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Photoperiod-Dependent Effects of Grape-Seed Proanthocyanidins on Adipose Tissue Metabolic Markers in Healthy Rats

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Scope: Variations in photoperiod patterns drive metabolic adaptations in mammals, involving important changes in body weight and adiposity. Moreover, (poly)phenols can help heterotrophs adopt metabolic adaptations to face the upcoming environmental conditions. Particularly, proanthocyanidins from grape-seeds show photoperiod-dependent effects on different metabolic parameters. The present study aims to explore whether grape-seed proanthocyanidin extract (GSPE) consumption differently affects the expression of metabolic markers in WAT (subcutaneous and visceral depots) and BAT in a photoperiod-dependent manner.

Methods and results: GSPE (25 mg kg^{-1} day^{-1}) is orally administrated for 4 weeks to healthy rats exposed to three photoperiods (L6, L12, and L18). In WAT, GSPE consumption significantly upregulates the expression of lipolytic genes in all photoperiods, being accompanied by increased serum concentrations of glycerol and corticosterone only under the L6 photoperiod. Moreover, adiponectin mRNA levels are significantly upregulated in response to GSPE regardless of the photoperiod, whereas *Tnfα* and *Il6* expression are only downregulated in L6 and L18 photoperiods but not in L12. In BAT, GSPE upregulates *Pgc1α* expression in all groups, whereas the expression of *Pparα* is only increased in L18.

Conclusions: The results indicate that GSPE modulates the expression of important metabolic markers of WAT and BAT in a photoperiod-dependent manner.

1. Introduction

Metabolic adaptations to seasonal rhythms are observed in specific species of mammals, that are capable of developing distinct metabolic features depending on the time of year.[1] For instance, Fischer 344 rats are considered photoperiod-sensitive animals and are supposed to lose weight during winter-like conditions, while their food intake is diminished, and their energy expenditure increases.[2] Moreover, they develop molecular adaptations in brown and white adipose tissues (BAT and WAT, respectively), which are relevant for the whole-body metabolism.[3]

WAT is crucial to maintain energy homeostasis in the organism, as its metabolic function allows energy storage and release when needed.[4] Particularly, adipocytes uptake circulating fatty acids (FAs), glucose, and amino acids to synthetize triglycerides through lipogenesis, involving important transporters and enzymes such as the lipid transporter cluster of differentiation 36 (CD36), fatty acid synthase (FASN), or acetyl-CoA carboxylase (ACAC).[5]

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Table 1. Biometric parameters.

	L12-VH	L12-GSPE	*p*	L18-VH	L18-GSPE	*p*	L6-VH	L6-GSPE	*p*	ANOVA[1]
Body weight [g]	479.75 ± 12.2	489.50 ± 10.4	*0.919*	501.00 ± 10.6	484.75 ± 10.9	*0.739*	491.37 ± 12.0	488.12 ± 15.5	*0.997*	*Ns*
Body weight gain [g]	96.50 ± 8.9	112.38 ± 6.5	*0.349*	112.71 ± 5.1	95.12 ± 7.2	*0.292*	106.12 ± 8.1	97.50 ± 7.6	*0.795*	*Ns*
Weekly caloric intake [kcal]	76.05 ± 2.0	77.20 ± 5.3	*0.992*	80.11 ± 4.0	77.50 ± 2.8	*0.921*	82.86 ± 1.3	82.02 ± 2.4	*0.997*	*Ns*
Total fat mass [g]	35.32 ± 3.4^{a}	36.28 ± 1.9^{a}	*0.997*	46.54 ± 3.6^{b}	48.88 ± 4.1^{b}	*0.958*	40.09 ± 3.7^{ab}	46.09 ± 4.2^{ab}	*0.556*	*P*
iWAT mass [g]	4.05 ± 0.4	3.23 ± 0.4	*0.760*	4.84 ± 0.1	5.20 ± 1.2	*0.972*	4.20 ± 0.6	4.78 ± 0.7	*0.895*	*Ns*
eWAT mass [g]	7.50 ± 0.9^{a}	7.92 ± 0.5^{a}	*0.980*	10.99 ± 0.5^{b}	9.89 ± 0.7^{b}	*0.767*	9.31 ± 1.0^{ab}	9.60 ± 1.3^{ab}	*0.993*	*P*
BAT mass [g]	0.55 ± 0.1^{ab}	0.53 ± 0.0^{ab}	*0.913*	0.50 ± 0.1^{ab}	0.38 ± 0.0^{a}	*0.318*	0.65 ± 0.1^{b}	0.57 ± 0.1^{b}	*0.687*	*P*

Values are presented as the mean ± SEM of eight animals per group. [1] Denotes two-way ANOVA analysis: P: photoperiod effect ($p < 0.05$), ns: no significant differences. Different letters indicate significant differences by Šídák's *post hoc* test for multiple comparisons. [a,b,c] Different letters indicate significant differences by *Sídák's post hoc* test for multiple comparisons.

On the other hand, fasting or exercise stimulate the lipolysis pathway in WAT, which is monitored by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), and releases glycerol and FAs to the bloodstream so that it can be used by other tissues.[6] Importantly, adipocyte formation is mainly driven by peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα), which are considered the master regulators of adipogenesis.[7] Moreover, WAT also shows a relevant endocrine role, represented by its secretion of endocrine factors (mostly adipokines) that strongly modulate important metabolic processes of the whole organism, such as the feeding behavior, glucose homeostasis, or inflammation.[8] The most studied adipokines are leptin and adiponectin, which are positively and negatively correlated with the WAT reservoir, respectively. Thus, leptin levels are overexpressed under obesity, whereas adiponectin shows anti-inflammatory properties, and its levels are associated to a healthy metabolic profile.[9,10] Importantly, an obesogenic environment provokes an exacerbated WAT expansion that can alter its functionality and lead to metabolic disruption.[11] Moreover, the dysfunctional WAT secretes chemoattractant molecules that increase macrophage infiltration and generate a low-grade proinflammatory state.[12]

The main function of BAT is the non-shivering thermogenesis, which consists of using glucose and FAs to produce heat, through the action of uncoupling protein 1 (UCP1).[13] BAT is particularly important in rodents, and its activity is regulated by several factors and is associated to glucose and lipid homeostasis.[14] BAT also shows an endocrine function, through secreting batokines that regulate metabolic functions such as glucose utilization, browning or food intake.[15] Similar to WAT, its functionality is altered by obesity, but the contribution of BAT to inflammation is much lower than WAT.[16]

Importantly, both WAT and BAT are seasonally regulated, and their functionality is modified along the year, depending on the environmental conditions.[17–19] In this context, (poly)phenols and, specifically, proanthocyanidins, the most structurally complex subclass of flavonoids, have been shown to modulate metabolism and provide global protection to the organism, preventing from multiple metabolic alterations caused by oxidative stress and inflammation.[20,21] However, the effects of (poly)phenols in the organism are, in turn, photoperiod-dependent, as the metabolic impact of their consumption changes depending on the season of the year.[22] In fact, grape-seed proanthocyanidin extract (GSPE) was recently reported to modulate metabolic parameters in liver in a photoperiod-dependent manner, suggesting that under long photoperiod (18:6 light:dark hours), GSPE consumption could induce glycolysis.[23] Moreover, the effects of GSPE supplementation on gut microbiota in obese rats were recently found to be significantly dependent on the photoperiod in which GSPE was consumed, being the long photoperiod (18:6 light:dark hours) the most receptive to proanthocyanidins.[24] Considering this background, we can speculate that GSPE supplementation could affect the metabolic adaptations of WAT and BAT to seasonal changes. Thus, the main aim of this study was to determine the impact of GSPE consumption on the expression of the most important metabolic markers of WAT (subcutaneous and visceral depots) and BAT in healthy Fischer 344 rats exposed to different photoperiodic conditions.

2. Results

2.1. BAT Weight Increased in L6 and GSPE Consumption Tended to Reduce it Only in L18

After 9 weeks of study, no significant differences in body weight and food intake were observed between experimental groups (**Table 1**), and only a significant photoperiod effect on total fat mass ($p = 0.007$) was detected, which was especially increased in animals subjected to L18 compared to L12 animals ($p = 0.047$). Particularly, epididymal WAT (eWAT) weight significantly increased in animals subjected to L18 photoperiod compared to the L12 group ($p = 0.024$). In addition, a significant increase in BAT mass was observed in L6 compared to L12, and even more when it was compared to L18, suggesting that short photoperiods might modulate BAT activity and energy expenditure in these animals. However, GSPE consumption did not exert any effects on these biometric variables, and only a trend to reduce BAT mass was detected when GSPE was consumed at L18 photoperiod (students' *t* test $p = 0.073$).

2.2. GSPE Modulated Corticosterone Concentrations in a Photoperiod-Dependent Manner

Serum concentrations of glucose were increased under L6 photoperiod compared to both L12 and L18 animals, while

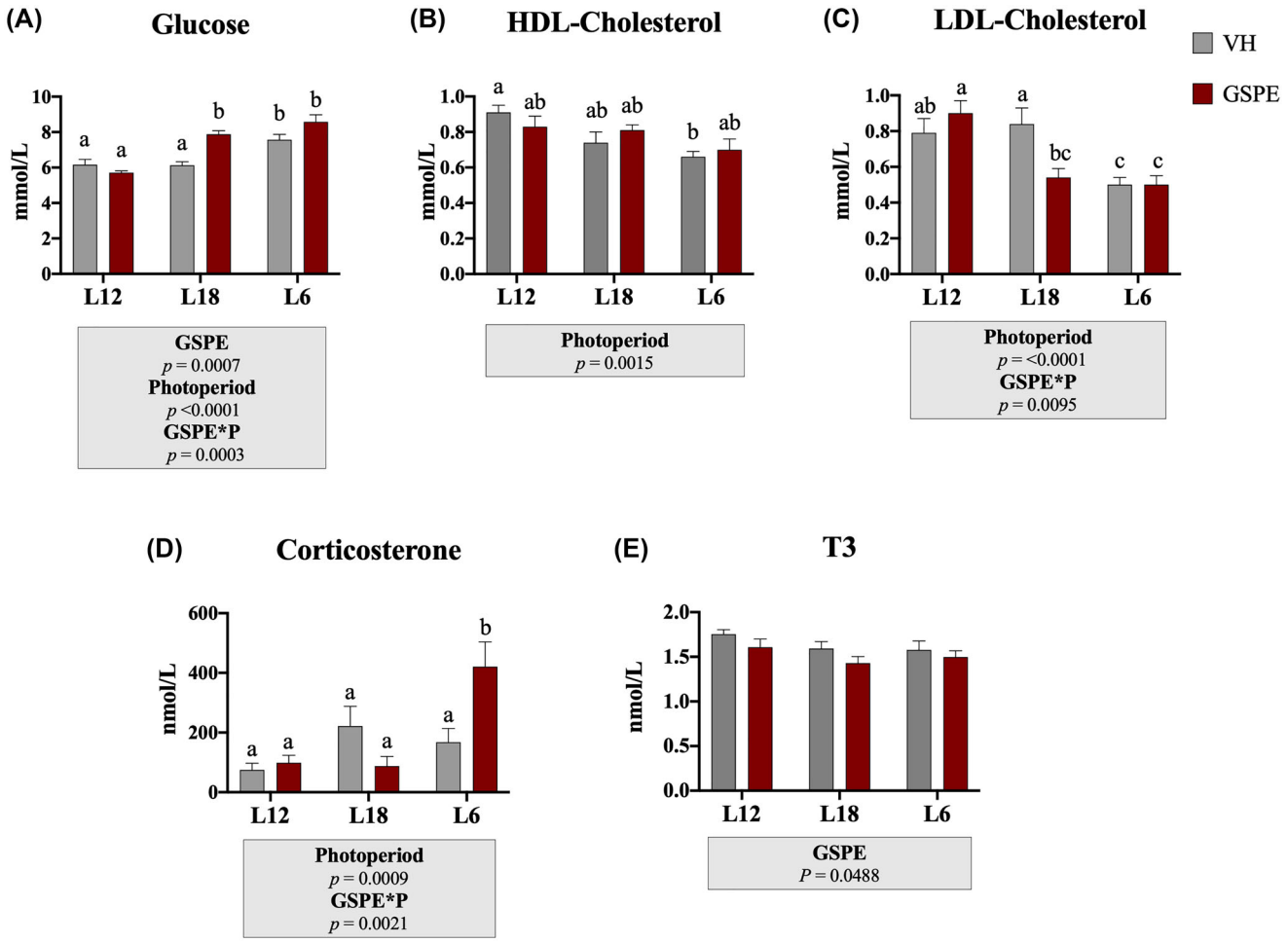

Figure 1. Impact of GSPE supplementation under different photoperiodic conditions on serum concentrations of glucose, LDL-cholesterol, HDL-cholesterol, corticosterone, and T3. Circulating glucose (A), LDL-cholesterol (B), HDL-cholesterol (C), corticosterone (D), and T3 (E) in the serum of Fischer 344 rats treated with VH or GSPE exposed to standard (12 h light:12 h dark), long (18 h light:6 h dark), or short (6 h light:18 h dark) photoperiods fed with STD diet ($n = 8$). The results are presented as the mean ± SEM. Two-way ANOVA followed by Šídák's *post hoc* test ($p < 0.05$) was performed to compare the values between groups. GSPE, GSPE effect; GSPE * P, interaction effect between GSPE and photoperiod; Photoperiod, photoperiod effect; different letters indicate significant differences.

LDL-cholesterol and HDL-cholesterol were reduced in L6 compared to L12 animals (**Figure 1**A–C). In addition, GSPE supplementation significantly increased glucose values and decreased LDL-cholesterol levels only in L18 groups (Figure 1A,C). Corticosterone levels were also affected by photoperiod, being increased in L18 photoperiod compared to L12 and L6 groups. Interestingly, the consumption of GSPE produced a photoperiod-dependent effect on serum concentrations of corticosterone ($p = 0.0021$), which was reflected by an interaction effect between GSPE and photoperiod (Figure 1D). Hence, GSPE consumption significantly enhanced serum corticosterone values in L6 ($p = 0.003$), while in L18 GSPE tended to decrease them (student's *t*-test $p = 0.088$). In addition, GSPE significantly reduced circulating concentrations of T3 ($p = 0.048$) regardless of the photoperiod in which animals were exposed (Figure 1E). No effects on circulating triglycerides, HDL-cholesterol, melatonin, T4 or testosterone were observed in response to GSPE consumption (Figure 1B and Table S3, Supporting Information).

2.3. GSPE Significantly Altered Serum Metabolite Concentrations in a Photoperiod-Dependent Manner

To deepen in the description of the photoperiod-dependent effects of GSPE on the metabolic markers of adipose tissue, concentrations of serum metabolites involved in its functionality were explored by mass spectrometry (**Figure 2** and Table S4, Supporting Information). As shown in Figure 2A–D, an important photoperiod effect was detected in the serum levels of glycerol, glyceric acid, glycolic acid, and 3-hydroxybutiric acid/3-hydroxyisobutyric acid (3-HBA/3-HBIA). Particularly, animals exposed to L18 presented significantly higher levels of glycerol compared to L12 and L6 animals, and lower serum concentrations of glyceric acid compared to L12 animals. In addition, animals exposed to L6 showed higher levels of 3-HBA/3-HBIA compared to L12 animals. However, the impact of GSPE was mostly detected in L6 photoperiod. In fact, the serum concentrations of glycerol, 3-HBA/3-HBIA, glutamine,

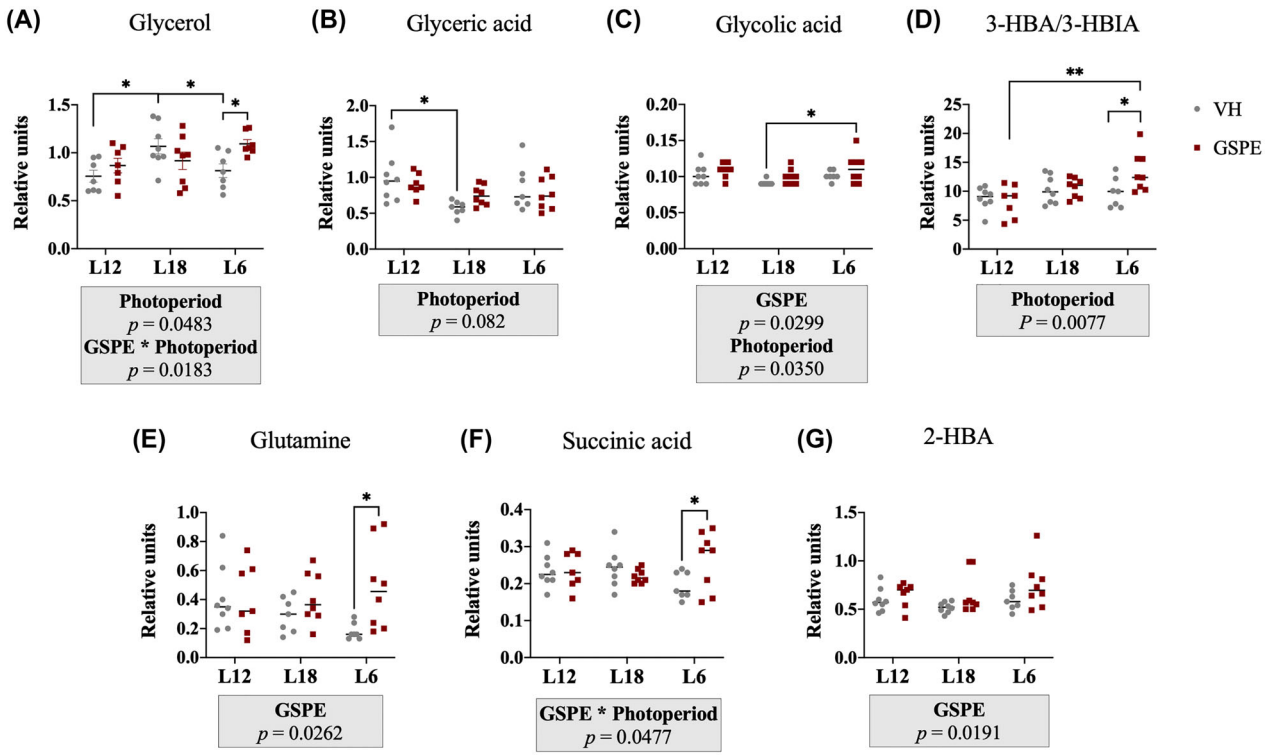

Figure 2. Impact of GSPE supplementation under different photoperiodic conditions on serum concentrations of glycerol, glyceric acid, glycolic acid, 3-HBA/HBIA, glutamine, succinic acid, and 2-HBA. Circulating glycerol (A), glyceric acid (B), glycolic acid (C), 3-HBA/HBIA (D), glutamine (E), succinic acid (F), and 2-HBA (G) in the serum of Fischer 344 rats treated with VH or GSPE exposed to standard (12 h light:12 h dark), long (18 h light:6 h dark), or short (6 h light:18 h dark) photoperiods fed with STD diet ($n = 8$). The results are presented as the mean ± SEM. Two-way ANOVA followed by Šídák's *post hoc* test ($p < 0.05$) was performed to compare the values between groups. GSPE, GSPE effect; **GSPE * P**: interaction effect between GSPE and photoperiod; **Photoperiod**: photoperiod effect. * indicates significant differences; # denotes tendency ($p < 0.1$).

and succinic acid were increased in response to GSPE only when it was consumed under L6 (Figure 2A,D–F). Moreover, increased serum concentrations of 2-hydroxybutyric acid (2-HBA) were also observed in response to GSPE consumption (Figure 2G). Finally, serum concentrations of branched-chain amino acids (BCAAs) were also affected by photoperiod (Table S4, Supporting Information). Particularly, serum leucine and isoleucine concentrations were notably reduced in animals exposed to both L18 and L6 photoperiods compared to L12 animals, but no significant effects were observed in response to GSPE consumption.

2.4. GSPE Upregulated *Hsl* and *Adipoq* Gene Expression Only When it was Consumed Under L6

We next investigated the gene expression profile of several key regulators of metabolism in iWAT. No significant changes induced neither by photoperiod nor by GSPE were observed in the expression of *Pparγ*, but a downregulation of the gene expression of *Cebpα* was detected in L6 (**Figure 3**A) while GSPE consumption reverted this effect (Šídák *post hoc* test $p = 0.0983$; student's *t*-test $p = 0.025$).

Furthermore, GSPE consumption upregulated the gene expression profile of Hsl (Figure 3B) in all animals, with the effect being most evident in animals exposed to L6 (student's *t*-test $p = 0.016$). In addition, mRNA levels of the lipogenic gene *Fasn* were also downregulated in response to GSPE consumption in this tissue, especially under L18 and L6 photoperiods (Figure 3C). These findings suggested that, in response to GSPE consumption, short photoperiods could stimulate lipolysis and reduce anabolic pathways in iWAT.

Finally, leptin (*Lep*) mRNA levels showed a significant effect of photoperiod, being upregulated in animals exposed to L18 photoperiod, but no significant effects were observed in response to GSPE consumption (Figure 3D). However, adiponectin (*Adipoq*) mRNA was significantly upregulated by GSPE consumption with the effects being most evident in animals exposed to L6 (student's *t*-test $p = 0.02$). No effects were detected in the expression of browning-related genes in this tissue (Figure 3E).

2.5. L6 Photoperiod Repressed GSPE-Induced Upregulation of *Cebpα* and *Acacα*

Contrary to the results observed in iWAT, a significant photoperiod effect on the expression profile of genes involved in lipid metabolism was observed in eWAT, indicating that this tissue may be more susceptible to seasonal changes. Specifically, the mRNA levels of *Pparγ* and *Hsl* were significantly upregulated in animals exposed to L6 photoperiod (**Figure 4**A,B). On the other hand, *C/ebpα* showed the opposite pattern, being significantly downregulated in L6. Interestingly, an interaction effect

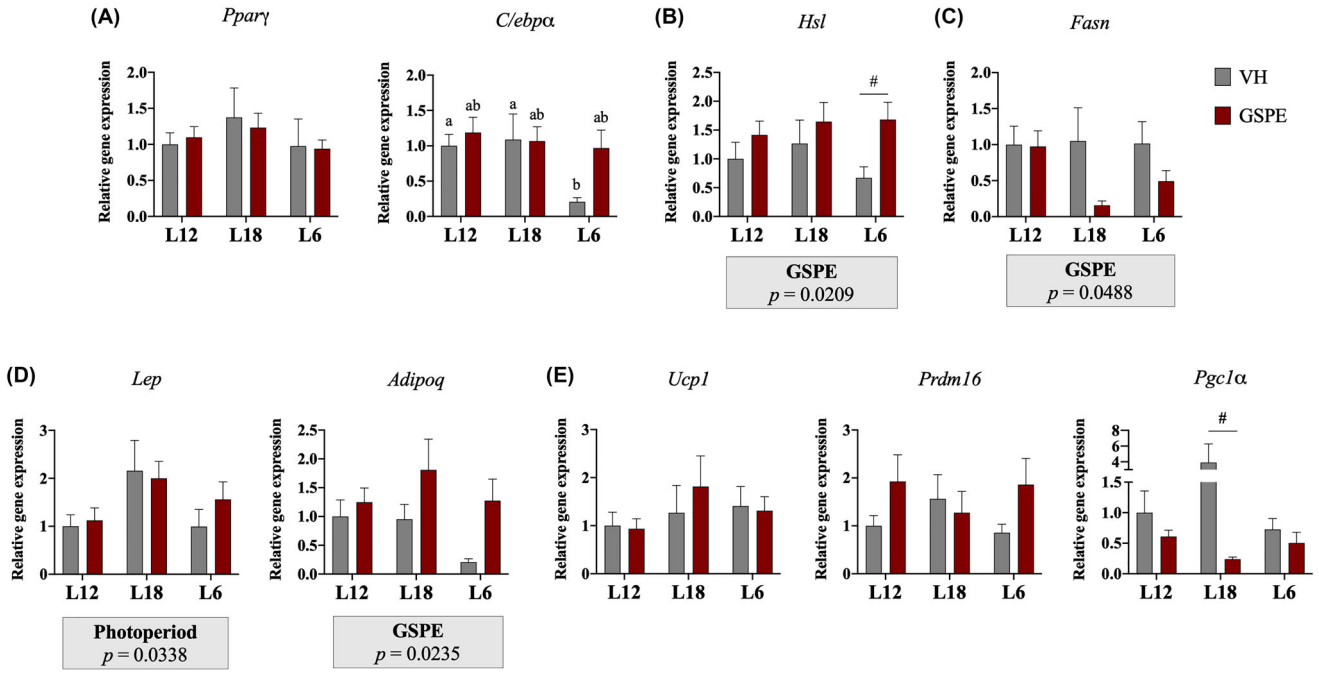

Figure 3. Effect of GSPE supplementation under different photoperiodic conditions on the gene expression of key regulators of the main metabolic pathways in iWAT. Gene expression of *Pparγ* and *C/ebpα* (A), *Hsl* (B) and *Fasn* (C), *Lep* and *Adipoq* (D) and browning markers, *Ucp1*, *Prdm16*, and *Pgc1α* (E) was evaluated by quantitative PCR on animals fed standard chow diet + VH or GSPE for 4-week-period and exposed to standard (L12), long day (L18) or short day (L6,) photoperiods. Values are referred to the L12-VH group. Data represent mean ± SEM (n = 5–6). Two-way ANOVA followed by Šídák's *post hoc* test ($p < 0.05$) was performed to compare the values between groups. **GSPE**, GSPE effect; **Photoperiod**, photoperiod effect. Different letters denote significant differences; # denotes tendency ($p < 0.1$).

was detected on the mRNA levels of *C/ebpα*, where GSPE supplementation upregulated its expression specifically in L12 and L18 groups, but the exposure to a short photoperiod prevented this increase. Similar results were detected in the mRNA levels of *Acacα*, suggesting that L6 photoperiod might repress the upregulation of both adipogenic and lipogenic genes induced by GSPE in longer photoperiods. In addition, the mRNA levels of *Cd36* were significantly downregulated in L6 as well as in response to GSPE consumption (Figure 4C). Moreover, similar to iWAT, a photoperiod-independent upregulation in the gene expression of *Atgl* was detected in response to GSPE consumption in this tissue (Figure 4B).

Finally, mRNA levels of *Adipoq* were again affected by GSPE consumption, particularly in L12 and L18 photoperiods (student's *t*-test: $p = 0.027$ in L12 and $p = 0.041$ in L18), whereas leptin mRNA levels were not affected neither by the photoperiod nor by GSPE consumption in this tissue. (Figure 4D).

2.6. GSPE Downregulated *Tnfα* and *Il6* Gene Expression in a Photoperiod-Dependent Manner

Visceral adipose tissue is the adipose depot most prone to induce systemic metabolic disturbances in case it becomes proinflammatory. Therefore, the gene expression profile of *tumor necrosis factor alpha* (*Tnfα*) and *interleukine 6* (*Il6*) was assessed in eWAT. Remarkably, GSPE consumption significantly downregulated the gene expression of *Tnfα* and *Il6* only when GSPE was consumed under L6 and L18, respectively, compared to L12 photoperiod (**Figure 5**A,B).

2.7. GSPE Upregulated *Pparα* Gene Expression in BAT only in Animals Exposed to L18

Finally, to assess potential changes in the metabolic markers of BAT in response to GSPE consumption under different photoperiods, the expression profile of different genes involved in thermogenesis, adipogenesis, and lipid transport was also investigated in this tissue. Although no effects on the mRNA levels of *Ucp1* were detected, a significant photoperiod effect was observed for most of the genes involved in BAT activation. Particularly, the mRNA levels of *deiodinase 2* (*Dio2*), a direct regulator of Ucp1, were upregulated in L18 groups. In turn, an upregulation of *carnitine palmitoyltransferase 1 beta* (*Cpt1β*) gene expression, which is involved in mitochondrial oxidation, was observed in L6 groups (**Figure 6**A).

In addition, the mRNA levels of brown adipogenesis-related genes were also affected in this study (Figure 6B). Interestingly, an interaction effect between photoperiod and GSPE was observed on *Pparα* gene expression, being upregulated only when GSPE was consumed at L18 (student's *t*-test: $p = 0.011$). On the other hand, *Pparγ* gene expression was not affected by GSPE consumption, although its values were significantly upregulated in response to L18 and L6 exposure. Nevertheless, the expression of *Prdm16* was not affected neither by photoperiod nor by GSPE

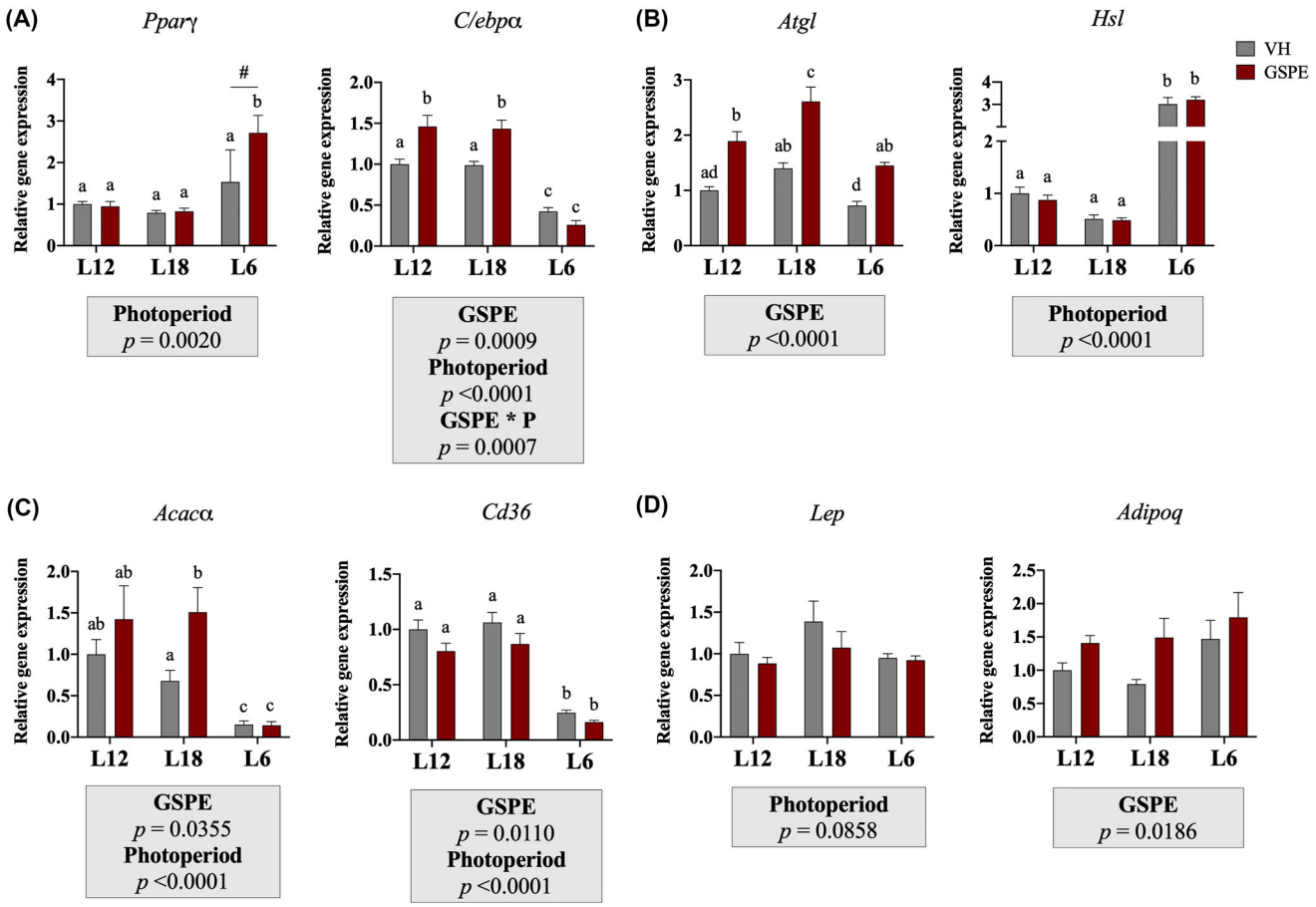

Figure 4. Effect of GSPE supplementation under different photoperiodic conditions on the gene expression of key regulators of the main metabolic pathways in eWAT. Gene expression of *Pparγ* and *C/ebpα* (A), *Atgl* and *Hsl* (B), *Acacα* and *Cd36* (C), *Lep* and *Adipoq* (D) was evaluated by quantitative PCR on animals fed standard chow diet + VH or GSPE for 4-week-period and exposed to standard (L12), long day (L18), or short day (L6,) photoperiods. Values are referred to the L12-VH group. Data represent mean ± SEM (n = 5-6). Two-way ANOVA followed by Šídák's *post hoc* test ($p < 0.05$) was performed to compare the values between groups. **GSPE**, GSPE effect; **GSPE * P**, interaction between GSPE and photoperiod; **Photoperiod,** photoperiod effect. Different letters denote significant changes, # denotes tendency ($p < 0.1$).

consumption. Finally, GSPE consumption induced an increase in the mRNA levels of *Cd36* only in L6 animals, which enables the entrance of fatty acids into the brown adipocytes.

3. Discussion

Seasonal rhythms influence diverse features of mammals' metabolism to adapt to the environmental conditions and favor their survival.[25] Particularly, photoperiod is a crucial cue that informs about the circannual clock, and photoperiod-sensitive mammals respond to this cue and develop the necessary metabolic adaptations to face the upcoming conditions.[1] WAT and BAT are key metabolic tissues that show molecular adaptations to photoperiod, involving changes on their morphology and gene expression.[3] In this context, (poly)phenol consumption can help organisms adapt to the upcoming environmental conditions,[26] also affecting the adipose tissue,[27] which can be beneficial for their global metabolism. In our study, we focused on the effects of GSPE supplementation under different photoperiod conditions on the expression of different metabolic and endocrine markers of WAT (considering subcutaneous and visceral depots) and BAT. GSPE is a proanthocyanidin-rich extract obtained from grape seeds, which has been widely used in research given its beneficial effects on metabolic-related alterations, such as glucose and lipid metabolism.[28] Therefore, we hypothesized that GSPE consumption would affect the gene expression profile of WAT and BAT in a different way depending on the photoperiod under which it was consumed.

After 9 weeks of study, we observed an increase in the total fat mass of animals subjected to L18, which agrees with previous studies reporting increased adiposity in longer photoperiods.[29] However, despite short photoperiods often drive to reduced body weight, food intake, and adiposity,[3] in our study we did not observe these photoperiodic changes. This could be explained because of the sub strain of Fischer 344 rats used in our study was F344/NCrHsd, which typically shows a reduced response to photoperiod compared to the sub strain F344/NHsd.[30] Nevertheless, BAT mass and circulating glucose concentrations were notably increased in animals exposed to L6, as previously reported,[31] and GSPE consumption tended to reduce BAT mass only in L18 animals. Moreover, in the present study, LDL-cholesterol levels significantly dropped in L6. LDL is one

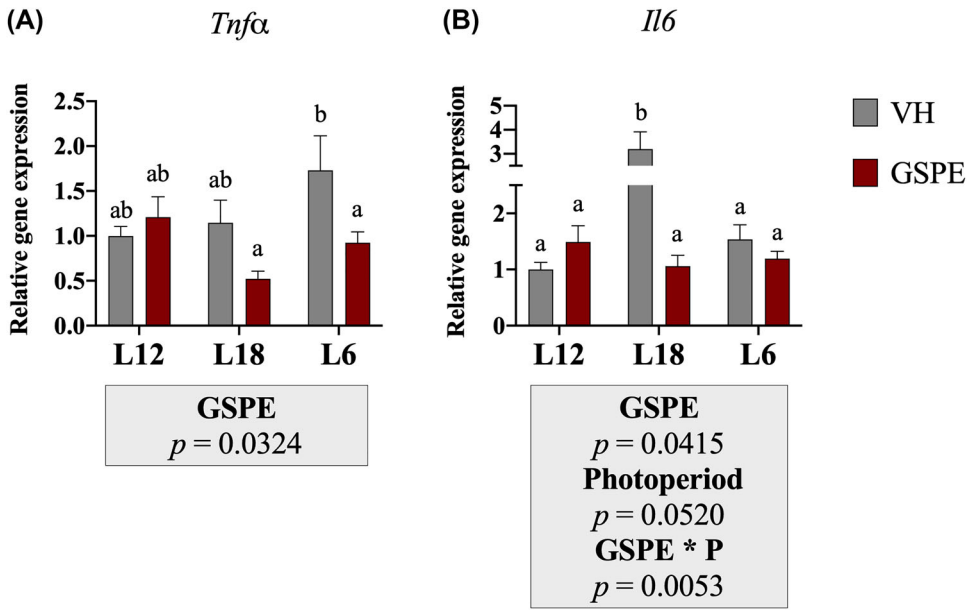

Figure 5. Effect of GSPE supplementation under different photoperiodic conditions on the gene expression of pro-inflammatory cytokines in eWAT. Gene expression of *Tnfα* (A) and *Il6* (B) was evaluated by quantitative PCR on animals fed standard chow diet + VH or GSPE for 4-week-period and exposed to standard (L12), long day (L18), or short day (L6,) photoperiods. Values are referred to the L12-VH group. Data represent mean ± SEM (n = 5–6). Two-way ANOVA followed by Šídák's *post hoc* test ($p < 0.05$) was performed to compare the values between groups. **GSPE**, GSPE effect; **GSPE * P,** interaction between GSPE and photoperiod; **Photoperiod,** photoperiod effect. Different letters denote significant changes.

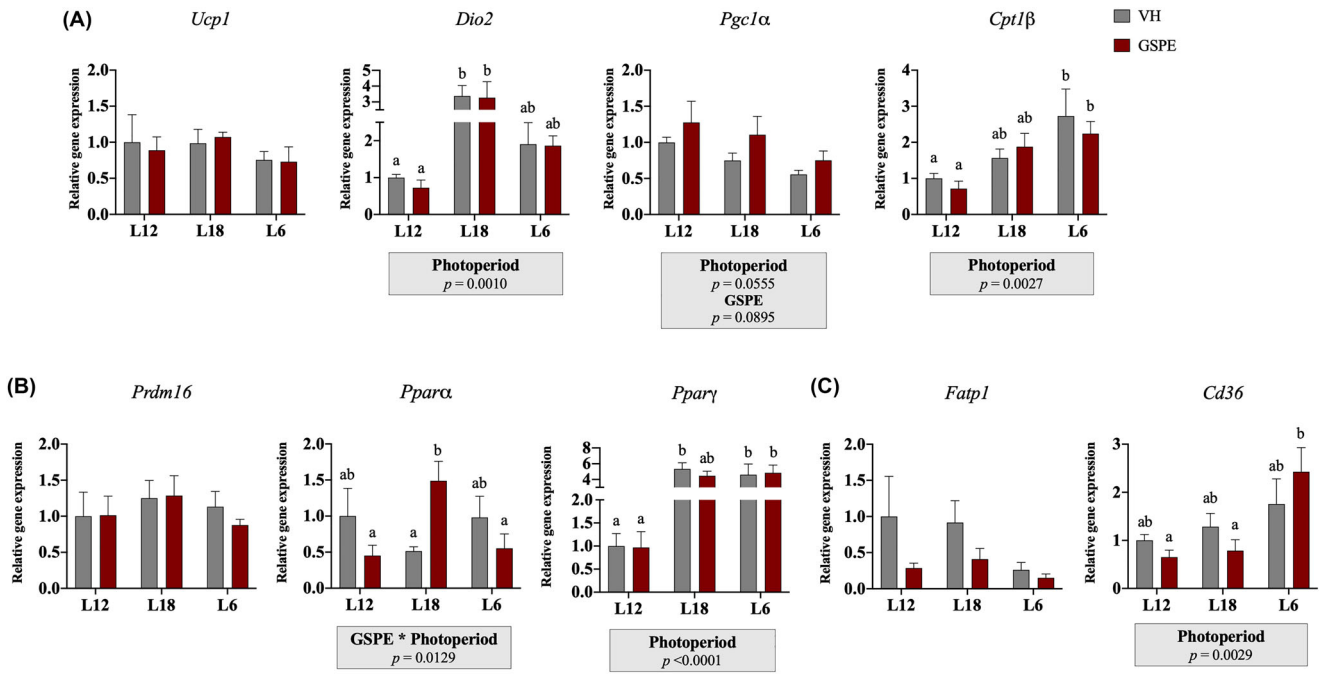

Figure 6. Effect of GSPE supplementation under different photoperiodic conditions on the gene expression of key regulators of the main metabolic pathways in BAT. Gene expression of thermogenic genes *Ucp1*, *Dio2*, *Pgc1α*, and *Cpt1β* (A), adipogenic genes *Prdm16*, *Pparα*, and *Pparγ* (B), and lipid transporters *Fatp1* and *Cd36* (C) was evaluated by quantitative PCR on animals fed standard chow diet + VH or GSPE for 4-week-period and exposed to standard (L12), long day (L18) or short day (L6,) photoperiods. Values are referred to the L12-VH group. Data represent mean ± SEM (n = 5–6). Two-way ANOVA followed by Šídák's *post hoc* test ($p < 0.05$) was performed to compare the values between groups. **GSPE,** GSPE effect; **GSPE * P**, interaction between GSPE and photoperiod; **Photoperiod,** photoperiod effect. Different letters denote significant changes.

of the main cholesterol transporters in mammals, and high LDL-cholesterol levels are closely related to unhealthy habits and lifestyle, therefore it is often assessed as a risk factor for atherosclerosis and cardiovascular disease.[32] In response to GSPE consumption, LDL-cholesterol levels were significantly decreased in animals exposed to L18, suggesting that GSPE could reduce LDL-cholesterol levels, to similar values as those detected in short photoperiod, when it was consumed at L18.

To study the photoperiodic effects of GSPE on adiposity, we focused on the gene expression profile of different models of subcutaneous and visceral WAT depots (iWAT and eWAT, respectively) and also on BAT. Interestingly, although BAT mass was reduced in L18 animals after GSPE consumption, the gene expression of an important marker of brown adipogenesis, *Pparα*, was strongly increased in these animals compared to the animals exposed to L18, which could indicate that despite the smaller size of this tissue, it might be intending to be more active. However, PPAR*α* is directly related to the expression of PGC1*α* and PRDM16, which are responsible for upregulating the expression of thermogenic-related molecules such as CPT1*β* and UCP1,[33] and we could not detect consistent changes in the gene expression of these molecules. Therefore, it could be possible that the functionality of *Pparα* was not fully induced posttranscriptionally, or that its ligand RXR-*α* was not accessible.[34,35] On the other hand, we could not observe a significant impact of GSPE supplementation in the BAT of animals exposed to the L6 photoperiod. Nevertheless, consistently with the increased weight of this tissue, *Pparγ* gene expression was increased under L6 photoperiod when compared to L12 animals. Besides that, the lipid transporter *Cd36* and the fatty acid oxidation-related gene *Cpt1β* were upregulated in animals exposed to the short-day schedule, suggesting higher fatty acid mobilization and mitochondrial activity rates only under this photoperiod.[36] These results are in agreement with a previous study that reported increased energy expenditure in animals exposed to short photoperiods,[37] supporting the involvement of BAT in the photoperiodic modulation of energy metabolism.

Interestingly, GSPE did affect gene expression in WAT under L6. Hence, after analyzing the results in iWAT, we detected that GSPE upregulated *Hsl* mRNA levels, especially in animals subjected to L6 photoperiod, suggesting high lipolytic activity in this tissue. Similar findings were obtained in eWAT, where *Atgl* mRNA levels were also upregulated in response to GSPE. This potential activation of lipolysis in WAT was directly associated with serum concentrations of glycerol, that were significantly increased only when GSPE was consumed under L6, indicating that higher levels of glycerol might be released by WAT in response to GSPE consumption when consumed under the short photoperiod. Although we could not fully confirm these findings, it is assumed that WAT is the main responsible for regulating glycerol circulating levels, which are notably released after adipocyte lipolysis, and converted into glucose by liver and kidney.[38] Indeed, it was previously reported that the 80–85% of circulating glycerol in humans during fasting was released from the adipose tissue, and an in vivo study showed that depletion of aquaporin 7 (AQP7), which is an adipose-specific membrane protein that controls glycerol release, resulted in significantly reduced glycerol levels in blood, indicating that the WAT was the most important tissue responsible for the regulation of serum glycerol levels.[39,40] Moreover, evidence exists regarding the stimulating effects of corticosterone in gene expression of lipolysis-related enzymes in the adipose tissue.[41,42] Considering that in our study serum corticosterone levels were four times higher in L6 animals compared to their counterparts, it would be possible that this hormone influences the WAT functionality in response to GSPE consumption. Therefore, our results suggested that, in a photoperiod-dependent manner, GSPE can induce lipolysis in the adipose tissue by increasing circulating levels of corticosterone, but further studies are needed to confirm this hypothesis.

Noteworthy, a strong photoperiod effect was also detected in eWAT. Paradoxically, eWAT mass was significantly increased in L18 and in L6 groups (compared to L12 and statistically significant only in L18), but the expression of *C/ebpα*, *Acacα* and *Cd36*, genes importantly involved in adipogenesis and lipid storage, was strongly downregulated only under L6. Moreover, the short photoperiod effect also prevented the GSPE-induced rise in *C/ebpα* and *Acacα* observed in L12 and L18 animals. Moreover, the short photoperiod effect also prevented the GSPE-induced rise in *C/ebpα* and *Acacα* observed in L12 and L18 animals. Otherwise, the master regulator of adipogenesis *Pparγ*, was upregulated in L6, which could partly explain the unexpected increase in eWAT mass under the short photoperiod. Considering the overexpression of *Hsl* under L6, we can speculate that eWAT was more prone to fat oxidation under L6 than under L18, trying to compensate the increased tissue weight. The photoperiodic adaptation of WAT was assessed previously, but only the retroperitoneal WAT depot was analyzed,[18] and it was reported that the expression of most of the metabolic genes was downregulated under the short photoperiod. Therefore, the localization of the adipose depot might be crucial in WAT photoperiodic adaptation. Contrarily, we did not observe significant changes in the gene expression of eWAT in L18 animals compared to L12 animals, reinforcing the natural impact of the long photoperiod in accumulating fat mass.

Another remarkable finding was that *adiponectin* mRNA levels were also increased in response to GSPE consumption in both iWAT and eWAT. Previous studies reported increased serum levels of adiponectin in obese rodents after GSPE supplementation,[43,44] and similar results were also observed in vitro, after inflammatory stimulus.[45] These studies used much higher doses of GSPE and, additionally, no data were found correlating GSPE administration and adiponectin levels in healthy conditions. Therefore, in this study we demonstrated that *Adipoq* mRNA levels in both subcutaneous and visceral depots of the WAT were upregulated in response to chronic administration of low doses of GSPE regardless of the photoperiod under which GSPE was consumed.

Adiponectin is an adipokine that has an important anti-inflammatory role. Its circulating levels are highly correlated with decreased levels of systemic inflammation and reduced risk for several diseases such as obesity, atherosclerosis, or diabetes.[46] Interestingly, our results also reflected reduced inflammatory levels in the eWAT in response to GSPE consumption, reinforcing the anti-inflammatory role of adiponectin in this tissue. Moreover, serum glutamine concentrations were also significantly increased in response to GSPE only when it was consumed at L6 photoperiod, and previous studies showed an inverse correlation in glutamine levels and proinflammatory status in the adipose tissue.[47] In our study, animals consuming GSPE under L6

showed the most significant effect in reducing *Tnfα* mRNA levels. Inflammation in the visceral adipose tissue is particularly important, as this adipose depot is the most involved in metabolic alterations under inflammatory conditions.[48] The antioxidant and anti-inflammatory properties of GSPE have been shown under inflammatory circumstances, but our results suggested that GSPE could also prevent the proinflammatory response in the visceral adipose tissue in healthy conditions, probably by increasing the serum concentrations of both adiponectin and glutamine.

The impact of photoperiod in inflammatory-related genes was also observed mainly in Il6 mRNA levels, suggesting that the exposure to standard and short photoperiods, but not to a long photoperiod, could be protective against visceral WAT inflammation. A recent study using squirrels indicated a strong correlation between the short photoperiod-induced increased levels of melatonin and the reduced levels of inflammatory markers such as IL-6 and TNFα.[49] In our study, we could not detect significant differences in melatonin levels, which could be explained by the time point at which animals were sacrificed (9 a.m.). In fact, in the aforementioned study, significant differences in melatonin levels were mainly detected during the dark period. However, melatonin supplementation was positively associated to reduced inflammation in WAT,[50] and another study indicated that IL-6 levels in WAT were reduced at night.[51] Considering that our results revealed that *Il6* expression was increased in the eWAT of L18 animals during the day, it could be possible that the reduction in *Il6* levels induced by standard and short photoperiods were not fully dependent on melatonin, but other mechanisms were involved in the photoperiodic modulation of the inflammatory response in the eWAT.

Overall, the exploratory and preliminary nature of this study must be considered, and future research should focus on the mechanisms that confirm the results presented, in order to draw firm conclusions about the photoperiod-dependent effects of GSPE on WAT and BAT.

4. Concluding Remarks

Under the short photoperiod, genes related to adipogenesis, fatty acid transport, and oxidation were upregulated in BAT, but no effects were detected after GSPE administration. However, GSPE increased the gene expression of *Pparα* only in animals under the long photoperiod, which in turn induced reduced BAT mass.

Under the short photoperiod, GSPE strongly enhanced lipolytic gene expression in WAT, which was accompanied by increased serum concentrations of corticosterone and glycerol.

GSPE also protected against proinflammatory markers in WAT and elevated the serum concentrations of glutamine under the short photoperiod.

Finally, GSPE strongly upregulated *adiponectin* mRNA levels under all photoperiods, suggesting a healthier metabolic profile in the WAT of all animals.

5. Experimental Section

5.0.0.1. Grape-Seed Proanthocyanidin Extract: GSPE was kindly provided by Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE composition used in this study contained monomers (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%), and oligomers (5–13 units; 31.7%) of proanthocyanidins.

5.0.0.2. Characterization of the Phenolic Profile of GSPE: The exact phenolic composition of GSPE was determined by HPLC-MS/MS (TOF 6210, Agilent),[52] according to what was described by Quiñones et al.,[53] and can be found in Table S1, Supporting Information. Briefly, the flow rate and injection volume were 0.4 mL min^{-1} and 5 µL, respectively. Ionization in the mass spectrometer was performed by electrospray (ESI) in the negative mode, and the source parameters were as follows: capillary voltage, 3.5 kV; fragmentor, 120 V; source temperature, 150 °C; desolvation gas temperature, 350 °C, with a drying gas flow rate of 12 L min^{-1}. Nitrogen was used as the cone gas. Individual phenols were quantified with a five-point regression curve by using standard compounds obtained from commercial suppliers and analysis with Agilent Mass Hunter Qualitative Analysis software for extracted ion chromatogram (EIC) of each individual phenolic compound.

5.0.0.3. Experimental Design: Forty-eight (n = 48) male Fischer 344 rats, 12-weeks of age, were purchased from Charles River Laboratories (Barcelona, Spain). The animals were pair-housed and randomly distributed in three different rooms according to photoperiod (n = 16, each). They were fed a standard chow diet (Panlab A04, Barcelona, Spain) and had access to tap water ad libitum. Photoperiod groups consisted of a L6 (6:18 h light:dark cycle), L12 (12:12 h light:dark cycle), and L18 (18:6 h light:dark cycle), emulating winter, fall/spring, and summer, respectively. Temperature was kept at 22 °C, and animals were housed in animal quarters. After an adaptation period of 5 weeks, animals in each photoperiod were randomly distributed into two subgroups (n = 8, each): vehicle (VH), and GSPE administration. In all cases, lights were turned on at 9.00 a.m. and treatments were orally administered between 9.00 and 10.00 a.m. for a period of 4 weeks. Body weight and food intake were measured weekly. All animals were sacrificed by decapitation at the start of the light cycle (09:00 am), after 3 h fasting. Serum was obtained after blood clotting and centrifugation (15 000 × g, 10 min, 4 °C), and stored at −80 °C for further analysis. WAT and BAT were rapidly collected weighted and frozen immediately in liquid nitrogen and stored at −80 °C for further analysis.

The Animal Ethics Committee of the Universitat Rovira i Virgili and Generalitat de Catalunya approved all the procedures (reference number 9495), and they were carried out in accordance with relevant guidelines and regulations.

5.0.0.4. Dosage Regimen: GSPE was administrated for 4 weeks at a dose of 25 mg kg^{-1} day, diluted in the VH solution, which consisted in condensed milk diluted in water (1:5). GSPE and VH were administrated orally using a syringe, 1 h after lights were turned on. The dose of GSPE was chosen based on previous studies, in which the same doses showed significant effects on metabolism.[54] The human equivalent dose (HED) was calculated based on body surface area,[55] and corresponded to 4.73 mg kg^{-1} day^{-1} of extract for a 70 kg adult. This amount of (poly)phenols can be rapidly achieved with a healthy diet including fruits, tea, or nuts. In fact, it was estimated that the average amount of (poly)phenols consumed with fruit in Spain corresponded to 360 ± 127 mg day^{-1}.[56]

5.0.0.5. Serum Analysis: Circulating levels of glucose, total cholesterol, and HDL and LDL cholesterol and triglycerides were analyzed by colorimetric enzymatic assay kits (QCA, Barcelona, Spain) according to the manufacturer's instructions.

5.0.0.6. Hormone Analysis: Determination of circulating melatonin, corticosterone, triiodothyronine (T3), and thyroxine (T4) was achieved using liquid chromatography coupled to a triple quadrupole mass spectrometry (LC-QqQ). Serum samples were thawed at 4 °C. Then, 50 µL of serum were mixed with 250 µL of methanol containing the internal standard (2 ng mL^{-1}). The mixture was vortexed and centrifuged for 5 min at 4 °C and 27 000 × g. The supernatant was transferred to a new tube and mixed with 700 µL of 0.1% formic acid in water. The sample was loaded to an SPE tube, previously conditioned with methanol and 0.1% formic acid in water. The cartridge was washed with 0.1% formic acid in water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45 °C, reconstituted with 50 µL of water:methanol (60:40, v/v) and transferred to a glass vial for

analysis. The analytical column was a Zorbax Eclipse C18 (150 × 2.1 mm) from Agilent Technologies.

5.0.0.7. Metabolomic Analysis: Metabolomic analysis in serum samples was performed using gas chromatography coupled to a quadrupole time-of-flight mass spectrometry (GC-qTOF). The extraction was performed by adding 400 µL of methanol:water (8:2) containing internal STD mixture to serum samples (approx. 100 µL). Then, samples were mixed, incubated at 4 °C for 10 min, centrifuged at 27 000 × *g* 4 °C for 10 min, and the supernatant was evaporated to dryness before compound derivatization (methoximation and silylation). The derivatized compounds were analyzed by GC-qTOF (model 7200, Agilent, USA). The chromatographic separation was based on Fiehn Method[57] using a J&W Scientific HP5-MS (30 m × 0.25 mm i.d., 0.25 µm film capillary column) and helium as carrier gas using an oven program from 60 to 325 °C. Ionization was done by electronic impact, with electron energy of 70 eV and operating in full scan mode. Identification of metabolites was performed using commercial standards and by matching their electronic impact mass spectrum and retention time to metabolomics Fiehn library (from Agilent) which contains more than 1400 metabolites. After putative identification of metabolites, these were semi-quantified in terms of internal standard response ratio.

5.0.0.8. Gene Expression Analysis: Inguinal WAT (iWAT, as a model of subcutaneous WAT depot), epididymal WAT (eWAT, as a model of visceral WAT depot), and BAT were processed to extract total RNA using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain), according to manufacturer's protocol. RNA quantity and purity were measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Madrid, Spain). Reverse transcription was performed to obtain cDNA using the High-Capacity Complementary DNA Reverse Transcription Kit (Thermo Fisher). Gene expression was analyzed by quantitative PCR using the iTaq Universal SYBR Green Supermix (Bio-Rad) in the ABI prism 7900HT real-time PCR system (Applied Biosystems) using primers obtained from Biomers.net (Ulm, Germany). The primer sequences used in this study were presented in Table S2, Supporting Information. The relative expression of each gene was calculated in reference to *Ppia* housekeeping gene and normalized to the L12-VH group. The $^{\Delta\Delta}$Ct method was used and corrected for primer efficiency.[58]

5.0.0.9. Statistical Analysis: The effects of photoperiod, GSPE consumption and the interaction effects between GSPE and photoperiod were evaluated by two-way ANOVA with Šídák's *post hoc* test for multiple comparisons, after checking for normality and homogeneity between samples through Levane's test. GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. The values were expressed as means ± SEM. $p < 0.05$ was considered significant. Statistics in gene expression were referred to L12-VH group. In the figures, significant main effects of either photoperiod, GSPE or the interaction between both (GSPE * *P*) were indicated at the down part of each graph, together with the *p* value. Moreover, the significant effects between individual groups (obtained with two-way ANOVA Šídák's *post hoc* test for multiple comparisons) were indicated with either different letters or asterisks (*).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, F.I.B., C.T.-F., B.M. and G.A.; formal analysis, È.N.-M.; funding acquisition, F.I.B., C.T.-F., B.M., and G.A; investigation, È.N.-M., R.M.R., and F.M.; methodology, È.N.-M., R.M.R., and F.M.; supervision, G.A.; writing—original draft, È.N.-M., G.A.; writing—review and editing, È.N.-M. and G.A. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

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

Keywords

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