.05, two-way ANOVA) (**Figures 5C and 7C**).

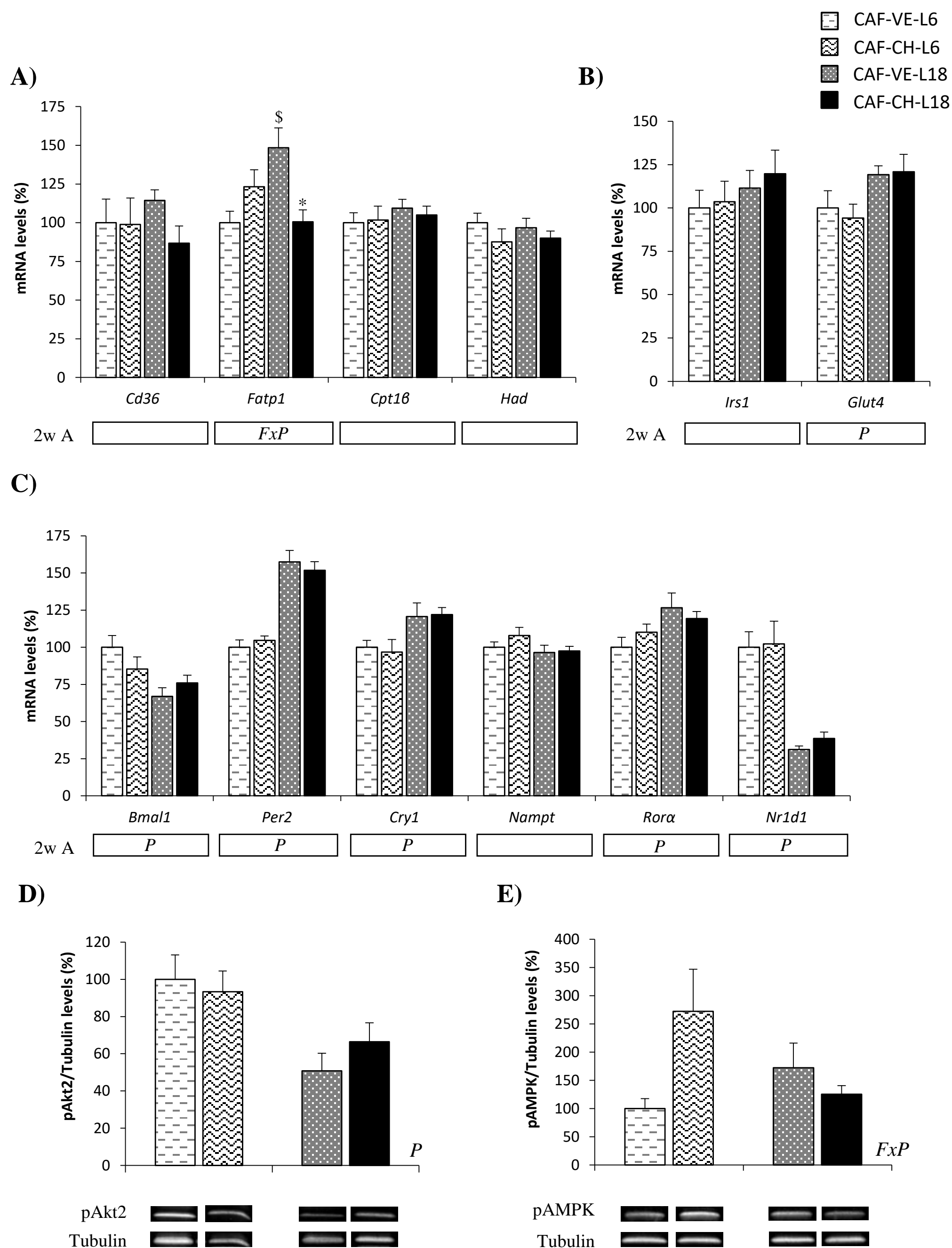

Figure 5. The mRNA levels of lipid metabolism (**A**), glucose metabolism (**B**) and circadian rhythm-related genes (**C**), and pAkt2 (**D**) and pAMPK protein levels (**E**) in the soleus muscle of cafeteria diet-fed male Fischer 344 rats exposed to two different photoperiods for 11 weeks and supplemented with vehicle or lyophilized cherry for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). *P*, photoperiod effect; *FxP*, fruit x photoperiod interaction effect (two-

way ANOVA analysis, p<0.05). * The effect of cherry consumption within photoperiod groups (Student's t test, p<0.05); $ The effect of photoperiod within vehicle groups (Student's t test, p<0.05). The genes and proteins analyzed have already been described in Figure 1.

3.11. Cherry consumption out of season significantly raised the circulating levels of glucose and insulin in CAF-fed obese rats

Both CAF-CH-L6 and CAF-CH-L18 groups exhibited a significantly lower cumulative food intake and an increase in the feed efficiency ratio (*F* effect, p<0.05, two-way ANOVA), although these changes were not translated into lower final body weight or body weight gain (**Table 2**). Relevantly, a clear interaction effect was reported for the circulating glucose levels (*FxP* effect, p=0.045, two-way ANOVA), which were significantly higher only in the CAF-CH-L6 group in comparison with their nontreated counterparts (p=0.041, Student's t test) (**Table 2**). In addition, although an overall fruit effect was obtained for the circulating insulin levels (*F* effect, p=0.019, two-way ANOVA), the reported increase was mainly observed in the CAF-CH-L6 rats (25.5% higher than the CAF-VE-L6 group) (**Table 2**).

3.12. Cherry supplementation enhanced lipid oxidation and pAMPK expression in obese rats exposed to the short photoperiod

CAF-CH-L6 animals exhibited residually lower RQ than those in the CAF-VE-L6 group (p=0.030, Student's t test) (**Figure 4A**), shifting towards a significantly higher lipid oxidation rate (p=0.015, Student's t test) (**Figure 4B**) and decreased carbohydrate utilization (p=0.047, Student's t test) (**Figure 4C**). These results suggest that, in diet-induced obese animals, cherry intake out of its season resembled the whole-body energy catabolism behavior observed in L18 photoperiod-exposed rats.

Two-way ANOVA revealed a clear interaction effect between fruit and photoperiod in the pAMPK levels of the soleus muscle (*FxP* effect, p=0.034, two-

way ANOVA). Relevantly, the active form levels of this protein were higher only when cherry was consumed out of its season (172.3% higher in CAF-CH-L6 than in CAF-VE-L6 animals, p=0.056, Student's t test) (**Figure 5E**).

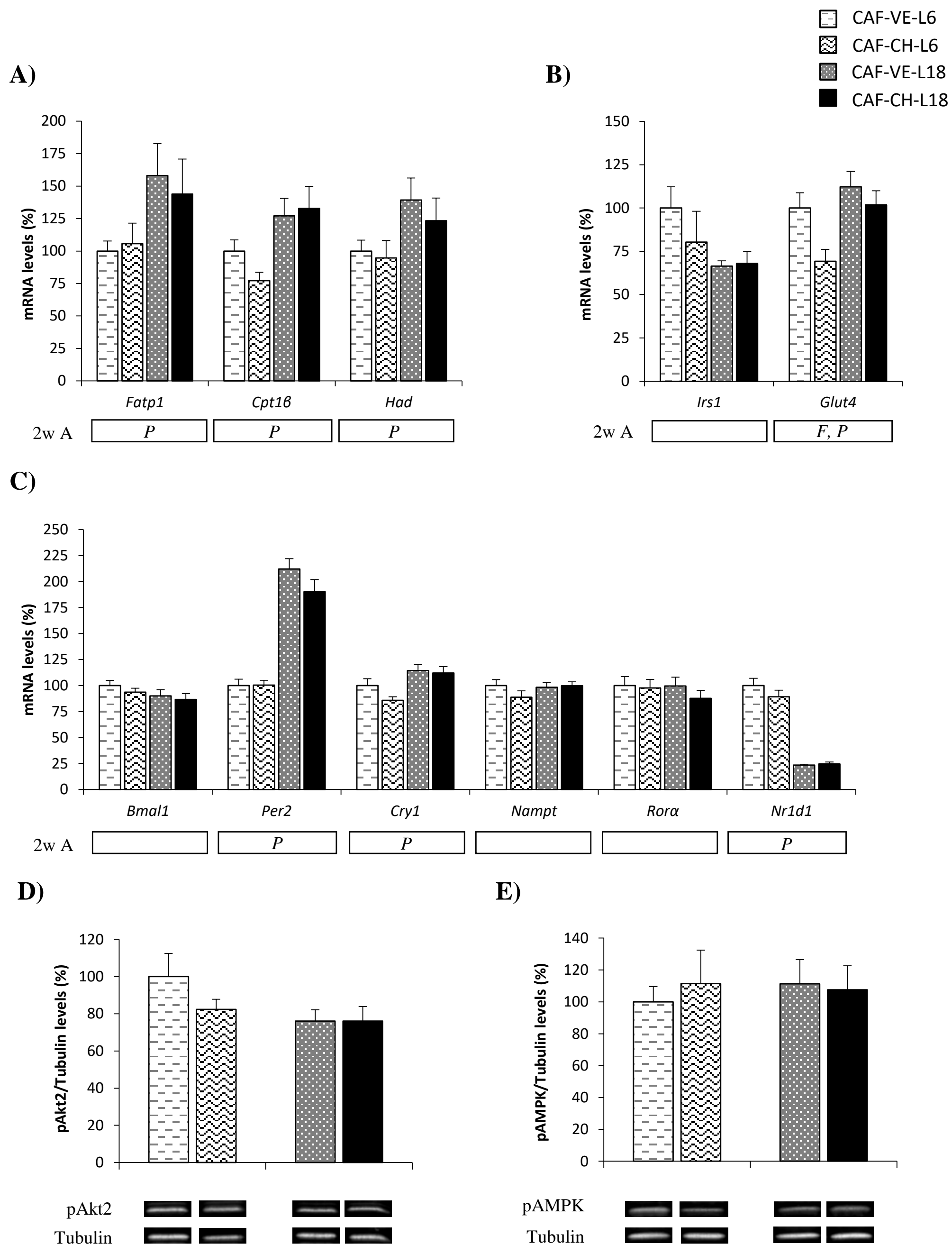

Figure 6. The mRNA levels of lipid metabolism (**A**), glucose metabolism (**B**) and circadian rhythm-related genes (**C**), and pAkt2 (**D**) and pAMPK protein levels (**E**) in the gastrocnemius

muscle of cafeteria diet-fed male Fischer 344 rats exposed to two different photoperiods for 11 weeks and supplemented with vehicle or lyophilized cherry for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). *F*, fruit effect; *P*, photoperiod effect (two-way ANOVA analysis, p<0.05). The genes and proteins analyzed have already been described in Figure 1.

3.13. Cherry consumption out of season decreased Glut4 mRNA levels in the gastrocnemius muscle of obese rats

Cherry consumption produced scarce changes in lipid metabolism-related genes in the skeletal muscle, only decreasing the *Fatp1* mRNA levels in the soleus muscle of CAF-CH-L18 animals in comparison with their nontreated controls (p=0.006, Student's t test) (**Figure 5A**).

A clear cherry consumption effect was reported for *Glut4* mRNA levels in the gastrocnemius muscle (*F* effect, p=0.018, two-way ANOVA), although this effect was mainly attributed to the downregulation of this gene in the CAF-CH-L6 group compared to the non-treated L6 group (30.8% lower) (**Figure 6B**).

4. DISCUSSION

In the present study, we reported that the consumption of cherry produced differential physiological and metabolic responses in both normoweight and diet-induced obese F344 rats, depending on the photoperiod to which the animals were chronically exposed. Relevantly, in agreement with our hypothesis, these effects were more accentuated and to some extent, more deleterious, when cherry was consumed out of its harvesting season (in the animals that were exposed to the short photoperiod). Thus, in STD-fed normoweight rats, cherry consumption out of season induced a) a more pronounced increase in the mRNA levels of fatty acid transport- and β-oxidation-related genes in the soleus muscle compared to those observed in STD-CH-L18 animals; b) a sharp upregulation of the energetic sensor pAMPK [34] in both the soleus and gastrocnemius muscles; and c) a decrease in the

downstream postreceptor target of insulin, pAkt2 [35], in the soleus muscle. Under obesogenic conditions in L6 but not in L18 rats, the intake of cherry produced a) a significant increase in circulating glucose and insulin levels accompanied by a decrease in *Glut4* mRNA levels in the gastrocnemius muscle; b) an enhancement of whole-body lipid utilization; and c) an increase in the pAMPK protein levels in the soleus muscle.

Sweet cherries are rich in anthocyanins, a family of flavonoids, which are well-known antioxidants present in a wide variety of fruits such as berries and grapes [36,37] that can ameliorate risk factors for cardiovascular disease, such as dyslipidemia [38], glucose intolerance and obesity [39,40]. In this study, normoweight rats supplemented with cherry displayed decreased circulating cholesterol levels regardless of the photoperiod to which they were subjected. These results were in agreement with Graf and collaborators, who reported a significant decrease in serum cholesterol concentrations after 10 weeks of an anthocyanin-rich juice treatment in F344 rats [41]. Some proposed mechanisms to explain this cholesterol-lowering effect of anthocyanins could be the increase in cholesterol reverse transport, the inhibition of cholesterol absorption and its elimination through bile acids [42]. Further analyses focused on key proteins and genes involved in these pathways would be of value to elucidate the molecular mechanisms by which cherry induced this effect. In addition, in CAF-induced obese rats, cherry consumption significantly lowered the cumulative caloric intake in both the L18 and L6 groups, although this effect was not translated into changes in body weight gain. This finding is coincident with those obtained by Han and collaborators, which showed that supplementation with anthocyanin-rich purple potato flakes for 28 days in F344 rats fed a cholesterol-rich diet tended to decrease food intake, although no changes in body weight gain were reported [43]. In our study, this lack of body weight-lowering effects may be due to the increased feed efficiency observed in both photoperiod-exposed groups that were supplemented with

cherry, which would indicate that the intake of this fruit induced a higher efficiency of energy intake conversion into body weight gain [44]. Additional analysis would be needed to elucidate the mechanisms responsible for this effect, such as a lower activation of thermogenesis in the brown adipose tissue [45].

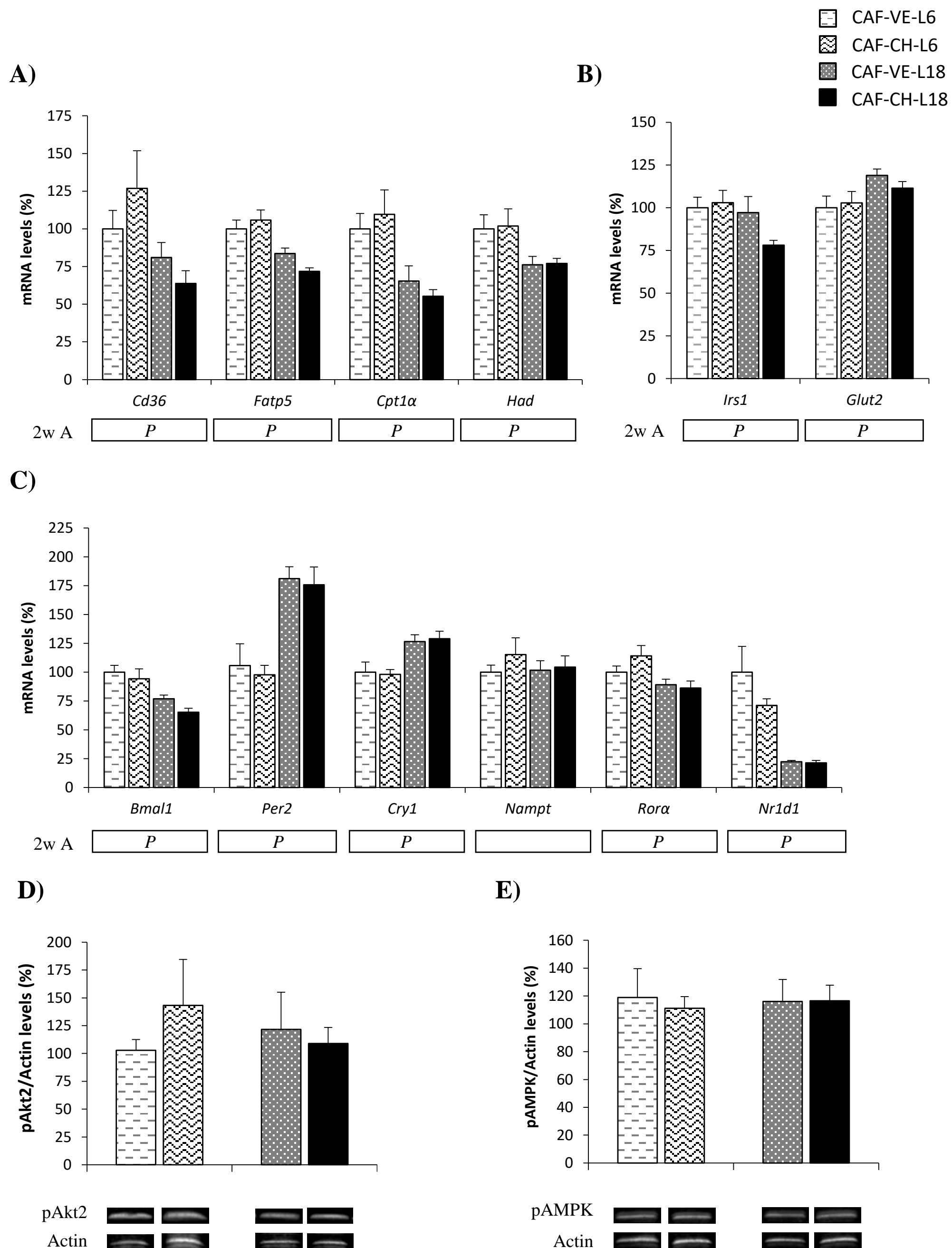

Figure 7. The mRNA levels of lipid metabolism (**A**), glucose metabolism (**B**) and circadian rhythm-related genes (**C**), and pAkt2 (**D**) and pAMPK protein levels (**E**) in the liver of cafeteria diet-fed male Fischer 344 rats exposed to two different photoperiods for 11 weeks and supplemented with vehicle or lyophilized cherry for the last 7 weeks. Data are expressed as the mean ± SEM (n=10). *P*, photoperiod effect (two-way ANOVA analysis, $p<0.05$). The genes and proteins analyzed have already been described in Figures 1 and 3.

Interestingly, chronic cherry intake produced profound changes in fatty acid metabolism, whole-body substrate utilization and glucose homeostasis, which in some cases, were highly influenced by the circannual endogenous metabolism in both STD-fed and CAF-fed rats. We previously showed that chronic exposure to 6 h of light was characterized by a marked alteration in fatty acid metabolism in normoweight F344 rats [16]. This seasonal adaptive response was evidenced by higher serum levels of NEFAs and lower soleus mRNA levels of the genes involved in β-oxidation (*Had* and *Cpt1β*) and fatty acid transport (*Cd36*) in the soleus muscle and liver when compared with animals exposed to 12 h of light [16]. In the present study, at first glance, the exposure to a long photoperiod seemed to exacerbate the negative impact on lipid catabolism formerly observed in L6 animals because both STD-VE-L18 and STD-CH-L18 rats displayed a significant decrease in the fatty oxidation enhancer p-AMPK [46] in both skeletal muscles and a hepatic downregulation of the genes encoding HAD, CPT1β and the fatty acid transporter FATP5. However, these STD-fed L18 rats showed significantly lower blood NEFAs levels than those exposed to the short photoperiod, which could be tentatively attributed to a lower lipolytic activity in fat depots or/and to a higher fatty acid uptake in white adipose tissues or skeletal muscles [32]. If this were true, it could be understood as an adaptive mechanism addressed to counteract the detrimental effects observed in muscular and hepatic lipid-related molecular pathways in order to maintain their metabolic homeostasis. Additional research is needed to clarify this issue. Remarkably, cherry consumption triggered

relevant changes in lipid catabolism of normoweight rats exposed to both short and long photoperiods. Thus, the significant increase in *Cd36* and *Cpt1β* mRNA levels in the soleus muscle of STD-CH-L6 and STD-CH-L18 rats would suggest an enhancement of fatty acid uptake and β-oxidation in these animals in response to cherry consumption. These lipid catabolic effects observed at the molecular level in response to cherry intake have not been previously described and could be tentatively attributed to its polyphenol content since different authors have shown very similar effects in animals supplemented with other polyphenols, such as grape seed procyanidins [47]. Interestingly, only cherry-supplemented normoweight rats exposed to the short photoperiod displayed a significant upregulation of *Had* gene expression in the soleus muscle and a sharp increase in the skeletal muscle levels of phosphorylated AMPK. Taking into account that the activation of AMPK can mediate the upregulation of the β-oxidation-related genes *Had* and *Cpt1β* through the activation of PPARα [34,48], we suggest that cherry consumption out of season would enhance the use of fat as an energy substrate in the STD-fed animals through the activation of AMPK. These results would strongly suggest that some molecular effects triggered by this fruit were highly influenced by seasonal endogenous metabolism, at least in lipid catabolism-related pathways. In agreement with this hypothesis, in the present study, we also observed that cherry-treated CAF-fed rats exposed to the short photoperiod displayed a significant decrease in the RQ and consequently, showed increased lipid utilization, which could be driven by the upregulation of the pAMPK levels in the soleus muscle. Remarkably, in these animals, the intake of cherry out of season resembled the long photoperiod phenotype in terms of whole-body substrate utilization, which in our study, was characterized by increased lipid oxidation that could be partly explained by a significant overexpression of the genes encoding key enzymes involved in fatty acid transport (FATP1) and β-oxidation (CPT1β and HAD) in skeletal muscles, when compared with their L6-

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exposed counterparts. Since this lipid catabolic enhancement effect was not observed when this fruit was consumed by CAF-CH-L18 animals, our findings reinforce the idea that this physiological change triggered by the bioactive compounds present in fruits can be highly dependent on the mammal's seasonality, even in the obese state. Nevertheless, no differences in the circulating NEFAs levels were observed either in STD-CH-L6 or in CAF-CH-L6 animals. Additional studies focused on key lipid metabolism-related proteins and genes in white adipose tissue, which is considered the main contributor of NEFAs to the bloodstream [49], would be necessary to clarify this issue.

We previously demonstrated that, in normoweight F344 rats, the exposure to different photoperiods profoundly affected glucose metabolism and insulin signaling [16]. Thus, compared with L12 animals, L6 rats displayed a marked increase in circulating glucose levels that was accompanied by a downregulation of pAkt2 protein levels in the soleus and gastrocnemius muscles and by decreased mRNA levels of the genes encoding IRS1 and GLUT2 in the soleus and liver, respectively [16]. In line with our findings and other *in vivo* evidence [9,10] pointing towards an insulin resistance-like phenotype in animals exposed to short day lengths, in the present study, the chronic exposure to 6 h of light was also characterized by a marked alteration in glucose homeostasis, which was evidenced by increased circulating levels of glucose, decreased pAkt2 protein levels in both the soleus and gastrocnemius and lower soleus *Irs1* mRNA levels in STD-fed animals compared with their L18 counterparts. Intriguingly, as opposed to what was observed in normoweight rats, CAF-fed animals held under the short photoperiod and supplemented with the vehicle showed a decrease in blood glucose levels compared with their nontreated L18 counterparts. This change was accompanied by an upregulation of the soleus pAkt2 protein levels and by an overexpression of the gastrocnemius *Irs1* gene, which would suggest an enhancement of the insulin-dependent glucose uptake in these rats [35]. Nevertheless, the slight

downregulation of the hepatic and muscular *Glut2* and *Glut4* mRNA levels, respectively, observed in CAF-VE-L6 rats would not be in agreement with this hypothesis and would point toward a decreased glucose uptake in the liver and skeletal muscle. However, since mRNA levels do not always parallel protein data, additional measurements focused on the analysis of the protein levels of these key glucose transporters would be useful to clarify this issue. Altogether, these results indicate that seasonal glucose metabolism can be modified by obesity and/or by the consumption of the obesogenic CAF diet, which includes highly palatable and energy-dense foods consumed by humans [50]. Whether this differential seasonal glucose metabolism response induced by diet and/or the obese status can also occur in humans deserves further research.

Interestingly, chronic cherry intake exerted profound changes in glucose metabolism and insulin signaling in both normoweight and CAF-fed obese rats. Thus, the decrease in the levels of the phosphorylated downstream postreceptor target of insulin Akt2 [35] observed in the gastrocnemius muscle in both the STD-CH-L6 and STD-CH-L18 groups would suggest that cherry supplementation induced a lower muscular glucose uptake in animals exposed to both L18 and L6 photoperiods. However, Yan *et al.* reported a significant increase in the Akt mRNA levels and a higher pAkt/Akt protein ratio in the skeletal muscle of db/db mice supplemented with a mulberry anthocyanin extract for 7 weeks [51]. This discrepancy between both studies could be attributed to differences in the animal model used and/or in the photoperiod to which animals were exposed, since Yan and collaborators' animal models were subjected to a 12 h light/dark schedule [51]. Interestingly, cherry-supplemented STD-fed animals exposed to the short photoperiod displayed a significant decrease in pAkt2 levels in the soleus muscle, an effect that was not observed in the normoweight rats chronically exposed to a long day length that received the fruit. These findings also contribute to strengthening the idea that the effects produced by cherry depend on the rats' seasonal metabolism.

Despite the fact that insulin-induced activation of Akt2 in the skeletal muscle stimulates glucose uptake via GLUT4 translocation from intracellular vesicles to the plasma membrane [15], no differences either in circulating glucose or insulin levels were observed among STD-fed groups supplemented with cherry. This lack of physiological effects observed in STD-CH-L6 animals could be explained, at least in part, by the phenotypic flexibility that characterizes an optimal metabolic health and allows the normoweight rats to buffer external challenges through the modulation of a wide range of metabolic processes and molecular mechanisms in order to maintain their homeostatic robustness [52]. It has been suggested that most chronic metabolic diseases are triggered by an impairment of phenotypic flexibility [52]. In agreement with this hypothesis, in our second experiment, cherry supplementation out of season indeed caused a significant increase in glycemia and insulinemia in CAF-CH-L6 rats, which developed obesity and MetS-like alterations. Relevantly, these animals only exhibited slight molecular changes related to the insulin-dependent glucose uptake pathway, such as the downregulation of *Glut4* mRNA levels in the gastrocnemius muscle, suggesting a lower capability to address external challenges (i.e., cherry intake) through the activation of this key molecular pathway, which resulted in an impairment of glucose homeostasis.

We previously described marked changes in circadian rhythm-related genes in the liver and skeletal muscles of normoweight rats chronically exposed to different photoperiods [16]. At the same line, in the present study, a clear photoperiod effect was observed in the hepatic and muscular expression patterns of several circadian rhythm genes in both STD- and CAF-fed rats. Some studies have shown that polyphenols [53,54] and exogenous melatonin [55], which are present in considerable amounts in cherry [28,56], can highly modulate biological rhythms, affecting the circadian regulation in target tissues. In the present study, we observed that cherry consumption out of season was able to modulate the mRNA levels of different circadian rhythm-related genes,

such as *Nr1d1* in the gastrocnemius muscle and *Per2* and *Cry*1 in the liver of STD-fed rats. This photoperiod-dependent response could be tentatively attributed to an interaction between the effects produced by these bioactive compounds and the animal's seasonal adaptive response. Despite the limitation of these single-point (ZT 1-2) measurements, these results would suggest that, in normoweight animals, the physiological and molecular changes triggered by chronic cherry intake out of season could be partly driven by modulation of the expression of genes encoding clock-related nuclear receptors that play a relevant role in lipid and glucose metabolism regulation [57,58]. In contrast, cafeteria feeding dampened the modulatory effects of cherry consumption on circadian rhythms in L6 animals, suggesting that the cherry effects observed in these rats would not be attributed to changes in these key modulators of metabolism.

5. CONCLUSION

In conclusion, we demonstrated that cherry consumption exerted a marked photoperiod-dependent effect, inducing more pronounced changes when it was consumed out of season in both normoweight and diet-induced obese F344 rats. Nevertheless, the physiological and metabolic responses significantly differed depending on the obese status and/or dietary pattern. Thus, in STD-fed rats exposed to the short photoperiod, cherry intake exacerbated the activation of fatty acid transport and β-oxidation-related pathways observed in the L18 animals supplemented with this fruit, which was evidenced by an upregulation of *Had* mRNA levels (soleus) and p-AMPK (soleus and gastrocnemius), and decreased the levels of the key activator of insulin-stimulated glucose uptake pAkt2 (soleus). Nevertheless, these molecular changes were not translated into changes in blood markers of glucose and lipid metabolism, which could be tentatively attributed to the phenotypic flexibility associated with the normoweight status. In contrast, in CAF-fed obese rats, cherry consumption

triggered detrimental changes related to glucose metabolism in L6 but not in L18 rats, as evidenced by increased glycemia and insulinemia. In addition, these rats also showed enhanced whole-body lipid utilization, resembling the substrate oxidation pattern observed in the animals exposed to 18 h of light. Although the pathophysiological significance of these results requires further investigation and keeping in mind that the modern lifestyle attenuates the impact of seasonal variations in day length on human health, these results could contribute to highlighting the importance of the consumption of proximal and seasonal fruits in order to maintain optimal health and/or to design nutritional strategies addressed to ameliorate obesity and its related disorders.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Cinta Bladé (CB), Lluís Arola (LA), Antoni Caimari (AC) and Josep del Bas (JdB) designed the studies; Roger Mariné (RM), Cristina Domenech (CD), AC and JdB performed the experiments and analyzed the data; RM, AC and LA wrote the manuscript. All the authors read, discussed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary table 1. Nucleotide sequences of primers used for real time quantitative PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Tissue
β-actin	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	*L*
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC	*G, L, S*
Cd36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG	*G, L, S*
Cpt1α	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG	*L*
Cpt1β	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	*G, S*
Cry1	TGGAAGGTATGCGTGTCCTC	TCCAGGAGAACCTCCTCACG	*G, L, S*
Fatp1	TGCTCAAGTTCTGCTCTGGA	CATGCTGTAGGAATGGTGGC	*G, S*
Fatp5	CCTGCCAAGCTTCGTGCTAAT	GCTCATGTGATAGGATGGCTGG	*L*
Glut2	AGTCACACCAGCACATACGA	TGGCTTTGATCCTTCCGAGT	*L*
Glut4	CCATTGCTTCTGGCTATCAC	TCCGTTTCTCATCCTTCAGC	*G, S*
Had	ATCGTGAACCGTCTCTTGGT	AGGACTGGGCTGAAATAAGG	*G, L, S*
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG	*G, L, S*
Irs1	CTACACCCGAGACGAACACT	TAACCTGCCAGACCTCCTTG	*G, L, S*
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG	*G, L, S*
Nr1d1	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT	*G, L, S*
Per2	CGGACCTGGCTTCAGTTCAT	AGGATCCAAGAACGGCACAG	*G, L, S*
Ppia	CCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCAAAACGCT	*G, S*
Rorα	CCCGATGTCTTCAAATCCTTAGG	TCAGTCAGATGCATAGAACACAAACTC	*G, L, S*
Tfrc	ATCATCAAGCAGCTGAGCCAG	CTCGCCAGACTTTGCTGAATTT	*S*

The table shows the nucleotide sequences of primers used for PCR amplification. Primer pairs for PCR were designed using Primer3 software and the sequence information were obtained from Genbank. *β-actin*, actin beta; *Bmal1,* brain and muscle Arnt-like protein-1; *Cd36*, fatty acid translocase, homologue of CD36; *Cpt1α*, carnitine palmitoyltransferase 1 alpha; *Cpt1β*, carnitine palmitoyltransferase 1 beta; *Cry1,* cryptochrome circadian clock 1; *Fatp1*, fatty acid transport protein 1; *Fatp5*, fatty acid transport protein 5; *Glut2*, glucose transporter 2; *Glut4,* glucose transporter 4; *Had*, hydroxyacyl-CoA dehydrogenase; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Irs1,* insulin receptor substrate 1; *Nampt,*

nicotinamide phosphoribosyltransferase; *Nr1d1,* nuclear receptor subfamily 1, group D, member 1; *Per2,* period circadian clock 2; *Ppia*, peptidylprolyl isomerase A; *Rorα*, RAR-related orphan receptor A; *Tfrc*, transferrin receptor. Gene expression levels were analyzed in the liver (L) and the gastrocnemius (G) and soleus (S) muscles.

IV. GENERAL DISCUSSION

Throughout evolutionary history, animals and plants have developed several strategies in order to adapt to the continuous variations in the environment, improving the probabilities to enlarge their lifespan.

Almost all kind of animal species have developed a complex regulatory mechanism in which physiology, behavior, metabolism and other body functions are integrated and synchronized in order to anticipate the external changes that take place over the 24 hours of the day. Mainly modulated by the light and dark phases, the persistent daily fluctuations of all these functions are known as circadian rhythms [1–4]. Moreover, evolution has permitted not only the anticipation of daily predictable changes but also the ability to predict annual variations and favorably adapt the physiology and reproduction towards them [5–8]. Although the mechanisms involved in these circannual rhythms are not fully elucidated, robust responses to seasonal variations in day length have been reported in several species, including humans [9–12].

By following different evolutionary strategies, plants have been able to inhabit the vast majority of the terrestrial surface. To achieve it, they have developed a secondary metabolism in which they synthesize non-nutritive and bioactive compounds, known as phytochemicals, which exert numerous functions in order to ensure plants' survival, such as protective roles against abiotic conditions, pathogens or herbivore attacks and reproductive functions by attracting pollinators and ensuring seed dispersal [13,14].

The coexistence between plants and animals has also favored the coevolutionary development of regulatory mechanisms in which these phytochemicals can be recognized as signals by heterotrophs that consume them, informing about the environmental conditions and allowing animals to favorably adapt their body functions. These mechanisms were proposed by Howitz and collaborators and postulated in the xenohormesis theory [15–17]. Indeed, during last decades, phytochemicals have been emerged as useful bioactive compounds in the

prevention and amelioration of several diseases with a high prevalence in our society, such as cardiovascular disease [18,19], insulin resistance [20,21] and other obesity-related pathologies [22,23].

Currently, world's globalization ensures the commercialization of all kind of plant-derived foods, such as fruits and vegetables, allowing people to consume products from other hemispheres throughout the year. Taking into account that each fruit contains a distinctive composition of phytochemicals informing about the environmental status in which they were harvested, the main aim of the present thesis was to determine whether fruit consumption out of season could prompt an illegitimate signaling, leading to physiological and metabolic detrimental effects that could contribute to the development and exacerbation of obesity and/or metabolic disorders, such as dyslipidemia and insulin resistance. Among different fruits, we chose the sweet cherry (*Prunus avium L.*) as it is a seasonal fruit typically consumed worldwide in the LD season spring and that has an interesting composition of phytochemicals.

To achieve this aim, we firstly studied the physiological and metabolic adaptations to the chronic exposure to a short (L6, 6 h of light/day, simulating winter) and a long (L18, 18 h of light/day, simulating summer) photoperiod in comparison with a normal photoperiod (L12, 12 h of light/day) in normoweight F344 rats (**Manuscript 1**), which was crucial to further understand the effects of sweet cherry consumption in the different seasons. Previous studies in this rat strain have evidenced a differential physiological adaptation in response to different photoperiod exposure, in which LD photoperiods induced a stimulatory response in terms of food intake, body weight and reproductive parameters in comparison with SD photoperiods, which induced a regressive phenotype [24–26]. Nevertheless, the metabolic adjustments promoted by day length variations have not been previously described. In our study, after 14 weeks of photoperiod exposure, rats displayed a markedly differential phenotype in terms of

physiology and metabolism, as it was illustrated by the clear clustering of the three analyzed groups obtained in a multivariate analysis carried out with 239 parameters. Relevantly, the lack of effects that we reported in cumulative food intake, body weight and testis size was interpreted as a refractory response to SD photoperiods, which is considered a physiological phenomenon induced by the endogenous circannual rhythms in order to anticipate seasonal changes [27]. However, this trend was not observed in the circulating levels of several parameters involved in glucose and lipid metabolism. Concretely, we reported that the exposure to the L6 photoperiod induced an increase of the circulating glucose levels in comparison with the L12 photoperiod. This higher glycemia was mainly attributed to the sharp downregulation of the phosphorylated levels of the post-receptor target of insulin Akt2 in the soleus and gastrocnemius muscles. The decreased levels of this key protein in the regulation of glucose, lipid and protein metabolism in postprandial conditions [28,29] were accompanied by a downregulation of the mRNA levels of its activator *Irs1* in the soleus muscle [30], the main hepatic glucose transporter *Glut2* [31] and a muscular microRNA that has been reported to be decreased in diabetic rodents and humans, miR-194 [32]. Hence, in accordance with previous results in C57BL/6J mice, in which the exposure to a short photoperiod induced higher glycemia due to a lower muscular glucose uptake via GLUT4 [33], we suggested that the chronic exposure to the L6 photoperiod induced a lower insulin sensitive phenotype in normoweight F344 rats when compared with the rats held under the L12 photoperiod.

In addition, FA metabolism was also affected by the chronic exposure to different photoperiods. This finding was evidenced by the increased circulating NEFAs in L6 animals, which were tentatively attributed to the decreased FA transport, as was strongly suggested by the downregulation of the genes encoding the FA transporter CD36 in the liver and the soleus muscle and the lower mRNA levels of the β-oxidation-related genes *Had* and *Cpt1β* in the soleus muscle. Taking into

consideration that Akt2 can modulate the insulin-mediated translocation of FA transporters and consequently, FA uptake and oxidation [34], we suggested that these effects could also be a consequence of the lower insulin sensitivity reported in L6 rats. Intriguingly, despite the fact that several studies have reported that AMPK, the key energy sensor [35,36], is reduced in insulin resistance states [37], in this study we observed a sharp upregulation of the phosphorylated and active form of this protein in animals exposed to the L6 photoperiod. It has been described that AMPK is directly involved in muscular glucose uptake through the stimulation of GLUT4 translocation [38], which would not be in agreement with the higher glycemia levels reported in L6 animals. Nevertheless, taking into account that AMPK effects on glucose metabolism are produced in energy-demanding conditions such as starving [39], and that our animals were sacrificed in post-prandial conditions (1 h of fasting), we suggested that the increased levels of AMPK did not relevantly contribute to enhance glucose uptake in the skeletal muscle. In addition, this protein has been reported to inhibit protein synthesis and stimulate protein degradation by inhibiting mTORC1 via TSC2 phosphorylation [40] and activating FOXO3, which increases the expression of the E3 ligases MAFbx and MuRF1[41]. Hence, the higher amount of some circulating amino acids (alanine, glutamine, isoleucine, threonine and tryptophan) and the lower protein content in both soleus and gastrocnemius muscles reported in L6 animals, prompted us to hypothesize that the higher pAMPK levels could account for a higher protein degradation in the skeletal muscle. Similarly, other authors have previously described that the exposure to a SD photoperiod induced a higher degradation of muscle proteins in mice [42].

In this study, we also reported a higher glycemia in animals chronically exposed to the L18 photoperiod compared to the L12 group, although the molecular mechanisms involved in these effects were not as evident as those reported in L6 animals, since no changes in Akt2 and AMPK protein levels were observed. Nevertheless, these animals displayed higher levels of DAG and

glycerophosphocholine in the liver. Taking into account that the accumulation of DAG in the liver can induce a pro-inflammatory response that impairs insulin signaling [43,44], we hypothesized that the increased DAG and glycerophosphocholine levels could account for the hyperglycemia reported in L18 rats. Moreover, these animals also displayed significant molecular changes in the regulation of FA metabolism, displaying lower gene expression of FA transporters either in the liver (*Cd36* and *Fatp5*) and the soleus muscle (*Cd36*), as well as decreased *Cpt1β* mRNA levels in the soleus muscle than L12 animals. Nevertheless, these molecular alterations were not translated into variations in blood NEFAs levels, which could be tentatively explained by a lower release of FA by the white adipose tissue or a higher FA uptake by other tissues so as to compensate the decreased uptake of this metabolites in the liver and the skeletal muscle [45,46].

In last decades, it has been elucidated that circannual and circadian rhythms are interconnected by a complex regulatory system in which day length acts as the main environmental modulator [7,47]. In addition, peripheral core-clock genes daily fluctuations have been directly associated with the modulation of several vital metabolic processes in the liver and skeletal muscle, such as lipogenesis *de novo*, insulin signaling, glucose uptake and glycolysis, through the modulation of key genes and proteins, such as FASN, Akt, *Glut4* and *Hk*, respectively [48–50]. Relevantly, after analyzing the peripheral clock genes in the liver and both soleus and gastrocnemius muscles, we reported clear variations in *Bmal1, Per2* and *Nr1d1* mRNA levels in all three tissues in L18 animals, whereas L6 and L12 animals did not exhibit any differences. Hence, we suggested that these variations could have also accounted to the metabolic responses observed in L18 rats.

Altogether, these results highlight the impact of the chronic exposure to both short and long photoperiods on glucose and lipid metabolism, especially in the

L6 photoperiod, which, in our study, induced a marked disruption of insulin sensitivity. Moreover, these innovative findings pave the way for the use of the rat strain F344 in the study of several diseases that can be associated with impairments of glucose and lipid metabolism and that show rising peaks of prevalence in winter, such as obesity, SAD and CVD [22,51–53].

Once we characterized the physiological and metabolic adaptations to the exposure to different photoperiods in normoweight conditions, we wanted to determine whether these effects were also observed in diet-induced obese rats (**Manuscript 2**). Hence, we carried out a study using F344 rats submitted to the same photoperiods described in the first study for 11 weeks. After 4 weeks of adaptation to each photoperiod, in which the animals were fed *ad libitum* with a STD, the rats were switched to a CAF for the last 7 weeks. CAF is characterized for containing several high-palatable and energy dense foods, such as cheese, bacon, muffins and milk with sugar, which are highly present in Western diets and which have been reported to induce obesity, insulin resistance and dyslipidemia [54,55].

As previously observed in our first study, the combination of different day length exposure and the CAF intervention induced a clear differential phenotype in the three analyzed groups, as was illustrated by a clear clustering in the multivariate analysis that we performed with 112 parameters. Interestingly, in contrast to what was observed in other studies [25,26], no differences were reported in biometric parameters among the three groups during the first 4 weeks of the study, in which animals were fed a STD diet. However, the inclusion of an obesogenic diet induced several changes in terms of physiology and metabolism.

CAF intervention induced a loss of body weight and body weight gain in both L6 and L18 groups when compared to L12 animals, which was mainly explained by a lower cumulative caloric intake. When we analyzed the hypothalamic expression of genes related with food intake regulation, we observed a sharp

upregulation of the orexigenic genes *Npy* and the ghrelin receptor *Ghsr* in both L6 and L18 animals. Taking into account that body weight is controlled by the balance between EE and food intake [56], we initially speculated that the decreased caloric intake reported in both L6 and L18 animals could be an adaptive response to deal with the lower EE observed in both groups in an attempt to maintain an optimal body weight. Nevertheless, the fact that the decrease of EE and caloric intake was accompanied by lower body weight suggested an impairment of body weight regulatory mechanisms. Thus, we hypothesized that the upregulation of hypothalamic orexigenic genes could be a compensatory action in order to increase food intake and maintain an optimal body weight and energy balance in both groups. In addition, the exposure to different day lengths induced significant changes in food preferences and macronutrient consumption. In a previous study, Togo *et al*. reported a significant photoperiod-dependent food preference, in which rats exposed to an LD photoperiod displayed a higher preference to a LFD than a HFD, whereas SD rats did not display any preference [57]. In contrast, we observed that L18 animals displayed a higher preference for fat-rich solid food than those exposed to the L6 photoperiod, as evidenced by the increased intake of bacon and muffins in the L18 group.

Furthermore, we reported a clear differential modulation of glucose and lipid metabolism between L6 and L18 rats in response to the intake of the CAF. Thus, the consumption of the CAF promoted a differential substrate utilization in a photoperiod-dependent manner, displaying L18 animals increased lipid oxidation rates and lower carbohydrate oxidation levels than L6 rats. The increased rates of whole-body lipid utilization observed in the animals held under the L18 could be partly explained by the higher lipid intake and by the upregulation of the mRNA levels of the genes encoding the FA transporter FATP1 and the β-oxidation related enzymes CPT1B and HAD in the skeletal muscle, which would enhance the FA supply and utilization in this tissue. Relevantly, in

contrast to what was observed in the first study, we also described a significant higher glycemia in L18 animals than in L6 rats, which could be mainly attributed to the decreased mRNA levels of the insulin signaling-related gene *Irs1* in the gastrocnemius muscle and the downregulation of the pAkt2 levels in the soleus muscle.

Previous studies have described that the intake of high caloric diets (e.g. HFD) can alter the regulation of circadian clocks in peripheral tissues [58]. Nevertheless, despite the fact that we just carried out a single measurement of the gene expression of the mammalian circadian core-clock components, we described a very similar expression pattern than those obtained in normoweight rats. Thus, although we cannot directly compare both studies because they were performed at different times, we suggested that the CAF did not dampen the mammalian circadian rhythm modulators.

Overall, we demonstrated that the consumption of a high-palatable CAF affects several parameters related with body weight, feeding behavior and glucose and lipid metabolism in a photoperiod-dependent manner. Moreover, in obesogenic conditions, we did not observe a more deleterious phenotype regarding glucose and lipid metabolism induced by the L6 photoperiod in comparison with the L18 photoperiod, as we previously described in normoweight F344 rats. Relevantly, these results could contribute to highlight the impact of the consumption of high-palatable and energy dense foods in the adaptations to seasonal day length variations, especially in those diseases that can be prompted by alterations in feeding behavior, such as SAD [53] and obesity [56].

Obesity is considered the global epidemic of the twenty-first century and it is estimated to affect the 58% of world's adult population by 2030 [59]. Although it is widely evidenced that the main etiology of obesity and its related disorders is an imbalance between caloric intake and energy expenditure [60], the preventive and therapeutic strategies in order to avoid their increasing

prevalence are not resulting effective. Thus, there is a pressing necessity to find new causes that can account for the development of obesity and related metabolic diseases.

In this sense, once we have described the physiological and metabolic adaptations in response to the chronic exposure to different day lengths under normoweight and obese conditions, we wanted to study whether fruit consumption out of its harvesting season could alter this metabolic adaptations contributing to the development of obesity and/or metabolic disorders, such as dyslipidemia or insulin resistance in animals fed a STD and to enhance the detrimental effects caused by the obesogenic CAF, which would be in line with our hypothesis that fruit intake out of season can lead to a disruption in characteristic seasonal physiology and metabolism (**Manuscript 3**).

For this purpose, we carried out a proof-of-concept study divided in two different experiments regarding the dietary intervention that was given to the animals. As was aforementioned, we selected sweet cherry due to its popularity as a spring/summer fresh fruit and its rich-phytochemical content [61,62]. This fruit is characterized by containing high levels of anthocyanins, which are synthesized due to its protective role in the plant and which have been reported to exert several beneficial effects on health, by decreasing the risk of CVD or insulin resistance in animals and humans [63–66].

In both studies animals were exposed to the most representative seasonal day length schedules (L6, simulating winter and L18, simulating summer), and were divided into two groups depending on the treatment received: a vehicle (20 mg of glucose:fructose 1:1 mixture per kg of body weight), or lyophilized sweet cherry (100 mg per kg of body weight, which represents a daily intake of 4.26 g of fresh cherry without pits for a 60-kg human) [67].

In STD-fed F344 rats, after 10 weeks of treatment, cherry consumption induced similar effects in lipid metabolism-related parameters in both L6 and L18

animals, such as decreased circulating cholesterol levels and an upregulation of genes involved in FA uptake (*Cd36*) and β-oxidation (*Cpt1β*) in the skeletal muscle. However, cherry consumption induced more changes when it was consumed out of season, exhibiting L6 animals that received this fruit, but not L18-treated rats, an enhancement of FA transport and β-oxidation-related pathways, which was evidenced by increased *Had* gene expression in the soleus muscle and by elevated levels of the FA oxidation enhancer pAMPK in both soleus and gastrocnemius muscles, when compared to the non-treated L6 animals. Thus, we suggested that cherry consumption out of season enhanced lipid utilization as an energy substrate through activating AMPK. Moreover, concerning glucose metabolism, we reported a global lower Akt2 activation in the skeletal muscle only when cherry was consumed out of season. Taking into account the key role of AMPK and Akt2 in glucose and lipid metabolism regulation, these results reinforce our hypothesis of a marked interaction between fruit intake out of season and seasonal characteristic metabolism. Relevantly, all these molecular alterations were not associated with changes in the blood levels of glucose or insulin. Considering the healthy status of STD-fed rats, we suggested that the lack of alterations in these parameters could be explained by the robust phenotypic flexibility associated with optimal health, which is capable of neutralizing external factors in order to maintain metabolic homeostasis [70].

Previous studies performed in the Nutrigenomics Research Group described a clear modulation of circadian clocks by the intake of polyphenols [71,72]. In addition, cherry is rich in anthocyanins and also contains high concentrations of melatonin [73], a hormone that is directly associated with the modulation of circadian and seasonal rhythms [74]. In the present study, only cherry consumption out of season induced significant changes in the mRNA levels of mammalian circadian clock components, such as *Nr1d1* in the gastrocnemius muscle and *Per2* and *Cry1* in the liver of normoweight animals, which would

emphasize the hypothesized interaction between seasonal fruit intake and body rhythms.

In CAF-fed rats, after 7 weeks of dietary intervention, cherry consumption out of season induced an increase of whole-body lipid utilization, which was mainly attributed to the upregulated pAMPK levels in the soleus muscle in comparison with L6 non-treated animals. Interestingly, we reported that when L6 animals were treated with cherry, they resembled the phenotype induced by the chronic exposure to the L18 photoperiod, which was characterized by an enhanced lipid utilization. This effect could be partly explained by the overexpression of genes involved in FA uptake (*Fatp1*) and β-oxidation (*Cpt1β* and *Had*) in the skeletal muscles. Moreover, cherry consumption out of season exacerbated the deleterious effects induced by the CAF, triggering in L6 animals that received this fruit, but not in L18-treated rats, hyperglycemia and hyperinsulinemia. Differently to what was described in normoweight animals, diet-induced obese rats exhibited slight molecular variations regarding insulin-mediated glucose uptake, such as decreased mRNA levels of the glucose transporter *Glut4* in the gastrocnemius muscle, indicating a lower capability to buffer external challenges through the activation of this key molecular pathway, which led to an impairment of glucose homeostasis. Moreover, in this experiment, the CAF intervention induced the loss of cherry modulation of the circadian clock machinery in peripheral tissues, not observing any difference among groups.

Altogether, although the consumption of sweet cherry out of season did not contribute to a higher fat mass accretion, and consequently, to the appearance and exacerbation of an obese phenotype in STD- and CAF-fed F344 rats, respectively, we demonstrated that the consumption of sweet cherry promotes strong photoperiod-dependent responses, causing more pronounced and, to some extent, more deleterious effects concerning glucose metabolism and insulin signaling, when it is consumed out of season in F344 rats. Hence, although

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more studies are necessary to determine the pathophysiological relevance of these results, this study could contribute to evidence the importance of seasonal and proximal fruit consumption in order to preserve an optimal health and/or to design nutritional strategies addressed to ameliorate metabolic disorders.

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V. CONCLUSIONS

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The main conclusions of the present thesis are:

1. The chronic exposure to the L6 photoperiod, which simulates winter-like day length, promotes a lower insulin sensitive phenotype in normoweight F344 rats, which was evidenced by hyperglycemia, decreased phosphorylated levels of the downstream post-receptor target of insulin Akt2 in both the soleus and gastrocnemius muscles and a downregulation of genes involved in insulin signaling and glucose metabolism (*Irs1*, soleus, and *Glut2*, liver).

2. L6 photoperiod exposure in normoweight F344 rats increases the circulating NEFAs levels, an effect that could be partly explained by the downregulation of key genes associated with muscular and hepatic fatty acid uptake (*Cd36*, soleus and liver) and β-oxidation (*Had* and *Cpt1β*, soleus).

3. In normoweight rats, the chronic exposure to the L18 photoperiod, which simulates summer-like day length, induces hyperglycemia and decreases the expression of genes involved in hepatic and muscular lipid utilization, which could be partly attributed to the hepatic accumulation of diacylglycerol and to the expression changes of key circadian clock genes.

4. The intake of the high-palatable CAF decreases cumulative caloric intake in both L6 and L18 animals and induces significant changes in feeding behavior in a photoperiod-dependent manner, inducing a higher preference for fat-rich food in the L18 photoperiod than in the L6 photoperiod.

5. The combination of the CAF intervention and the chronic exposure to different day lengths promotes a differential photoperiod-dependent substrate utilization, evidenced by the higher whole-body fat utilization and lower carbohydrate oxidation levels in L18 animals compared with L6 rats.

6. The consumption of sweet cherry, which is a seasonal fruit harvested in spring and summer, out of season induces an enhancement of fatty acid transport and β-oxidation-related genes in normoweight rats and triggers whole-body fat utilization as an energy substrate in CAF-fed F344 rats.

7. In normoweight conditions, the intake of cherry out of season decreases insulin-mediated activation of Akt2, which is not apparently translated into negative effects on health.

8. Cherry consumption out of season enhances the detrimental effects caused by the intake of the CAF, inducing hyperglycemia and hyperinsulinemia and decreasing muscular *Glut4* gene expression in F344 rats.

LIST OF PUBLICATIONS

Manuscripts included in the thesis

Mariné-Casadó R, Domenech-Coca C, del Bas JM, Bladé C, Caimari A, Arola L (2018). Cherry consumption out of season alters lipid and glucose homeostasis in normoweight and cafeteria-fed obese Fischer 344 rats. In press, The Journal of Nutritional Biochemistry.

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PHYSIOLOGICAL AND METABOLIC ADAPTATIONS TO DIFFERENT PHOTOPERIODS: EFFECTS OF OBESITY AND
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Through evolutionary history, the coexistence of animals and plants have led to the development of an adaptive phenomenon that has been recently explained by the xenohormesis theory. This theory postulates that the phytochemicals synthesized by stressed plants could be recognized as signals by the heterotrophs that consume them, being informed about the external conditions in which plants were harvested and allowing them to favorably adapt to unpredictable changes in the environment. Thus, each plant contains a distinctive phytochemical composition informing about the environmental status. In this framework, the main aim of the present thesis was to evaluate whether fruit consumption out of season would induce an erroneous signaling, leading to detrimental effects on physiology and metabolism of normoweight and cafeteria-fed obese Fischer 344 rats, by analyzing glucose and lipid metabolism-related parameters in blood, liver and skeletal muscle. To achieve this objective, we firstly characterized the physiological and metabolic adaptations to the chronic exposure to different photoperiods, which resembled seasonal variations in day length, in normoweight and obesogenic conditions. Once characterized, we evaluated the effects of the consumption of sweet cherry, a popular anthocyanin-rich fruit harvested in spring/summer, in short and long photoperiods resembling winter and summer, respectively. Firstly, we reported that the chronic exposure to different photoperiods induces several variations in physiological and metabolic parameters in normoweight and diet-induced obese rats, mainly affecting glucose and lipid metabolism and insulin signaling. Secondly, we revealed that cherry intake induces marked photoperiod-dependent effects, promoting more pronounced and, to some extent, more negative effects concerning glucose metabolism and insulin signaling in normoweight and diet-induced obese F344 rats when it was consumed out of season. These findings could contribute to highlighting the importance of the consumption of seasonal fruits to maintain an optimal health.

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