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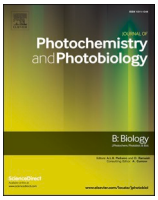

Fatty acid metabolism in liver and muscle is strongly modulated by photoperiod in Fischer 344 rats

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ABSTRACT

Circadian and seasonal variations produce variations in physiological processes throughout the day and the year, respectively. In this sense, both the light and the moment of feeding are strong modulators of the central and peripheral clocks. However, little is known about its influence on certain metabolic parameters and on the composition of liver and muscle fatty acids (FA). In the present study, 24 Fischer 344 rats were exposed for 11 weeks to different photoperiods, L6, L12 and L18, with 6, 12 and 18 h of light/day, respectively. They were fed a standard diet. Serum metabolic parameters, gene expression of liver enzymes and gastrocnemius muscle involved in the synthesis, elongation, desaturation and β-oxidation of FA were analyzed. We have found that exposure to different hours of light has a clear effect on FA composition and gene expression in the liver. Mainly, the biosynthesis of unsaturated FA was altered in the L18 animals with respect to those exposed to L12, while the L6 did not show significant changes. At the muscle level, differences were observed in the concentration of mono and polyunsaturated FA. A multivariate analysis confirmed the differences between L12 and L18 in a significant way. We conclude that exposure to long days produces changes in the composition of liver and muscle FA, as well as changes in the gene expression of oxidative enzymes compared to exposure to L12, which could be a consequence of different seasonal eating patterns.

1. Introduction

The rotational and translational movement of the Earth results in the variation of luminosity and temperature in either 24 h or seasonal variations during the year, called the circadian and circannual cycle, respectively. Chronobiology studies the adaptations and physiological changes of living beings in response to these variations [1]. There are different synchronizing agents that modulate the period of the circadian rhythm, such as lighting cycles (photoperiods), feeding, social stimulation [2,3] and temperature [4]. In addition, the organisms have a core of endogenous circadian rhythms that control genetic and molecular variations through its molecular components [5,6]. In mammals these rhythms are mainly regulated by the suprachiasmatic nucleus (SCN), which exerts a complex regulation by means of a system of transcription factors, allowing the generation and maintenance of oscillations in physiological processes such as the sleep cycle, hormone secretion, body temperature and metabolism. These rhythms mainly influence the homeostatic balance of feeding, the metabolism and obtaining of energy expenditure of nutrients, and the rest/activity behavior, among others [7]. In this sense, there is a communication between the SCN and autonomic clocks located in peripheral tissues that act directly or indirectly with the cycles of fasting and feeding [8]. Such feedback between SCN and peripheral clocks is carried out through behavioral, humoral and/or neuronal factors, while peripheral oscillators receive entrainment signals through serum metabolites [6]. These processes are part of an evolutionary process that has been preserved to provide adaptation to external environmental conditions [9].

It has been observed that the circadian rhythm of peripheral metabolic organs is affected by the feeding/fasting rhythm, meaning that tissues such as the liver respond rapidly to food availability, while the SCN is more sensitive to light/dark cycles [10]. In this regards, either the circadian clock, systemic signals from periodic food intake, or a combination of both mechanisms directly affect the expression of genes that control the peripheral clocks [5,11,12]. Specifically, in rodents it has

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been shown that during the illumination phase, that is, their resting period, lipogenesis is inhibited by an increase in the expression levels of REV-ERBα and REV-ERBβ. On the contrary, during darkness, their active period, the expression levels of these factors decrease, promoting the activation of lipogenic genes [13]. On the other hand, Brenner et al. (1981) stated that the activity of some hepatic desaturase enzymes also has a circadian activity, regulated mainly by adrenocorticotropic hormone and glucocorticoids [14]. In addition, the seasons of the year also influence these circadian rhythms, producing metabolic variations. For example, it has been observed that the rate of fatty acid (FA) desaturation is the same both in winter and in summer; however, the activity peak of the involved enzymes differs between one photoperiod and another. In this sense, it has been observed that the liver glycogen levels are strongly influenced by the time of year [15]. Mariné-Casadó et al. (2018), observed various metabolic consequences in rats exposed to different photoperiods (L6, L12 and L18, with 6, 12 and 18 h of light, respectively), simulating the seasons [16]. Specifically, they observed that those animals exposed to L6 presented altered glucose metabolism and lipid oxidation, due to a higher concentration of glucose and non-esterified fatty acids (NEFAs) than those exposed to L12. In addition, changes were also observed at the level of gene expression of enzymes related to the glucose metabolic pathway and in oxidative enzymes in liver and skeletal muscle, respectively [16]. Other authors also observed that exposure to L6 produces differential effects on biometric and serum parameters and on the gene expression of key enzymes in lipolysis, β-oxidation and adipogenesis in white and brown adipose tissues compared with L18 [17].Therefore, there is clear evidence of the impact of biological rhythms on circulating metabolites, in serum, as well as in peripheral tissues such as liver, skeletal muscle or adipose tissues. Likewise, the metabolic consequences of the same food may also be different depending on the photoperiod that is consumed. In this sense, Gilbert et al. (2020) observed that the consumption of oranges from the southern hemisphere at L6 not only increased the percentage of fat and adipocytes in white adipose tissue, but also decreased the mRNA concentration of genes related to uptake and β-oxidation in brown adipose tissue compared to VH. However, these changes were not observed when oranges were ingested at L18 [18].

Although there is evidence on the circadian and circannual rhythmicity of certain metabolic enzymes, hormones and nutrient availability both in serum and in various tissues, there is little information on how the chronic exposure to days with different hours of light and darkness affect metabolic parameters. Therefore, the purpose of this article is to analyze the effects of exposure to different photoperiods on serum markers, gene expression, and on the FA profile of the liver and skeletal muscle of Fischer 344 rats.

2. Materials and Methods

2.1. Animals

Twenty-four 8-week-old male Fischer 344 (F344) rats were randomly divided in one of three photoperiods, which simulated the season of the year: short photoperiod ($n = 8$, L6), standard (n = 8, L12) and long (n = 8, L18), with 6, 12 or 18 h of light (light density 700 lx). The animals were housed in cages and in pairs at 22 °C. They received standard diet (AO4, Panlab, Barcelona, Spain) and water ad libitum. As seen in Fig. 1. the light was turned on at 8:00 am for all groups. The amount of food intake was recorded fortnightly. After eleven weeks, the animals were sacrificed by decapitation. The experiment lasted a total of eleven weeks because in investigations carried out by the group it was established that this number of weeks were sufficient for the animals to adapt satisfactorily, for the metabolic changes to be established and for an refractory effect not to occur in them [19–21]. Blood was collected in non-heparinized tubes, incubated for 1 h at room temperature, and centrifuged (2000 ×*g*, 15 min, 4 °C) to obtain the serum. The liver and gastrocnemius muscles were rapidly weighed, frozen at liquid nitrogen and stored at −80 °C for further analysis. The Animals Ethics Committee of the Universitat Rovira I Virgili (Tarragona, Spain) approved all the procedures (Project identification code: 9495; file number: FUE-2017-00499873).

2.2. Calculation of Eating Pattern Index (EPI)

The kilocalories/day consumed by the animals were previously

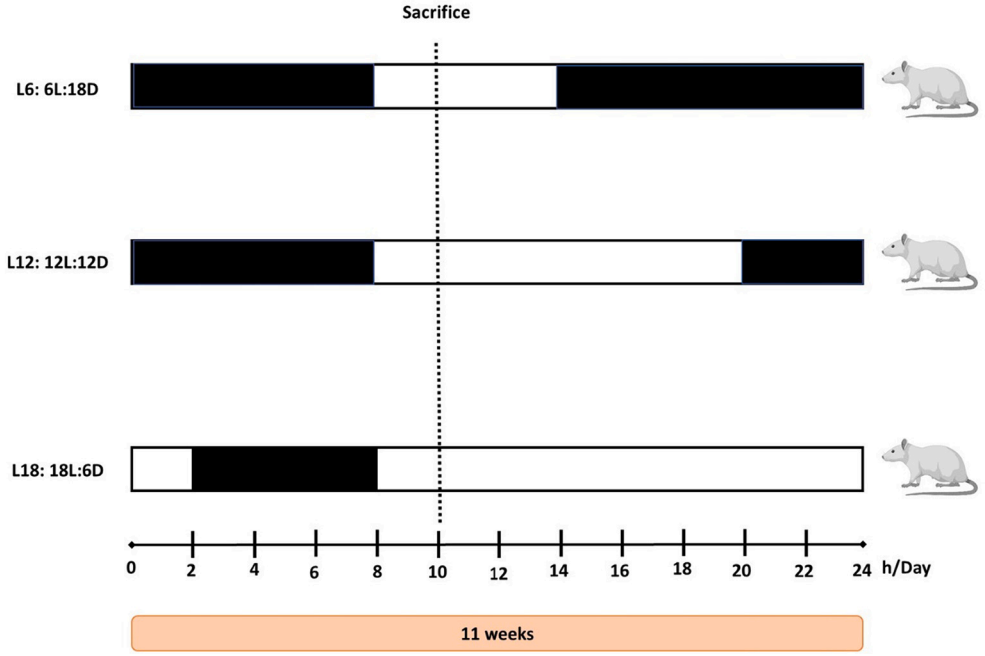


Fig. 1. Experimental model: twenty-four Fischer 344 rats were exposed to 6, 12 or 18 h of light (L6, L12 and L18, respectively) and fed ad libitum. After 11 weeks they were sacrificed 2 h after the light was turned on. The black color of the bars in each group indicates the time of darkness; the white color indicates the hours of light that the animals had in one day. [20].

provided by Cruz-Carrion et al. [20] Fortnightly records of the amount (gr) of food given and the remainder after 24 h were used to calculate them. Based on nutritional information provided by the commercial house, the caloric intake was determined. In this sense, for L6, L12 and L18 the kcal/day consumption was 54.1; 55.4 and 54.9. The food pattern index (EPI) has been calculated based on these data, as well as taking into consideration that the animals eat mainly during the hours of darkness:

EPI (kcal/h): (Kcal/day)/hours of darkness/day.

Being 18, 12 and 6 the hours of darkness for L6, L12 and L18 respectively.

2.3. Serum Analysis

Circulating levels of glucose, triacylglycerides (TAG), total cholesterol (TC), HDL cholesterol (HDL-c), LDL cholesterol (LDL-c) (QCA, Amposta, Spain) and NEFAs (WAKO, Neuss, Germany) were determined by enzymatic colorimetry. Serum insulin levels were determined using a rat insulin ELISA kit (Millipore, Barcelona, Spain). The homeostatic model assessment (HOMA) index was calculated, using the equation (Glycemia (mmol/L) * Insulinemia (mU/dL)/22.5).

2.4. Lipid Extraction and GC Analyses

The extraction of hepatic and muscle lipids was done following Blight and Dyer's protocol [22]. Briefly, either 400 mg or 500 mg of muscle or liver, respectively, were weighted. The samples were homogenized together with chloroform, methanol and water. After the successive steps of addition of chloroform and water, with vortex and centrifugation, the organic phase was separated and dried under stream of N_2. For the determination of lipid profile, the derivation of FA was done by methylation with methanol potassium hydroxide. The FA methyl esters (FAMEs) determination was carried out by gas chromatography (Shimadzu (GC-2014) equipped with FID detector and using the CP-Sil 88 (100 m × 0.25 mm × 0.2 μm, film thickness, Varian, Lake Forest, CA, USA). Four samples for group were utilized ($n = 4$). The FAMEs were identified by comparing their retention relative times of commercial standards, and the chromatogram analysis was done with the LabSolution Shimadzu Software. Values were expressed as a percentage of the total FAMEs.

Once determined the levels of the different FAMEs, the flow of the key enzymes involved in the biosynthesis of unsaturated FA was determined following the ratios described by Sain J [23].

2.4.1. Gene Expression

TRIzol Reagent (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) was used for the extraction of liver and muscle RNA, following the guidelines indicated by the supplier. The cDNA was synthesized by reverse transcription using High-Capacity cDNA Reverse Transcription (Thermo Fisher Scientific, Illkirch-Graffenstaden, France). The specific amplification of the cDNA was carried out by to the polymerase chain action in real time (RT-qPCR), using iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain). It was utilized eight samples for group ($n = 8$). Gene expression analysis was performed using primers obtained from Biomers.net (Ulm, Germany), which are detailed in Table 1**. Supplementary Material.** The genes of interest were those related to fatty acids metabolism in the liver and muscle: *Acc1* and *Acc2*, Acetyl-CoA carboxylase 1 and 2, catalyze the cytoplasmic formation of malonyl-CoA in liver and muscle, respectively; *AdipoR2*, Adiponectin receptor 2; *Cpt1α and Cpt1β*, carnitine palmitoyltransferase 1 alpha and beta, they catalyze the entry of FA into the mitochondria and is the main regulator of its oxidation in liver and muscle, respectively; *CS*, Citrate Synthase, catalyzes the condensation between oxaloacetate and acetyl-CoA to produce Citrate and CoA; *Elovl2*, Elongation of very-long-chain fatty acid enzyme 2, elongates docosapentaenoic acid (22:5n-3) to 24:5n-3, the precursor of docosahaexanoic acid (DHA, 22:6 n-3)*; Fads1*,

Table 1
Profile of hepatic fatty acids determined by GC/FID. Proportion of fatty acids in liver expressed as % FAME of animals exposed to different photoperiods (short, L6; standard, 12; long, L18, with 6, 12 and 18 h of light, respectively). Σ NI: sum of unidentified fatty acids; **ΣSFA**: saturated fatty acids **ΣMUFAs**: monounsaturated fatty acids; **ΣPUFAs**: polyunsaturated fatty acids; **AA**: arachidonic acid: cis-4,cis-8,cis-11,cis-14 20:4 (n-6); **ALA:** α-Linolenic acid: cis-9,cis-12,cis-15 18:3 (n-3); **DGLA**: Dihomo-γ-linolenic acid: cis-8,cis-11,cis-14 20:3 (n-6); **DHA**: Docosahexaenoic acid: cis-4,cis-7,cis-10,cis-12,cis16,cis-19 22:6 (n-3); **SA**: Stearic acid: 18:0; **EPA:** Eicosapentaenoic acid: cis-5,cis-8, cis-11,cis-14,cis-17 20:5 (n-3); **GLA:** gamma-linolenic acid: cis-6,cis-9,cis-12 18:3 (n-6); **LA**: Linoleic acid: cis-9,cis-12 18:2 (n-6); **OA**: Oleic Acid: cis-9 18:1; **PA**; Palmitic acid: 16:0. Values expressed as mean ± SEM ($n = 4$). P, photoperiod effect. (One-way ANOVA, $p < 0.05$); different letters indicate significant statistical differences p < 0.05; #indicates trend $0.05 < p < 0.1$ (Post-hoc DMS, one way ANOVA).

Fatty acid	L12	L6	L18	ANOVA
Σ SFA	41.86 ± 0.97 *a*	43.46 ± 0.28 *a#*	46.27 ± 0.34 *b*	P
12:0	ND	ND	ND	
14:0	0.89 ± 0.11	0.86 ± 0.10	0.93 ± 0.03	
15:0	0.17 ± 0.01	0.15 ± 0.02	0.16 ± 0.01	
PA	28.98 ± 1.22#	29.40 ± 0.85	31.42 ± 0.53#	P
17:0	0.23 ± 0.02	0.20 ± 0.04	0.22 ± 0.01	
SA	11.57 ± 0.61 *a*	12.85 ± 0.64 *ab*	13.52 ± 0.23 *b*	P
20:0	ND	ND	ND	
22:0	ND	ND	ND	
Σ MUFAs	22.82 ± 1.41#	19.65 ± 2.02	19.51 ± 0.46#	
cis-9 14:1	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.00	
Palmitoleic acid	5.34 ± 0.69	5.92 ± 0.70	5.46 ± 0.35	
cis-9 17:1	0.24 ± 0.03	0.19 ± 0.03	0.24 ± 0.01	
cis-6 18:1	0.09 ± 0.01	0.09 ± 0.01	0.15 ± 0.02	
OA	11.71 ± 1.03 *a*	9.46 ± 0.89 *a#b*	8.75 ± 0.27 *b*	P#
cis-11 18:1	5.09 ± 0.11 *a*	4.89 ± 0.05 *ab*	4.67 ± 0.16 *b*	
cis-12 18:1	0.17 ± 0.01	0.17 ± 0.03	0.16 ± 0.02	
cis-11 20:1	0.10 ± 0.01#	0.09 ± 0.01	0.08 ± 0.01#	
Σ PUFAS-cis	32.18 ± 0.46	33.84 ± 1.75	32.45 ± 0.86	
Σ PUFAS n-6	26.76 ± 0.47	27.53 ± 1.49	25.99 ± 0.76	
LA	12.32 ± 0.78 *a*	10.68 ± 0.38 *a#b*	9.61 ± 0.43 *b*	P
GLA	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	
cis-11, cis-14 20:2	0.19 ± 0.01 *a*	0.17 ± 0.01 *ab*	0.16 ± 0.01 *b*	P#
DGLA	0.63 ± 0.03	0.73 ± 0.06	0.72 ± 0.00	
AA	12.88 ± 0.54#*	15.39 ± 1.34#	15.22 ± 0.23	
cis-13, cis-16 22:2	0.07 ± 0.03	0.10 ± 0.03	0.05 ± 0.04	
cis-7,cis-10,cis-13,cis-16 22:4	0.40 ± 0.00	0.37 ± 0.04	0.41 ± 0.00	
Σ PUFAS n-3	5.42 ± 0.17 *a*	6.31 ± 0.28 *b*	6.40 ± 0.16 *b*	P
ALA (n-3)	0.25 ± 0.05 *a*	0.14 ± 0.02 *b*	0.14 ± 0.02 *b*	P#
EPA	0.28 ± 0.02 *a*	0.37 ± 0.02 *b*	0.36 ± 0.01 *b*	P
cis-13,cis-16,cis-19 22:3 (n-3)	0.08 ± 0.00	0.09 ± 0.00	0.12 ± 0.01	

(*continued on next page*)

Table 1 (*continued*)

Fatty acid	L12	L6	L18	ANOVA
cis-7,cis-10,cis-13,cis-16,cis-19 22:5	0.92 ± 0.07	0.91 ± 0.06	0.80 ± 0.07	
DHA	3.92 ± 0.10 *a*	4.75 ± 0.23 *b*	5.00 ± 0.20 *b*	P
Σ NI	0.73 ± 0.08	1.07 ± 0.15	0.99 ± 0.04	
Ratio n-6/n-3	4.95 ± 0.19 *a*	4.36 ± 0.08 *b*	4.06 ± 0.10 *b*	*P*
Palmitoleic Acid/PA	0.19 ± 0.00	0.20 ± 0.02	0.17 ± 0.00	
LA/ALA	40.76 ± 13.07 *a*	80.30 ± 9.34 *b*	71.17 ± 8.86 *ab*	*P#*
OA/EA	1.03 ± 0.13 *a*	0.75 ± 0.11 *a#b*	0.65 ± 0.03 b	P#
GLA/LA	0.07 ± 0.01#	0.09 ± 0.02	0.11 ± 0.01#	
AA/LA	1.12 ± 0.12 *a*	1.45 ± 0.13 *ab*	1.59 ± 0.06 *b*	*P#*
AA/DGLA	21.08 ± 0.26	21.37 ± 2.37	21.61 ± 0.38	
EPA/ ALA	1.38 ± 0.31 *a*	2.88 ± 0.62 *b*	2.86 ± 0.58 *b*	*P#*
DHA/ ALA	18.85 ± 3.36 *a*	36.52 ± 6.16 *b*	39.18 ± 7.15 *b*	*P*

Δ-5 desaturase, involved in the synthesis of essential FAs, catalyzes the desaturation of 20:4 n-3 to produce Eicosapentaenoic acid (EPA 20:5 n-3); *Fads2*, Δ-6 desaturase, limiting enzyme of the metabolic pathway of malnutrition and elongation of long-chain fatty acids; *Fas1*, Fatty acid synthase, catalyzes the synthesis of fatty acids; *FAT/CD36*, fatty acid translocase, homolog of CD36, membrane receptor that facilitates the absorption of most of the long-chain FA in metabolically active tissues; *Fatp5*, fatty acid transport protein 5, involved in cellular transport of FA in the liver; Had, hydroxyacyl-CoA dehydrogenase, is the third enzyme of the β-oxidation cycle; *Scd1*, Desaturase Δ-9, catalyzes the double bond in Stearoyl-CoA.; *Srebp1-c*, sterol regulatory element-binding protein 1c, a transcription factor that regulates metabolic processes. The relative expression of each mRNA level was calculated as a percentage of the L12 group using the Pfaffl method [24].

2.5. NMR Analysis and Data Processing

The dried hydrophilic and lipophilic extracts were reconstituted in 600 μL of deuterium oxide (D2O) phosphate buffer (0.05 mM PBS, pH 7.4, 99.5% D2O) with 0.73 mM trisilylpropionic acid (TSP) and in a deuterated chloroform (CDCl 3) / deuterated methanol (CD3OD) (2:1) solution with 1.18 mM tetramethylsilane (TMS), respectively for NMR measurement. Extracts were transferred to 5 mm glass NMR tubes for analysis. The detailed technical characteristics of the equipment can be consulted in the procedure carried out by Palacio et al. [25].

The acquired ^{1}H NMR spectra were compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, AN, Canada) and databases for metabolite identification. In addition, we assigned metabolites by 1H—1H homonuclear correlation (COSY and TOCSY) and 1H—13C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific 1H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package.

2.6. Statistics Analysis

The results were expressed as mean ± SEM. It was used SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA) for outliers' detection and statistics analyses. Normality and homogeneity were evaluated by using Shapiro–Wilk test and the Levene's test, respectively. For the values that met these criteria, a one-way ANOVA was utilized to determine effect of photoperiod. DMS post-hoc test was used to determine differences between the different groups. The analysis by the Student's *t*-test was used to compare different groups too. For the data that did not meet the normality criteria, the statistic for non-parametric Kruskall Wallis tests was performed.

2.7. Multivariate Analysis

After processing the original data, a multivariate statistical evaluation based on Sparse Partial Least Squares Discriminant Analysis (sPLSDA) and hierarchical clustering analyses were performed. Additionally, fatty acid differentials between photoperiods were used to determine which metabolic pathway is affected by chronic exposure to different hours of light in muscle and liver. The *p* value and the false discovery rate (FDR) were used to verify the affected pathways. The software MetaboAnalyst (version 5.0), available online, was used for doing theses analysis.

To determine how exposure to different hours of light affects hydro and lipophilic metabolites in serum and liver, sPLSDA and hierarchical clustering analyses were performed. All analyzes were performed after range scaling with the use of the software MetaboAnalyst (version 5.0), available online.

3. Results

3.1. Exposure to Different Photoperiods would Produce Differential Eating Patterns between Groups

Considering the kilocalories (kcal) consumed by the experimental groups, previously found in the group [20], and given that the hour of darkness is the time of ingestion of the rats, we propose a EPI. In this sense, for L6, L12 and L18 the calculated EPI in the week 11, was 2.92 kcal/h; 4.91 kcal/h; 9.2 kcal/h, respectively, where the L18 animals significantly consumed 3.17 times more kilocalories per hour than those L6 ($p = 0.00$) **(**Fig. 2**),**

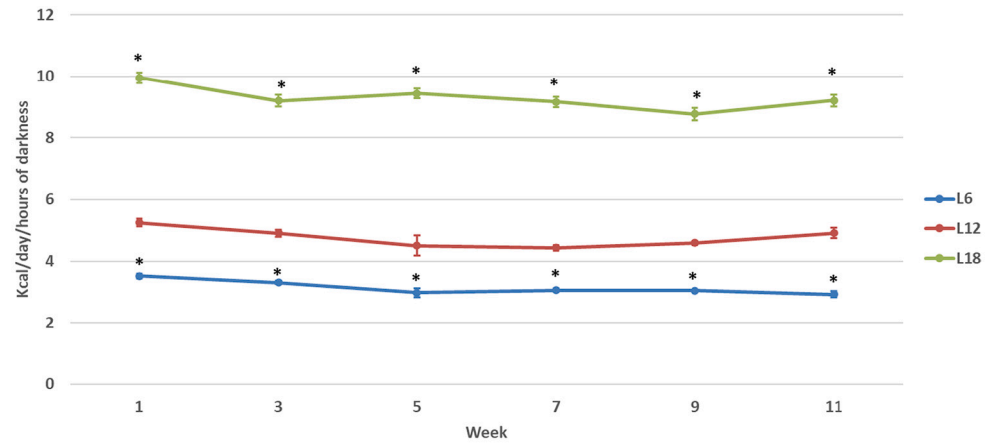
3.2. The Photoperiod Modulated Serum Parameters Related to Glucose Metabolism

ANOVA of the results showed that photoperiod significantly affected serum glucose levels ($p = 0.042$). Specifically, the animals exposed to L6 showed lower insulinemia (Fig. 3**-b)**, HOMA index (Fig. 3**-c)**, and a tendency to lower blood glucose (Fig. 3**-a)**, than those exposed to a standard photoperiod ($p = 0.048$; $p = 0.033$; $p = 0.055$; respectively). Interestingly, the animals exposed to L18 also presented lower glycemia, ($p = 0.017$) than the L12 group (Fig. 3**-d**).

On the other hand, no statistically significant differences were observed in the levels of TC, HDL-c, LDL-C and NEFAs between the three different groups evaluated. (Fig. 1 **Supplementary material).**

3.3. Exposure to L18 Produced a Higher Proportion of Hepatic Saturated Fatty Acids, but did not Affect its Muscle Level, Compared with Exposure to L12

The analysis of the FA profile in peripheral tissues, namely liver and muscle, showed an impact of the seasonality in some species of FA. Table 1 and Table 2 show the levels of FA in liver and muscle of the animals exposed to different photoperiods expressed as percentage of FAMEs. In this regard, an effect of the photoperiod on the content of total hepatic saturated fatty acids (SFA) was observed ($p = 0.02$) (Table 1). Specifically, the animals exposed to more hours of light showed a significantly higher proportion than the rest of the animals exposed to other photoperiods ($p = 0.005$; $p = 0.001$; L18 vs L6; L18 vs L12, respectively). In addition, the photoperiod tended to affect the level of stearic acid (SA; 18.0) ($p = 0.075$), since the L18 group presented a



p value	1	3	5	7	9	11
L6 vs L12	0.07	0.07	0.069	0.07	0.69	0.07
L18 vs L12	0.07	0.07	0.069	0.07	0.69	0.07
L6 vs L18	0.00	0.00	0.00	0.00	0.00	0.00

Fig. 2. Kilocalories consumed per hours of darkness in the different photoperiods. The data shown represents the feeding pattern index calculated for L6, L12 and L18, with 6, 12 and 18 h of light, respectively. * indicates a *p*-value <0.05 (Kruskall-Wallis).

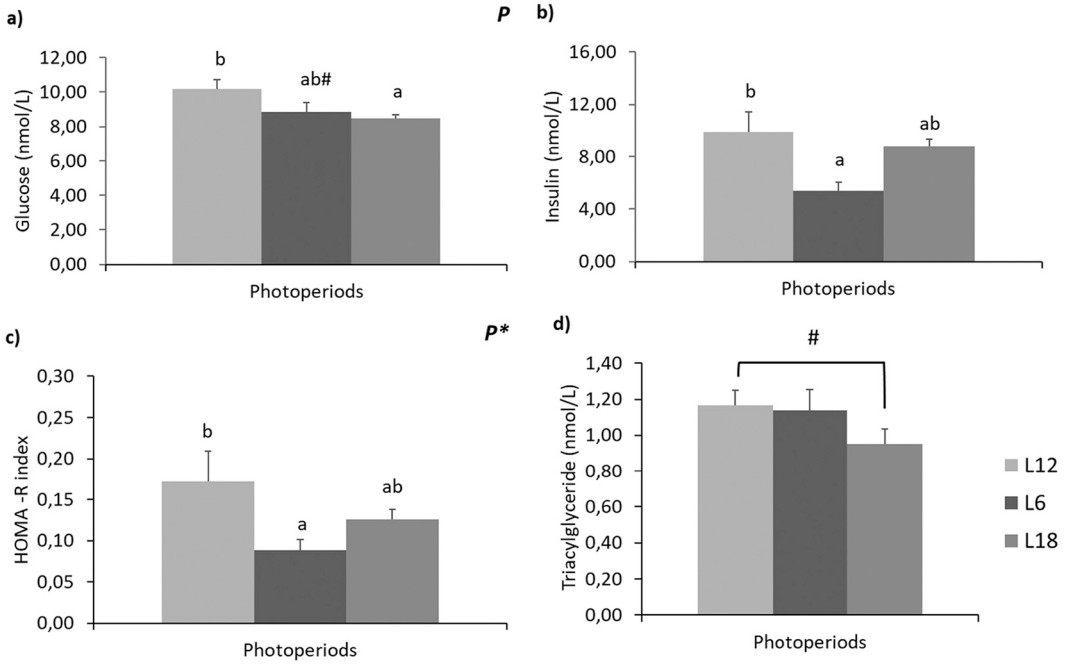


Fig. 3. Serum glucose (**a**), insulin (**b**), HOMA-R index (**c**) and triacylglycerides (**d**) in Fischer 344 rats exposed to a short, standard or long photoperiod, with 6 (L6), 12 (L12) or 18 (L18) hours of light, respectively for 11 weeks. Data are expressed as the mean ± SEM ($n = 8$). One-way ANOVA analysis was used to assess the differences between groups. P, photoperiod. Different letters above the bars indicate significant differences ($p < 0.05$) (post-hoc DMS, one-way ANOVA). # Indicates trend ($0.05 < p < 0.1$). HOMA-R, homeostatic model assessment.

significantly higher level than those L6 ($p = 0.025$). Similarly, the level of palmitic acid (PA; 16.0) was higher in the L18 animals compared to those exposed to L12 ($p = 0.018$, Student's *t*-test). No statistically significant differences were observed in the proportion of FA 14.0, 15.0 and 17.0, while FA 12.0 and 22.0 were not detected (Table 1).

In contrast, no effect of the photoperiod on the concentration of total SFA in muscle was observed. However, the animals exposed to L18 presented a lower level of SFA than the control group. ($p = 0.029$, student's t-test). Furthermore, exposure to different hours of light significantly affected the level of FA 12:0, PA and SA in the gastrocnemius muscle ($p = 0.014$; $p = 0.041$; $p = 0.03$; respectively). Specifically, the L18 and L6 groups showed a lower level of FA 12:0 and PA, while less SA and 22.0 than those L12 (Table 2).

3.4. Photoperiod Affected the Level of Certain Liver and Muscle MUFAs

Although the concentration of total hepatic MUFAs was not significantly affected by exposure to different hours of light, a trend towards a lower content of MUFAs was observed in L18 animals compared to control group (p = 0.08). This trend was evidenced in the level of hepatic oleic acid (OA, cis-9 18:1), since the animals exposed to L18 presented 2.95% less of this FA compared to those exposed to L12 ($p =$

Table 2
Profile of fatty acids in gastrocnemius muscle tissue determined by GC/FID. Proportion of fatty acids expressed as % FAME of animals exposed to different photoperiods (short, L6; standard, 12; long, L18, with 6, 12 and 18 h of light, respectively). **Σ NI**: sum of unidentified fatty acids; **ΣSFA:** saturated fatty acids; **ΣMUFAs**: monounsaturated fatty acids; **Σ PUFAs**: polyunsaturated fatty acids; **AA**: arachidonic acid: cis-4,cis-8,cis-11,cis-14 20:4 n-6; **ALA**: α-Linolenic acid: cis-9,cis-12,cis-15 18:3 (n-3); **DGLA:** Dihomo-γ-linolenic acid: cis-8,cis-11,cis-14 20:3 (n-6); **DHA**: Docosahexaenoic acid: cis-4,cis-7,cis-10,cis-12,cis16,cis-19 22:6 (n-3); **SA:** Stearic acid: 18:0; **EPA**: Eicosapentaenoic acid: cis-5,cis-8,cis-11,cis-14,cis-17 20:5 (n-3); **GLA:** gamma-linolenic acid: cis-6,cis-9,cis-12 18:3 (n-6); **LA**: Linoleic acid: cis-9,cis-12 18:2 (n-6); **OA**: Oleic Acid: cis-9 18:1; **PA**: Palmitic acid: 16:0. Values expressed as mean ± SEM ($n = 4$). P, photoperiod effect. (One-way ANOVA, $p < 0.05$); different letters indicate significant statistical differences $p < 0.05$; # indicates trend $0.05 < p < 0.1$ (Post-hoc DMS, one way ANOVA).

Fatty acid	L12	L6	L18	ANOVA
Σ SFA	40.05 ± 0.16	39.83 ± 0.10	39.22 ± 0.24	
12:0	0.10 ± 0.01 *a*	0.58 ± 0.00 *b*	0.07 ± 0.00 *b*	*P*
14:0	1.09 ± 0.19	0.83 ± 0.06	0.81 ± 0.00	
15:0	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	
PA	30.51 ± 0.14 *b*	28.08 ± 0.74 *a*	28.47 ± 0.46 *a*	P
17:0	0.18 ± 0.01	0.18 ± 0.02	0.22 ± 0.00	
SA	7.22 ± 0.27 *b*	9.30 ± 0.50 *a*	9.83 ± 0.17 *a*	P
22:0	0.01 ± 0.00#	0.03 ± 0.01#	0.02 ± 0.00	
Σ MUFAs	24.39 ± 1.80 *a*	18.15 ± 1.18 *b*	16.65 ± 0.77 *b*	P
cis-9 14:1	0.11 ± 0.03	0.04 ± 0.01	0.05 ± 0.00	
cis-7 16:1	0.27 ± 0.01	0.26 ± 0.02	0.27 ± 0.00	
Palmitoleic acid	5.63 ± 0.72 *a*	3.15 ± 0.26 *b*	3.08 ± 0.18 *b*	P
cis-9 17:1	0.16 ± 0.02	0.12 ± 0.02	0.15 ± 0.01	
cis-6 18:1	0.09 ± 0.01	0.08 ± 0.01	0.11 ± 0.02	
OA	13.39 ± 1.46 *a*	10.10 ± 1.04 *a#b*	8.49 ± 0.53 *b*	P
cis-11 18:1	4.44 ± 0.11	4.25 ± 0.08	4.34 ± 0.07	
cis 12 18:1	0.07 ± 0.00	0.07 ± 0.01	0.09 ± 0.01	
cis-11 20:1	0.10 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	
Σ PUFAS-cis	34.67 ± 1.64 *a*	40.13 ± 1.74 *b*	42.19 ± 0.52 *b*	P
PUFAS n-6	25.15 ± 0.95 *a*	27.66 ± 0.64 *b*	28.46 ± 0.17 *b*	P
LA	16.21 ± 0.39	16.66 ± 0.19	16.86 ± 0.47	
GLA	0.04 ± 0.00#	0.03 ± 0.01#	0.04 ± 0.01	
cis-11, cis14 20:2	0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.00	
DGLA	0.30 ± 0.04	0.33 ± 0.03	0.35 ± 0.02	
AA	7.46 ± 0.80 *a*	9.53 ± 0.67 *b*	10.03 ± 0.32 *b*	P
cis-7,cis-10,cis-13,cis-16 22:4	0.43 ± 0.04 *a*	0.53 ± 0.02 *b*	0.51 ± 0.01 *b*	P
cis-4,cis-7,cis-10,cis-13, cis-16 22:5	0.47 ± 0.07	0.48 ± 0.04	0.54 ± 0.03	
PUFAS n-3	9.52 ± 0.70 *a*	12.46 ± 1.10 *b*	13.73 ± 0.46 *b*	P
ALA	0.33 ± 0.05	0.29 ± 0.04	0.27 ± 0.04	
EPA		0.12 ± 0.01		
	0.11 ± 0.02		0.13 ± 0.01	
cis-7,cis-10,cis-13,cis16, cis19 22:5	1.89 ± 0.27#	2.35 ± 0.20	2.49 ± 0.08#	
DHA	7.15 ± 0.47 *a*	9.78 ± 0.84 *b*	10.85 ± 0.44 *b*	P
Σ NI	1.00 ± 0.17	1.13 ± 0.21	1.08 ± 0.09	

0.028), while the difference between the animal exposed to L6 and L12 of 2.25% did not reach statistical significance ($p = 0.078$) (Table 1). Similarly, it was observed that the L18 group presented a lower proportion of FA cis-11 18.1 and a tendency to have a lower level of FA cis-11 20.1 than the control group ($p = 0.038$; $p = 0.082$, respectively). It should be noted that no differences were found between palmitic acid levels due to variability between groups.

In the gastrocnemius muscle, the photoperiod significantly affected the concentration of total MUFAs, palmitic acid and OA ($p = 0.006$; $p = 0.003$; $p = 0.029$, respectively). In this sense, the animals exposed to L18 and L6 presented 7.74% and 6.23% less muscle MUFAs and 2.5% palmitoleic acid than those exposed to control group. (MUFAs; $p = 0.007$; 0.028; palmitoleic acid; $p = 0.015$; $p = 0.011$, respectively). It should be noted that the proportion of OA was less in those animals exposed to L18 compared to L12 ($p = 0.010$), while this difference did not reach statistical significance in relation to those exposed to fewer hours of light and control group ($p = 0.054$). It should be noted that the FA level of cis-11 18.1 and cis-11 20.1 did not differ between groups (Table 2).

3.5. The Photoperiod Modulated the Levels of Some Essential FA on Liver and Muscle

Although exposure to different hours of light did not affect the proportion of total hepatic PUFAs, it did statistically affect the level of n-3 PUFAs. As seen in Table 1, the animals exposed to L6 or L18 showed a higher content of PUFAs of the n-3 series than those L12. However, had lower ALA, but higher amounts of EPA and DHA compared to those exposed to control group (ALA: $p = 0.036$, $p = 0.041$; EPA: $p = 0.013$, $p = 0.024$; DHA: $p = 0.021$, $p = 0.006$, respectively). With regard to total hepatic n-6 PUFAs, no differences were found between the experimental groups. However, photoperiod significantly affected the level of essential FA LA ($p = 0.021$), while it tended to do so with cis-11, cis-14 20.2 ($p = 0.065$). Specifically, the L18 group showed a lower level of both FAs than the L12 group ($p = 0.007$; $p = 0.031$, respectively). It should be noted that it the animals exposed to L6, also presented a smaller proportion of LA than L12 group, but this difference did not reach statistical significance ($p = 0.065$).

Similarly, as shown in Table 2, in the gastrocnemius muscle photoperiod significantly affected both the concentration of total PUFAs, as well as the level of those from the n-3 and n-6 series ($p = 0.002$; $p = 0.003$; $p = 0.001$). Mainly, the animals that were exposed to L6 or L18, presented a higher concentration of these sums of PUFAs compared to the L12 animals. However, when analyzing each FA, only a significant effect of exposure to different hours of light was observed, on the level of DHA, with the L16 and L18 animals showing the higher level than the control group (L16 vs L12: $p = 0.023$; L18 vs L12: $p = 0.004$). In addition, the L18 animals tended to present a higher concentration of cis-7, cis-10, cis-13, cis-16, cis-19 22.5 than the L12 group ($p = 0.060$). On the contrary, no significant differences were observed in the concentration of either ALA or EPA. In relation to the n-6 PUFAs, the photoperiod affected the level of AA and FA cis-7, cis-10, cis-13, cis-16 22.4, where the L6 and L18 animals presented a significantly higher proportion of both FA than the control group.

3.6. L18 Affected the Expression of Hepatic Genes Related to Lipid Metabolism Compared to L12

Exposure to different photoperiods mainly affected the gene expression of *Fas1* and *Srebp-1c*, after performing a one-way ANOVA ($p = 0.04$; $p = 0.037$, respectively). As can be seen in Fig. 4**-a**, the animals exposed to L18 presented a higher expression of both genes compared to those animals exposed to L12 and L6. It should be noted that the difference in the level of *Srebp-1c* RNA between L18 and L12 did not reach statistical significance ($p = 0.083$). Contrarily, the L12 group showed a tendency to higher gene expression of *Cpt1α* and *FAT/Cd36* in relation to the L18 group ($p = 0.076$; $p = 0.075$, respectively). On the other hand, the concentration of mARN of *FATP5* transporter tended to be lower in animals exposed to the L6 photoperiod compared to the rest of the groups.

3.7. The Gene Expression of the Enzyme Scd1 was Modulated by Exposure to Different Photoperiods

As seen in Fig. 4**-b**, when analyzing expression of hepatic enzymes related to the onset of FA, only the expression of *Scd1* was statistically altered by exposure to different photoperiods ($p = 0.029$). Specifically, both L6 and L18 animals showed 34% and 33% lower mRNA concentration than those exposed to L12 ($p = 0.019$; $p = 0.021$, respectively). In addition, the analysis of the gene expression of the *Fads1, Fads2* and *Elovl2* enzymes was not altered by exposure to different hours of light.

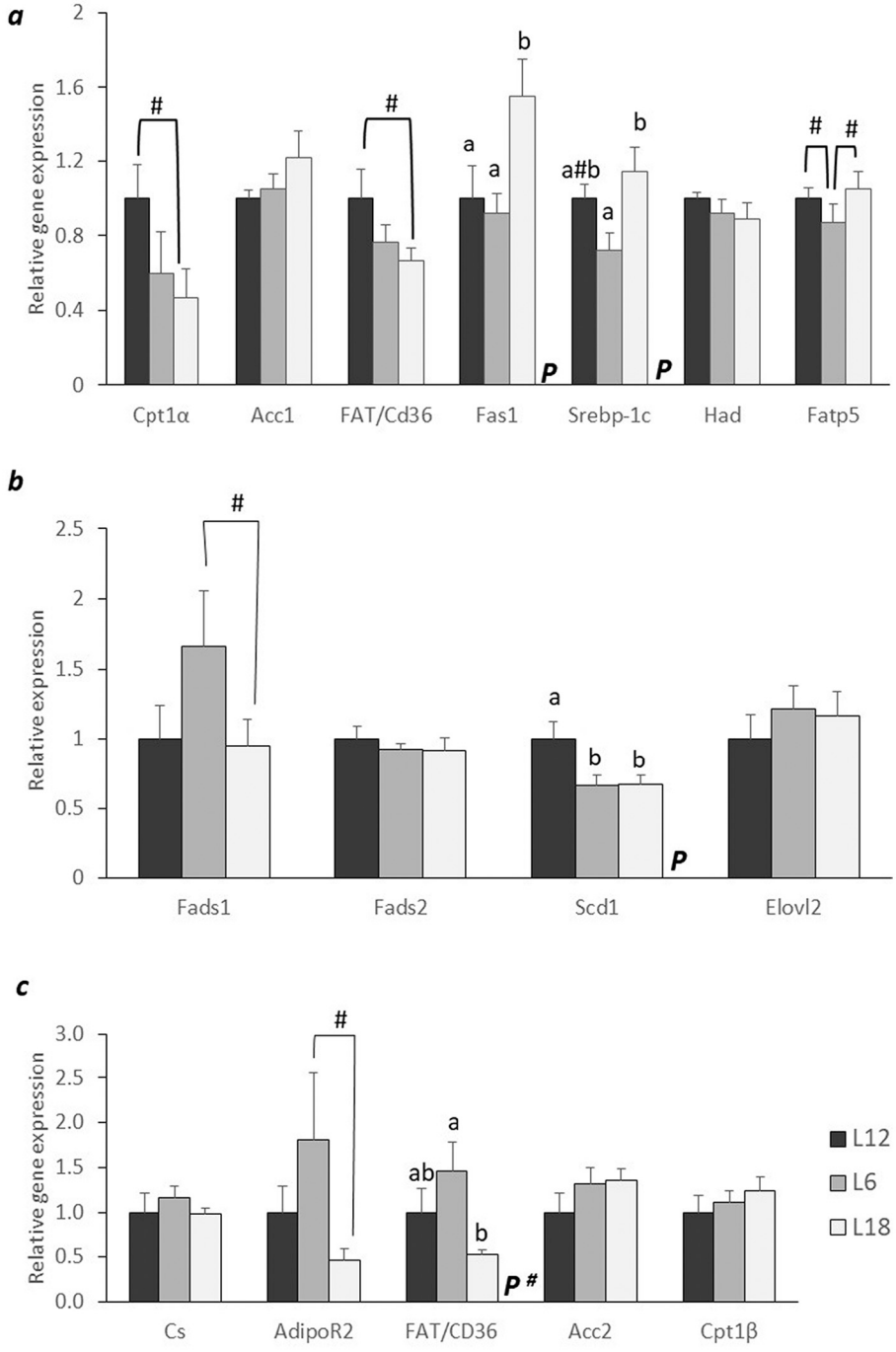


Fig. 4. Gene expression of hepatic lipogenic enzymes (**a**), hepatic enzymes related to the biosynthesis of unsaturated FA (**b**), and enzymes related to the metabolism of FA (**c**) in gastrocnemius muscle. The mRNA levels of *Cpt1α,* Carnitine palmitoyltransferase 1-α; *Acc1*, Acetyl-coenzyme A carboxylase; *FAT/Cd36*, fatty acid translocase homolog of CD36; *Fas1*, fatty acid synthase; *Srebp-1c*, sterol regulatory element-binding protein 1; *Had*, hydroxyacyl-CoA dehydrogenase; *Fatp5*, fatty acid transporter 5, of F*ads1*, Δ-5 desaturase; Fads2, Δ-6 desaturase; *Scd1*, Desaturase Δ-9; *Elovl2*, Elongation of very-long-chain fatty acid enzyme 2, Cs, Citrate Synthase; AdipoR2, Adiponectin receptor 2; Acc2, Acetyl-CoA carboxylase 2; Cpt1β, carnitine palmitoyl transferase 1 beta of male Fischer 344 rats exposed for 11 weeks to different photoperiods (short; L6, standard; L12 or long; L18). Values expressed as mean ± SEM ($n = 8$). The values were normalized by the L12 group. P, photoperiod effect (one-way ANOVA, $p < 0.05$). Different letters above the bars indicate significant differences ($p < 0.05$) (post-hoc DMS, one-way ANOVA). # Indicate trend ($0.05 < p < 0.1$).

3.8. Exposure to Different Photoperiods did not Modulate the Expression of Muscle Oxidative Enzymes, but it did affect that of the FA Transporter FAT/cd36

Exposure to different hours of daily light tended to affect the mRNA concentration of the FA transporter *FAT/cd36* in muscle ($p = 0.058$). As shown in Fig. 4-**c**, in animals exposed to L18, the gene expression of this transporter was only 36% of what was expressed in animals exposed to L6 ($p = 0.018$). Similarly, we found that the L18 group also tended to have a higher level of *AdipoR2* mRNA compared to the L6 group ($p =$

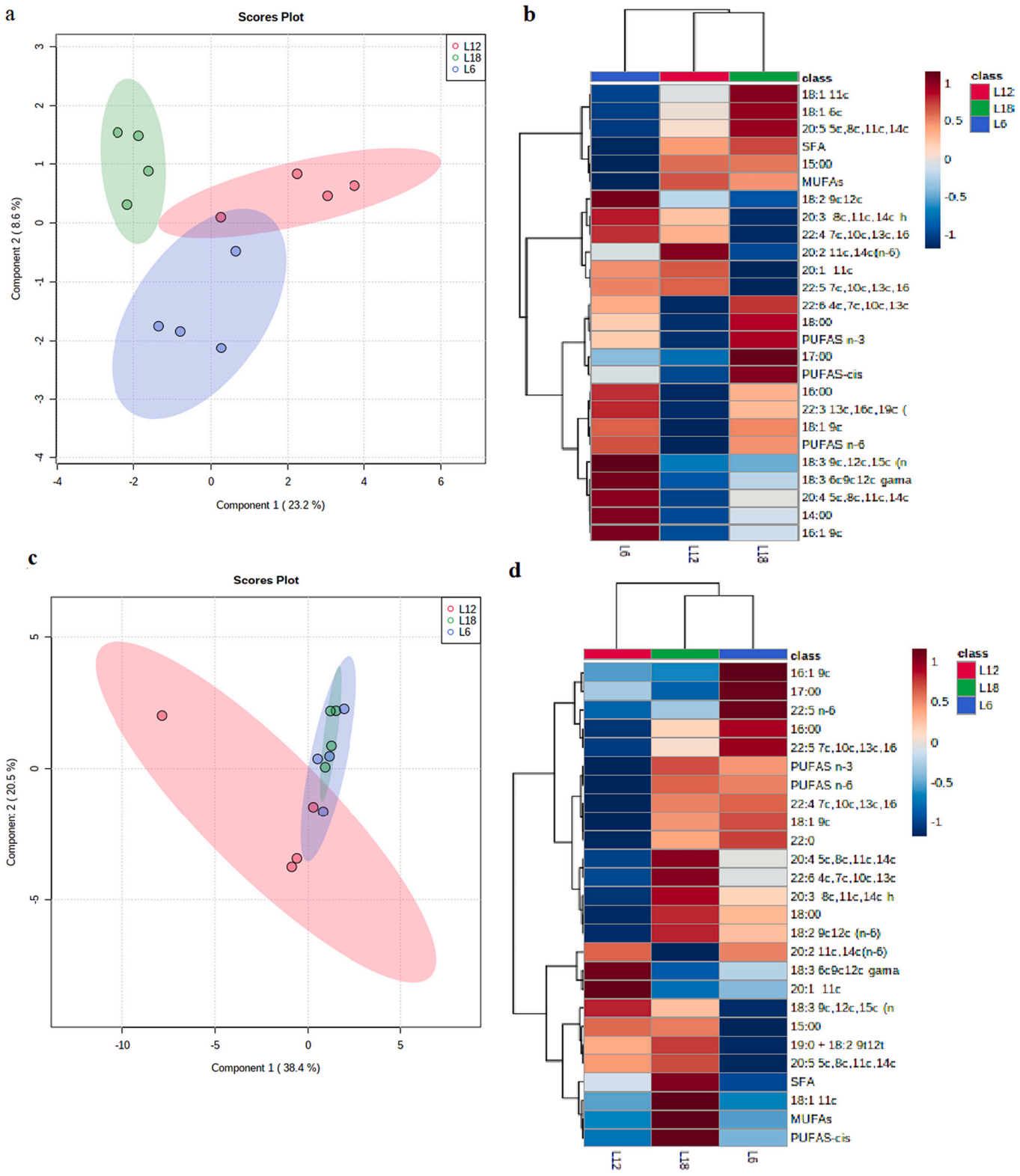


Fig. 5. Multivariate analysis of the effect of exposure to different photoperiods on hepatic and muscle fatty acid profile. Sparse Partial Least Squares- Discriminant Analysis (sPLSDA) (**a; c**) and heatmap plot of hierarchical clustering analysis (**b**; **d**) in liver and gastrocnemius muscle, respectively.

0.059). The gene expression of the rest of the muscle enzymes involved in FA oxidation did not show changes with the different photoperiods.

3.9. Multivariate Analysis Determined two FA Metabolic Pathways Affected between L18 Animals and Control Group

The GC data of twenty-eight FA hepatic and twenty-one FA of muscle, as well as the sum of SFA, MUFAs, PUFAs, PUFAs n-3 and n-6 were used to establish sPLSDA and heatmap (Fig. 5). These types of analysis allowed to obtain more reliable information about the intergroup differences in FA and the degree of correlation between the experimental groups. In Fig. 5-**a**, when comparing animal liver FAs between the photoperiods, a clear separation can be observed between those L18 animals, and the rest of the group.

The heatmap (Fig. 5-**b**) reflects that the L18 animals presented a differential proportion with respect to the control group, mainly in long-chain fatty acids. Although the profile of fatty acids between L6 and the control group show differences, the separation between them is not so defined. Likewise, when performing the multivariate analysis of muscle FA, we observed a greater separation between L18 and the control group (Fig. 5-**c**). The heatmap shows the differential FA between the groups (Fig. 5-**d**). However, the differences between L6 and L12 are less defined.

On the other hand, as shown in Fig. 6-**a**, when performing the pathway analysis, both the metabolism of LA (Fig. 6-**b**) and the biosynthesis of unsaturated FA (Fig. 6-**c**) were the main metabolic pathways affected by exposure to L18 compared to control group (L12). Table 3 shows the results obtained by the Metaboanalyst, as well as the different parameters for the validation of said routes. However, when making comparisons between L12 vs L6 or L18 vs L6, there were no significant differences in these metabolic pathways.

3.10. Multivariate Analysis Determines a Clear Effect on Hepatic Metabolites

As a result of NMR analysis, 33 hydrophilic and 14 lipophilic metabolites were found in serum, while 44 and 17 were found in liver tissue, respectively. The main affected serum and liver metabolites are shown in Table 4. All results can be found in supplemental material. (Tables 2, 3, 4 and 5). Specifically, photoperiod significantly affected serum levels of isoleucine ($p = 0.048$), lactate ($p = 0.04$), ornithine ($p = 0.004$), and choline ($p = 0.024$), while liver concentrations of valine (p

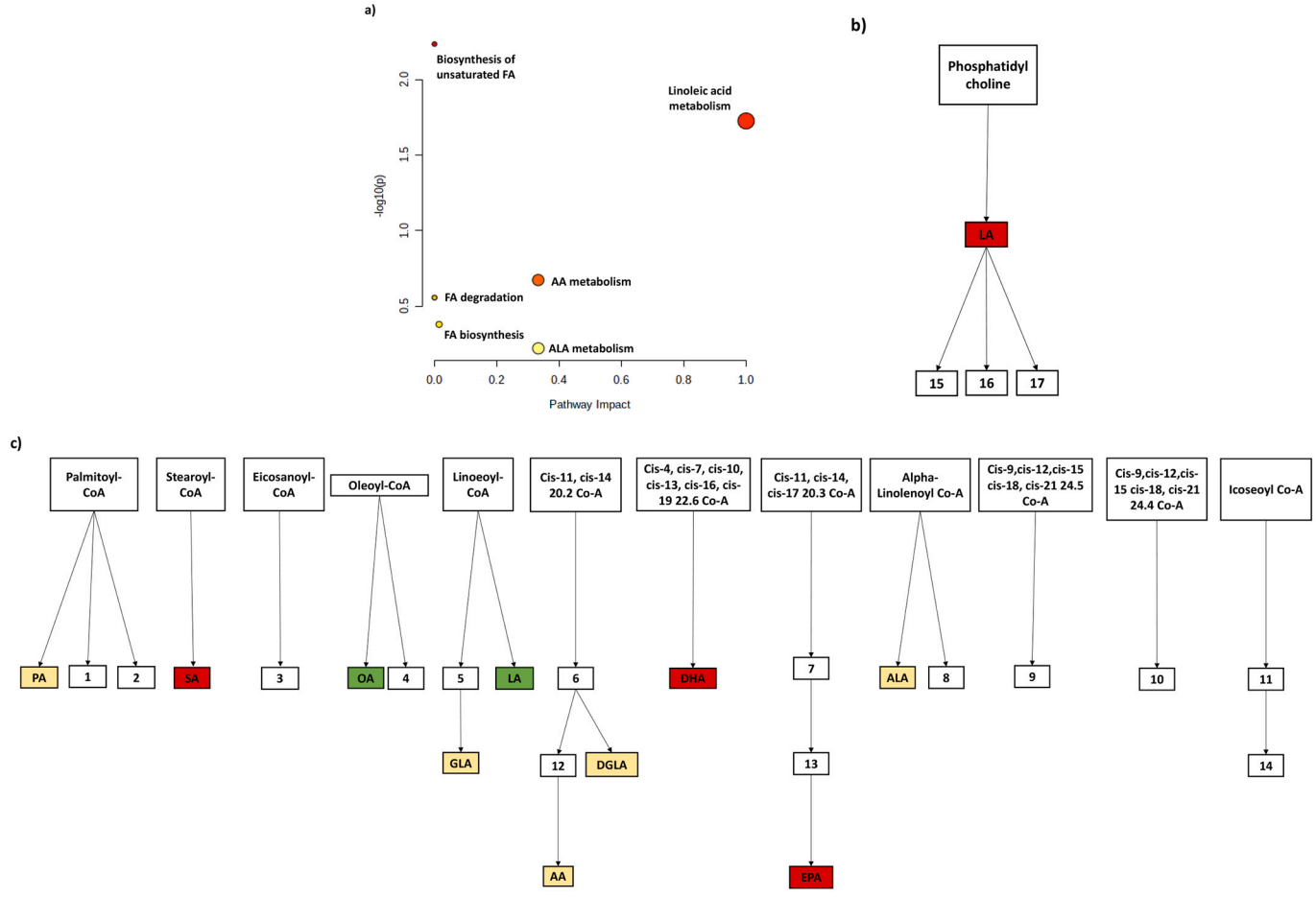


Fig. 6. Pathway analysis based on data from lipidic metabolites of liver samples of animals exposure to L18 compared to L12. Overview of pathway analysis (a) pathway of linoleic acid metabolism (**b**) and pathway of select biosynthesis of unsaturated fatty acid (FA) (**c**) from Kyoto encyclopedia of Genes and Genomes (KEGG) database. Red and green colored boxes indicate increased and decreased proportion of FA; yellow boxes indicate small variation; white boxes indicate no changes of lipid classes. **1**, Sapienoyl-CoA;**2**, Palmitoleoyl-CoA;**3**, Icosanoic acid; **4**, (6Z,9Z)-Octadecadienoyl-CoA; **5**, gamma-Linolenoyl-CoA; **6**, (8Z,11Z,14Z)-Icosatrienoyl-CoA; **7**, (8Z,11Z,14Z,17Z)-Icosatetraenoyl-CoA; **8**, (6Z,9Z,12Z,15Z)-Octadecatetraenoyl-CoA; **9**, (6Z,9Z,12Z,15Z,18Z,21Z)-Tetracosahexaenoyl-CoA; **10**, (6Z,9Z,12Z,15Z,18Z)-Tetracosapentaenoyl-CoA; **11**, (8Z,11Z)-Icosadienoyl-CoA; **12**, Arachidonoyl-CoA; **13**, (5Z,8Z,11Z,14Z,17Z)-Icosapentaenoyl-CoA; **14**, (5Z,8Z,11Z)-Icosatrienoyl-CoA; **15,** 9,10-Epoxyoctadecenoic acid; **16**, 12,13-Epoxyoctadecenoic acid; **17**, 13S-Hydroperoxy-9Z,11E-octadecadienoic acid. Abbreviations: AA: Arachidonic acid; ALA: alpha-Linolenic acid; DGLA: Dihomo-γ-linolenic acid; DHA: docosahexanoic acid; EPA: Eicosapentaenoic acid; GLA: gamma-linolenic acid; LA: Linoleic acid; OA: Oleic acid; PA: Palmitic acid; SA: Stearic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Analysis of metabolic pathways with hepatic FA. Liver fatty acids from animals exposed to 6, 12 and 18 h of light (L6, L12, L18, respectively) were exposed to pathway analysis using Metaboanalyst. Main metabolic pathways significantly affected by exposure to different photoperiods, the total amount of metabolites involved in the pathway, the amount of metabolites that have information, the *p* value, false discovery rate (FDR), and impact, are shown. *P* value <0.05 and FDR < 0.05 were considered significant (*); while a 0.05 < p value and/or FDR < 0.1 were considered a trend (#). Kyoto encyclopedia of Genes and Genomes (KEGG) was database used.

Pathway name	Total metabolites	Hits	Statistical comparison	p value	FDR	Impact
Biosynthesis Of Unsaturated Fatty Acids	36	10	L12 vs L18	0.006	0.040 *	0
			L12 vs L6	0.110	0.386	0
			L6 vs L18	0.375	0.438	0
Linoleic Acid Metabolism	5	1	L12 vs L18	0.018	0.065 #	1
			L12 vs L6	0.103	0.386	1
			L6 vs L18	0.112	0.263	1

Table 4
Concentration of major metabolites in serum and liver analyzed by nuclear magnetic resonance in male Fischer 344. Standard male Fischer 344 rats exposed to different photoperiods (short, L6; standard, 12; long, L18, with 6, 12 and 18 h of light, respectively). Data are expressed as the mean ± SEM ($n = 8$). All the serum and hepatic metabolites (hydrophilic and lipophilic) were obtained by performing a nuclear magnetic resonance (NMR) analysis. P, photoperiod effect. (one-way ANOVA, $p < 0.05$); different letters indicate significant statistical differences $p < 0.05$; # indicates trend $0.05 < p < 0.1$ (Post-hoc DMS, one way ANOVA).

		L6	L12	L18	*Anova*
Serum	Isoleucine	0,08 ± 0*a*	0,08 ± 0*a*	0,07 ± 0 *b*	P
	Lactate	5,11 ± 0,75 *a*	3,01 ± 0,22*b*	5,29 ± 0,35 *a*	P
	Pyruvate	0,05 ± 0 *ab*	0,04 ± 0 *a*	0,05 ± 0 *b*	P#
	Ornithine	0,1 ± 0,02 *a*	0,05 ± 0 *b*	0,05 ± 0 *b*	P
	Choline	0,02 ± 0 *a*	0,015 ± 0 *b*	0,015 ± 0 *b*	P
	Betaine	0,11 ± 0,01 *ab*	0,11 ± 0 *a*	0,13 ± 0,01 *b*	P#
	Theronine	0,11 ± 0,01 *a#b*	0,11 ± 0 *a*	0,09 ± 0,01 *b*	P#
	Formate	0,04 ± 0 *a*	0,04 ± 0 *ab*	0,03 ± 0 *b*	
	Sphingomyelin	0,09 ± 0,03 *a*	0,04 ± 0,01 *b*	0,06 ± 0,01 *ab*	
Liver	Betaine	1,63 ± 0,19 *a*	1,63 ± 0,12 *a*	2,08 ± 0,16 *b*	*P#*
	3-Hydroxybutyrate	0,3 ± 0,04 *ab#*	0,32 ± 0,02 *a*	0,26 ± 0,02 *b*	*P#*
	Valine	0,34 ± 0,03 *ab*	0,37 ± 0,03 *a*	0,29 ± 0,02 *b*	*P*
	Sarcosine	0,05 ± 0,01 *a*	0,07 ± 0,01 *b*	0,07 ± 0,01 *ab*	
	UDPs	0,59 ± 0,1 *a*	0,87 ± 0,1 *b*	0,74 ± 0,07 *ab*	
	Asparagine	0,04 ± 0,01 *a*	0,05 ± 0,01 *a*	0,03 ± 0 *b*	*P*
	Isoleucine	0,18 ± 0,02 *ab*	0,2 ± 0,01 *a*	0,16 ± 0,01 *b*	*P*
	Creatine	0,13 ± 0,02 *a*	0,14 ± 0,01 *a*	0,1 ± 0,01 *b*	*P*
	Carnitine	0,73 ± 0,09 *a*	1,04 ± 0,08 *b*	0,68 ± 0,07 *a*	*P*
	Free Cholesterol	3,44 ± 0,08 *a*	3,17 ± 0,07 *b*	3,31 ± 0,11 *ab*	
	Phophoethanolamine	8,05 ± 0,21 *a*	7,68 ± 0,39 *ab#*	6,75 ± 0,42 *b*	*P#*
	Sphingomyelin	1,05 ± 0,02 *a*	0,93 ± 0,04 *b*	0,94 ± 0,04 *b*	*P*

= 0.04), asparagine ($p = 0.003$), isoleucine ($p = 0.034$), creatine (p = 0.034), carnitine (p = 0.004), and sphingomyelin ($p = 0.017$) were affected. Due to the large number of quantified metabolites, we performed a multivariate analysis to observe the differences between the groups. Fig. 7**-a** shows the sPLSDA analysis of hepatic lipophilic and hydrophilic metabolites, where a clear photoperiod effect can be seen, with L18 showing a marked difference from all other exposure periods. The heatmap from hierarchical clustering analysis confirms these results, showing that the L18 group has a distinctly different pattern of metabolites than the L6 and L12 groups.

4. Discussion

It is now well documented that the circadian rhythm of physiological processes in mammals is mainly modulated by the amount of daily light [3,4,6,26]. In this sense, melanopsin activates neuronal pathways to establish the master clock of the hypothalamic SCN, depending on the wavelength of light captured by the retina [26,27]. Therefore, depending on the cyclical environmental changes, the central nervous system will orchestrate the clocks in the peripheral tissues and consequently their biological functions, such as the availability and metabolism of nutrients [5,8]. On the other hand, there is evidence in different animal models showing that through independent pathways, light can synchronize the peripheral clocks of tissues such as the liver, kidneys, hearts, adrenal glands and muscles, among others [5,28,29]. However, there is little evidence on the impact of seasonal rhythms and their consequences on the tissue lipid profile [15].

In the present study we have evaluated the effects of exposure to different photoperiods on various plasma metabolic parameters, gene expression of liver and muscle enzymes, as well as the content of the lipid profile of these tissues in Fischer 344 rats. Overall, exposure to different hours of daily light modulated the lipid profile of the animals.

We have previously documented that those animals exposed to different photoperiods do not show differences in body weight or in the amount of food consumed per day [20]. Therefore, analyzing the experimental design, and considering that the rats feed in the dark, these animals had different hours to eat the same amount of food, which is expressed by the EPI of each group. In this sense, we can observe different eating patterns. It should be noted that the timing of food intake functions as an activating signal for peripheral clocks in metabolic tissues, helping to maintain robust circadian rhythms [30–32].

4.1. Exposure to Different Hours of Light Mainly Modulates Lipid Metabolism in Liver and Muscle, Mainly in those Exposed to Long Days Compared to Standard Days

The multivariate analysis by sPLSDA, both of the FA profile and of the hydro and lipophilic metabolites in liver, show a clear separation between the L18 groups and the control group. Moreover, we have observed a trend towards lesser serum TAG, hepatic gene expression of the enzyme *Cpt1α* and *FAT/cd36* together with a greater expression of *Fas1* and a higher proportion of SFA in animals L18 with respect to those exposed to standard photoperiod. In this sense, the enzyme *Cpt1α* is the limiting enzyme for the passage of long-chain FA Co-A through the mitochondrial wall for its β-oxidation [33]. In turn, the FAT/cd36 transporter translocates to the plasma membrane of the hepatocyte, allowing the uptake of FA from the circulation [34]. However, it has recently been observed that FAT/cd36 is also found in the mitochondrial

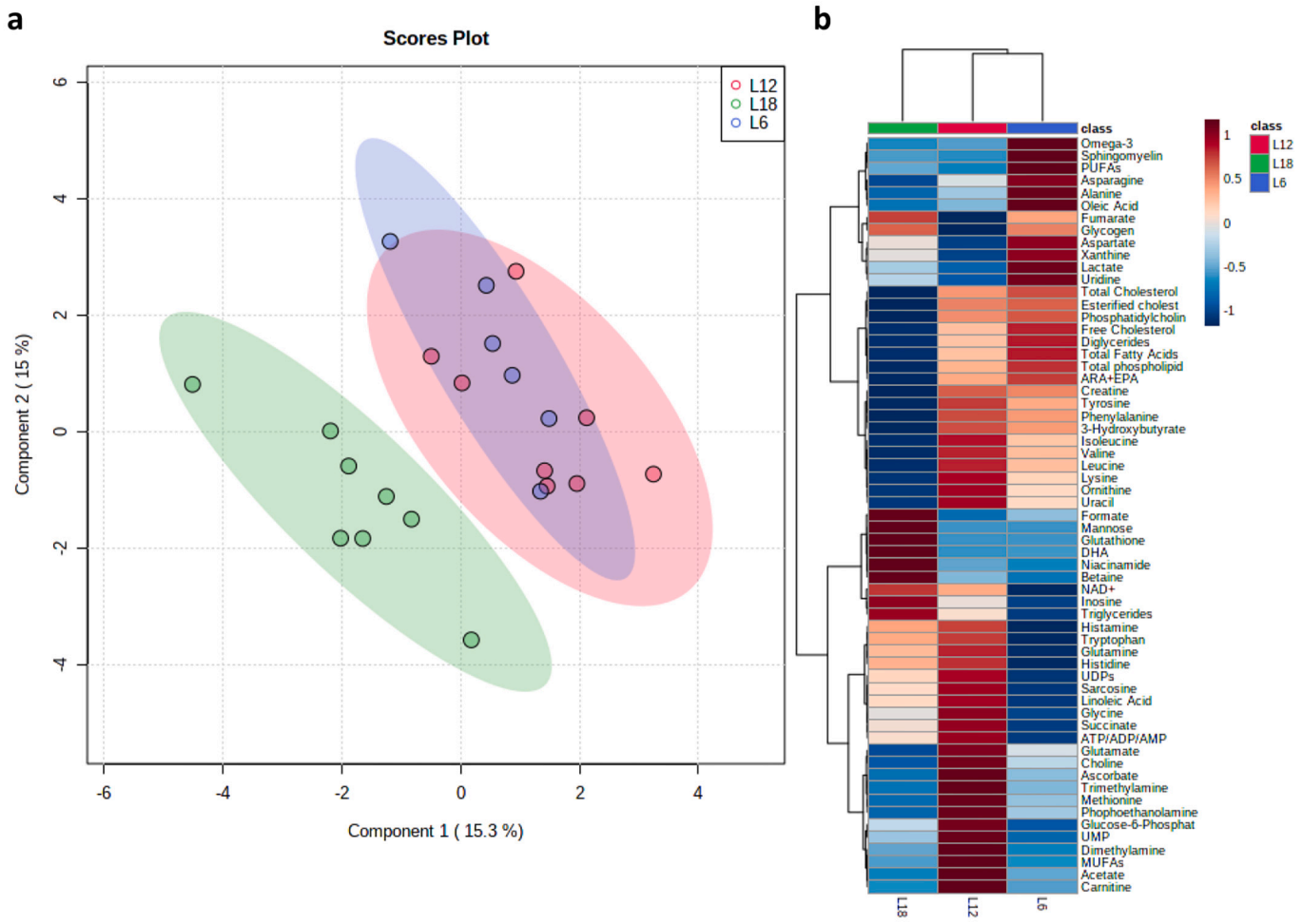


Fig. 7. Multivariate analysis of the effect of exposure to different photoperiods on hepatic hydrophilic and lipophilic metabolites. Sparse Partial Least Squares-Discriminant Analysis (sPLSDA) (a) and heatmap plot of hierarchical clustering analysis (b).

membrane, actively participating in the passage of FA for oxidation [35]. While increased expression of *FAT/Cd36* has been associated with metabolic diseases such as obesity, insulin resistance, and diabetes, increased *FAT/cd36* gene expression has also been shown to be associated with increased oxidation of long-chain FA [35,36]. In addition, the lipogenic enzyme Fas1 is responsible for the synthesis of palmitoyl-CoA from acetyl-CoA units in the liver [34]. Therefore, these results would explain the higher concentration of hepatic SFA, because of less mitochondrial oxidation and/or greater synthesis of FA in L18 animals compared to control group. It should be noted that these differences were not found between the L6 and L12 animals, indicating the importance of the light stimulus. Other authors also found in L18 animals a less expression of *FAT/Cd36* and the enzymes phosphoenolpyruvate carboxykinase 1 and fructose 1–6 biphosphatase 1, and lower concentration of liver glycogen, together with a greater concentration of glycerophosphocholine than in control group (L12), despite not having analyzed hepatic FA profile [16]. In addition, hepatic lipogenesis, as well as other metabolic enzymatic processes, have a regulated rhythmicity through several factors. In this sense, the state of feeding/fasting, certain hormones such as insulin, glucagon, glucocorticoids, or dietary composition directly influence the gene expression of transcription factors and key enzymes in lipid metabolism [14,37]. Although the concentration of *Srebp-1c* mRNA does not differ between these groups, it is known that this transcription factor is under circadian regulation, and that its oscillations also determine fluctuations in the expression of its target genes such as *Fas1*, *Scd1* and *Acc1*, throughout a day [38]. Considering that the treated animals were chronically exposed to days with different amounts of light hours, it can be expected that the moment of greater gene expression of key enzymes in lipid metabolism will be displaced, as well as differ in the moment of ingestion, which would grant seasonal variability and disruption to digestive processes. In this sense, other authors reported that animals exposed to L18 presented altered gene expression with respect to the other photoperiods, of genes that modulate the circadian clock, such as *Bmal1*, *Per2 Cry1* and *Nr1d1* in liver and muscle [16]. Since these clock genes influence lipid and carbohydrate metabolism, changes in FA profile and serum and liver metabolite concentration may relate to an disruption in circannual rhythm.

On the other hand, in the present study, we have observed greater differences in the hepatic FA profile between the L12 and L18 animals and to a lesser extent between the L12 and L6 groups. These differences were reflected in the analysis of the hepatic lipid profile by sPLSDA and the heatmap. The pathway analysis has shown that the biosynthesis of unsaturated FA is significantly affected by the L18 photoperiod compared to exposure to L12. As shown in **Fig. 2** of the **Supplementary material**, *Scd1* is a key enzyme at the beginning of the synthesis of unsaturated FA, since it introduces the first cis bond in its substrates, PA or SA, for the formation of palmitoleic acid and OA [39]. Since these FA are the major constituents of the plasma membrane, their proportion would give it fluidity and/or rigidity [39]. Therefore, a decreased ratio of OA/SA and/or palmitoleic acid/PA would indicate a greater presence of SFA, which is associated with certain pathologies such as cancer, diabetes, obesity, hypertension, among other chronic metabolic diseases [40]. Specifically, the L18 group presented a higher proportion of the

SFA PA and SA, together with a lower gene expression of *Scd1* and its desaturation product OA, compared to the L12 group, suggesting that exposure to long days would not only reduce the oxidation of FA, but also decreases the establishment of SFA, causing greater hepatic accumulation and providing greater rigidity to the plasma membrane. On the other hand, L6 animals also showed lower *Scd1* gene expression compared to L12 animals. However, this difference was not reflected in the proportion of SFA, palmitoleic acid or OA.

Leyton et al. (1987) stated that n-3 PUFAs have a higher rate of oxidation in 24 h than n-6 PUFAs or MUFAs [41]. We have found that although there are no differences between the amount of total PUFAs or those of the n-6 series, those animals exposed to L18 and L6 have a greater amount of hepatic n-3 PUFAs than the control group. Gibson et al. (2011), proposed a biosynthetic model of PUFAs, where OA, LA and ALA use the same metabolic pathway for their elongation and desaturation [42]. In this process, *Fads2* is considered the key enzyme for the biosynthesis of PUFAs, since it is involved in the first step and is where the three FAs compete for it. Furthermore, by introducing a double bond in the FA cis-9,cis-12,cis-15,cis-18 24.4 and cis-9,cis-12,cis-15,cis-18,cis-21 24.5, also there is competition with them. The metabolic pathway of unsaturated FA biosynthesis is summarized in **Fig. 2** of the **Supplementary material.** Therefore, since no differences were found in the proportion of PUFAs n-6 between the groups, we can establish that the higher level of PUFAs n-3 found in the L18 and L6 animals was not due to enzymatic competition between FA. Consequently, even though there are no differences in the gene expression of *Fads1, Fads2* and *Elovl2*, key in their synthesis, there are differences in the hepatic EPA/ALA, DHA/ALA ratios, indicating a greater flow towards the biosynthesis of PUFAs n- 3 in animals exposed to L18 and L6. In this sense, it is important to highlight that there are differences between the FA of the n-3 series between the experimental groups. Mainly, the L18 and L6 groups have a lower content of hepatic ALA and a higher proportion of its elongation and desaturation products, EPA and DHA than control group. Therefore, this difference in the proportion of n-3 PUFAs would indicate either and decrease in the rate of oxidation of the L18 group, which is accompanied by a tendency to lower expression of the enzymes involved in b-oxidation (compared to with the L12 photoperiod), or to a higher hepatic synthesis of PUFAs-n3. It should be noted that the oxidation of FA depends mainly on the ability to capture FA from the blood into the interior of the cell, as well as the intrinsic capacity of the tissue for oxidation [43]. In this sense, the differences between the L18 and L12 groups in the expression of the *Cpt1α* and the *FAT/cd36* transporter may support this theory.

Despite no differences in the proportion of hepatic n-6 PUFAs, we found a smaller content of LA, cis-11, cis-14 20.2 and a greater level of AA in animals exposed to L18 compared to standard group. Therefore, even though we did not find differences in the gene expression of key enzymes for their synthesis between both groups, we found a greater flow of synthesis towards these FA in the animals exposed to long days, given by the AA/LA and AA/GLA ratios. In this sense, the pathway analyzes showed a trend in a differential effect of the L18 photoperiod on the metabolism of LA. Although only one metabolite was involved in this pathway, more studies involving phosphatidyl choline and its metabolic cascade are suggested to understand its seasonal variation.

It has been shown that the dietary n-6/n-3 ratio is an important determinant in the state of inflammation, maintenance of homeostasis, and the development of chronic diseases such as cancer, rheumatoid arthritis, coronary heart disease, obesity, diabetes, etc. [44]. However, although all the experimental groups consumed the same type and amount of food daily, the L18 animals ingested double or triple the number of kcal per hour than those exposed to L12 or L6, respectively. Therefore, the different distribution of consumption times between the groups could determine the differences in the composition of hepatic FA.

On the other hand, skeletal muscle also plays an important role in FA uptake for oxidation and energy production. In this sense, the L18 group has shown a lower concentration of muscular SFA, together with a tendency to a smaller amount of plasmatic TAG, indicating that there could be a lower export of SFA from the liver to the gastrocnemius muscle compared to control group. Mariné-Casadó et al. (2018) did not observe changes in serum TAG in animals exposed to different photoperiods [16], however, Xie et al. (2017) stated that exposure to long days slightly increases this parameter [45]. Due to the discrepancy between the results, investigations in this regard are required. A higher proportion of muscle SFA, mainly PA, is associated with insulin resistance and higher blood glucose [46]. In this sense, the slight increase in SFA together with a higher proportion of PA found in the L12 animals could also explain their higher glycemia compared to the L18 animals. In addition, the L18 and L6 animals showed a higher proportion of both total PUFAs and those of the n-6 and n-3 in muscle than control group (L12). A higher level of 22.5 n-3 and DHA would also explain the lower amount of SFA and glycemia in L18 animals, since these FAs, mainly DHA, are associated with greater oxidation of glucose and lipids, and with anti-inflammatory effects, through AMPK phosphorylation [47]. A greater muscle β-oxidation of PUFAs over MUFAs or SFA could be hypothesized, however, the expression of key enzymes in muscle β-oxidation, *Cpt1β* and *FAT/cd36*, did not show differences between groups.

These results were also accompanied by a clear differentiation between groups when analyzing serum and liver metabolites. Specifically, when analyzing the hydro and lipophilic metabolites in the liver, we have observed that the L18 animals showed a lower concentration of betaine, 3-hydroxybutyrate, valine, asparagine, isoleucine, creatine, and carnitine compared to the control group, which would reflect an altered amino acid metabolism. However, we suggest future research on gene expression of enzymes, protein concentration and determination of metabolic intermediates to elucidate mechanisms involved.

4.2. Exposure to L18 Stimulates Lipogenesis through Srebp-1c Gene Expression and Key Enzymes in De Novo Synthesis

The SREBP family are master regulators of lipid metabolism [34]. Specifically, hepatic Srebp1-c activates genes related to TAG synthesis, such as *Acc1* and *Fas1*, as well as enzyme genes responsible for FA elongation and desaturation, such as *Scd1* and elongase complexes [48]. In agreement with our findings, the animals exposed to L18 presented a higher gene expression of *Srebp-1c*, together with a higher concentration of *Fas1* mRNA and a tendency to a higher expression of the FA transporter FATP5 in relation with the L6 animals. These results suggest a stimulation of lipogenesis, which is accompanied by a higher proportion of hepatic SFA, compared to animals exposed to L6. Specifically, this difference in total SFA content could be the result of a higher proportion of PA and SA in L18 animals. However, they do not reach statistical significance. InterestinglyInterestingly, when observing the sPLSDA and the heatmap we see a clear differentiation between the L18 and L6 groups, when analyzing the fatty acid profile, both liver and muscle. Although there are limited studies on the lipid profile and exposure to photoperiods, we previously observed that animals exposed to L18 have a higher % body fat than those exposed to L6 [20]. As rats are nocturnal animals, it makes sense that those animals that are active for more hours a day have a lower percentage of lipids. However, differences in the gene expression of hepatic oxidative enzymes were not found. In turn, the entry of adiponectin through its receptor produces the phosphorylation of Acc2, with the consequent decrease in malonyl-CoA and the activation of cpt1β, stimulating muscle β-oxidation [49,50]. In this sense, we have found a tendency to a greater expression of *AdipoR2*, accompanied by a greater concentration of mRNA of *FAT/cd36*, which would indicate a greater oxidation of muscle FA of animals L6, even though the expression of *Cpt1β* was not modified. In addition to the number of active or inactive hours of the animals, it is also important to note that those L18 animals have an EPI three times higher than the L6. Consumption of the same kcal, but in much less time could be responsible for these changes at the level of accumulation of SFA and in the

differentiation between.

4.3. Other Metabolic Parameters Differentially Affected by the Number of Hours of Light/Darkness

Based on serum metabolic parameters, the photoperiod significantly affects glucose and insulin levels, and therefore HOMA index. Interestingly, the lower concentration of insulin in the animals exposed to L6 is not reflected to the same extent in glucose levels, since only a tendency towards a lower amount is seen in these animals compared to the control group. In contrast, the animals exposed to L18 showed significantly lower glycemia, but the same insulinemia and HOMA index as the L12 group. These results suggest that the photoperiod differentially modulates glucose metabolism depending on the hours of light/darkness. Moreover, the L18 group had higher serum lactate and pyruvate concentrations than controls, suggesting greater glycolysis led to lower blood glucose levels. However, other authors have observed a higher glucose concentration in L6 and L18 animals, together with altered gene expression of glucose enzymes compared to the control group [16]. Therefore, we believe that further analysis of carbohydrate enzymes in liver and muscle, as well as gene expression of clock genes, activity tracking, and measurement of metabolic parameters in different point for 24 h, would be very useful to clarify the mechanisms involved. However, despite these weaknesses, to our knowledge, our study is the first to deep in the effects of circannual rhythms on the fatty acid profile of key metabolic tissues such as liver and gastrocnemius muscle. In this regard, Fig. 8 summarizes the main effects of chronic exposure to short and long photoperiods compared to an L12 photoperiod. Moreover, our findings reinforce the importance of knowing what happens in the metabolism with chronic exposure to different hours of light that could occur in certain situations in the life of humans. Of note, it has been observed in certain studies that the disruption of these rhythms can cause metabolic alterations that can lead to an increased risk of chronic non-communicable diseases, such as obesity, cardiovascular diseases or seasonal affective disorder [51].

5. Conclusion

In summary, this study provides evidence on the metabolic consequences of exposure to different hours of light. Specifically, the photoperiod modulates the lipid metabolism and could affect the metabolism of amino acids. A long photoperiod could be associated with less lipid oxidation, greater FA synthesis, and greater accumulation of SFA and PUFAs n-3 in the liver with respect to exposure to an L12 photoperiod. In

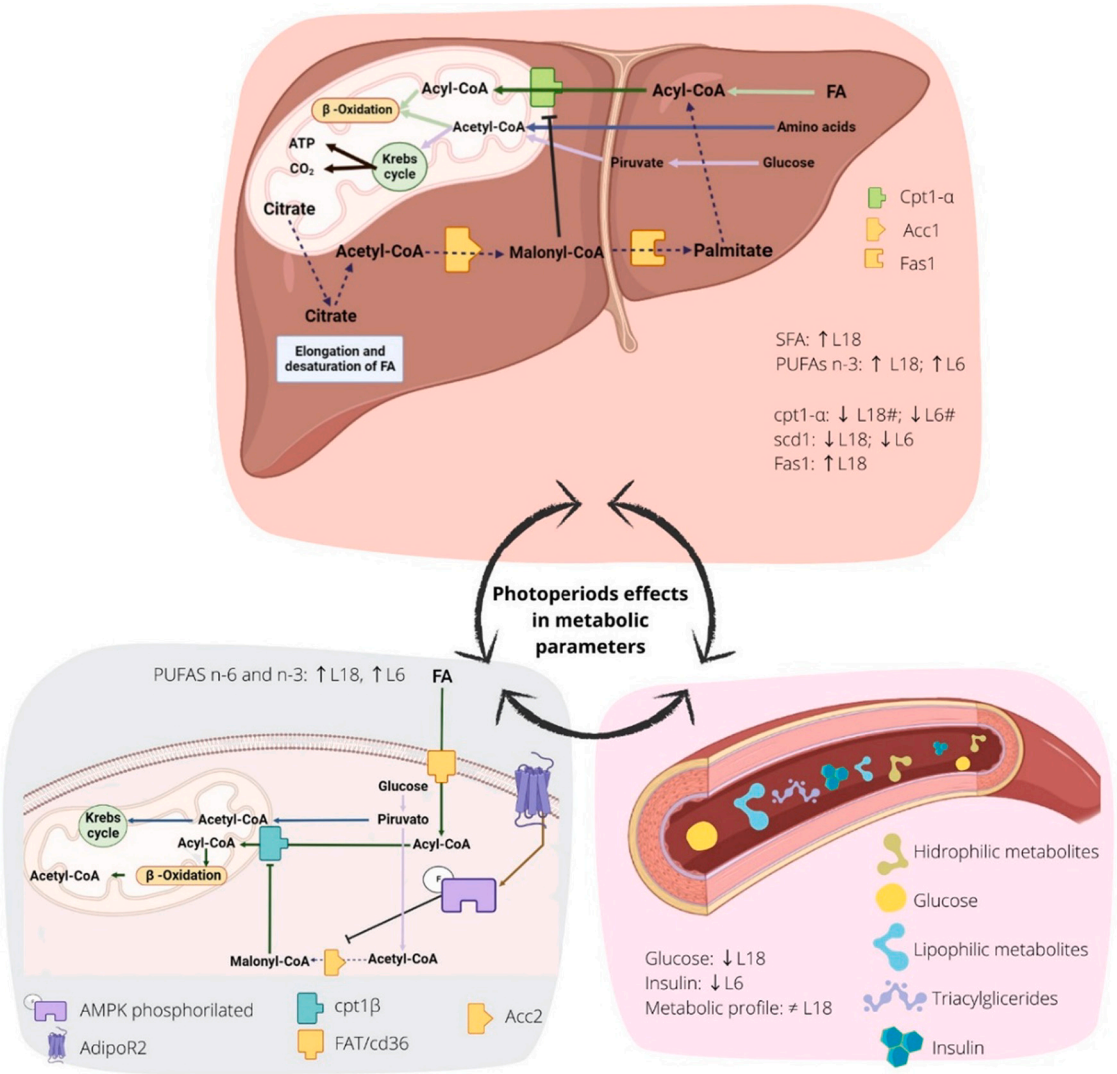


Fig. 8. Summary of the effects on enzyme gene expression, fatty acid profile, and hydrophilic and lipophilic metabolites of exposure to different photoperiods. The changes referred to are in comparison with the standard photoperiod (L12). L6 and L18, short and long photoperiod, with 6 and 18 h of light, respectively. ↑, indicate greater in; ↓ indicate lower in; # indicate tendency.

addition, L18 could be associated with alteration of hepatic hydrophilic metabolites. At the muscular level, the effects were less evident. However, a higher proportion of total MUFAs and PUFAs was also observed. A multivariate analysis confirms the differential effects of photoperiods. The consequences of exposure to L6 were less precise. However, animals exposed to L18 showed a higher gene expression of lipogenic enzymes together with a higher proportion of SFA with respect to those L6. These effects could be due to the different eating patterns that are established with the number of active hours of the animals, which produce the same energy consumption, but in different time intervals.

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Author Contributions

Conceptualization: RdA, M.J., C—C, A., M, B., A-A. A, T.F C, M. F and S, M.; methodology: RdA, M.J., C—C,A. S.J and S, M.; validation: S, M.; formal analysis: RdA, M.J. and M.F; investigation: RdA, M.J., C—C,A. and S, M.; resources: RdA, M.J., C—C,A.; writing original draft preparation: RdA, M.J.; writing—review and editing: C—C, A., B·C, and S, M.; visualization: RdA, M.J.; supervision: S, M.; project administration: C—C,A., A-A, A. and S, M.; funding acquisition: M. B and B.C. “All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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