INTERACTION OF PHENOLIC-FRUIT HALLMARKS AND SEASONAL RHYTHMS

Francesca Manocchio

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FAIG CONSTAR que aquest treball, titulat "Interaction of phenolic-fruit hallmarks and seasonal rhythms", que presenta Francesca Manocchio per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctoral

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HAGO CONSTAR que el presente trabajo, titulado "Interaction of phenolic-fruit hallmarks and seasonal rhythms", que presenta Francesca Manocchio para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el l Departamento de Bioquímica y Biotecnología de la universidad Rovira i Virgili y cumple con los requisitos para la obtención de la mención Internacional de Doctorado

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I STATE that the present study, entitled "Interaction of phenolic-fruit hallmarks and seasonal rhythms", presented by Francesca Manocchio for the award of the degree of Doctor, has been carried out under my supervision at the Department de Bioquímica i Biotecnologia from the University Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona,9 Mayo 2023

El/s director/s de la tesi doctoral

El/los director/es de la tesis doctoral

Doctoral Thesis Supervisor/s

Dr. Begoña Muguerza Marquínez Dr. Francisca Isabel Bravo Vázquez

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It has been a long journey but it's worth it.

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Summary

Seasonal animals have physiological and behavioural variations that allow them to adapt to annual environmental changes. To achieve optimal timing, seasonal rhythms need to be entrained by external cues, such as photoperiod and nutrition. Fruits rich in (poly)phenols are known for their numerous health benefits. According to xenohormesis theory, animals sense chemical cues in fruits to prepare them for the upcoming situation. (Poly)phenols could act as molecular signals because these secondary metabolites are produced in response to stress; therefore, fruits show distinctive phenolic hallmarks depending on the environmental conditions. The aim of this PhD thesis was to elucidate the differential healthy effects of specific phenolic-fruit hallmarks depending on the photoperiod of consumption. First, metabolic effects of new phenolic-enriched extracts from seasonal fruits on healthy F344 rats exposed to different photoperiods were investigated. Some extracts modulated several biochemical markers, such as blood glucose and lipid levels, in a photoperiod-dependent manner. Second, these fruit extracts were studied in F344 rats subjected to metabolic challenge tests to mimic metabolic disorders. The new extracts restored the disrupted homeostasis in F344 rats subjected to different metabolic challenges, depending on the composition of the administered fruit extracts and the photoperiod in which they were consumed. A higher inflammatory state was observed in animals exposed to short photoperiod than long photoperiod, which was counteracted by the consumption of winter-fruit extracts. Finally, two sweet cherries with different phenolic hallmarks, were tested in healthy F344 rats to better understand the mechanism underlying the crosstalk between seasonal rhythms and peripheral adaptation driven by fruit consumption. Hippocampal neuroprotective effects and circulating thyroid hormone levels were modulated by sweet cherries depending on the specific fruit composition and photoperiod. Overall, these results showed that there is a complex crosstalk between the consumption of fruits rich in (poly)phenols and seasonal rhythms, consumed either as phenolic-enriched fruit extracts or as whole fruit, and under healthy or metabolic stress conditions. Moreover, the time of year in which they are consumed plays a fundamental role.

Resumen

Los animales estacionales adaptan su fisiología a los cambios ambientales anuales. Los ritmos estacionales son guiados por señales externas, como el fotoperíodo y la nutrición. Las frutas ricas en (poli)fenoles son conocidas por sus beneficios para la salud. Según la teoría de la xenohormesis, los animales detectan señales químicas en las frutas para prepararse para la situación que se avecina. Los (poli)fenoles se producen en respuesta al estrés, y actúan como señales moleculares y las frutas mostrarían características fenólicas según las condiciones ambientales. El objetivo de la presente tesis doctoral fue dilucidar los efectos diferenciales de características fenólicas específicas de la fruta en función del fotoperiodo de consumo. En primer lugar, se investigaron los efectos metabólicos de nuevos extractos enriquecidos con (poli)fenoles de frutas estacionales en ratas F344 sanas expuestas a diferentes fotoperíodos. Algunos extractos modularon la glucemia y los niveles de circulantes de lípidos, de manera dependiente del fotoperíodo. En segundo lugar, estos extractos de frutas se estudiaron en ratas F344 sujetas a diferentes pruebas metabólicas para reproducir trastornos metabólicos. Los extractos restauraron la homeostasis en ratas F344 sujetas a diferentes pruebas metabólicas, según la composición de los extractos de frutas administrados y el fotoperíodo en el que se consumieron. Se observó un mayor estado inflamatorio en animales expuestos a fotoperíodo corto que a fotoperíodo largo, lo que fue contrarrestado con el consumo de extractos de frutas de invierno. Finalmente, se probaron dos tipos de cerezas con diferentes características en ratas F344 sanas para comprender la interacción entre los ritmos estacionales y la adaptación periférica debida al consumo de frutas. Los efectos neuroprotectores en hipocampo y los niveles circulantes de hormonas tiroideas fueron modulados por las cerezas según su composición y el fotoperiodo. Estos resultados mostraron que existe una interrelación compleja entre el consumo de frutas ricas en (poli)fenoles y los ritmos estacionales, ya sea su consumo como extractos de frutas enriquecidos con fenoles o como frutas enteras. A su vez, se ha demostrado que la época del año en la que se consumen juega un papel fundamental.

Resum

Els animals estacionals tenen variacions fisiològiques i de comportament que els permeten adaptar-se als canvis ambientals anuals. Els ritmes estacionals han de ser guiats per senyals externs, com ara el fotoperíode i la nutrició. Les fruites riques en (poli)fenols són conegudes pels seus nombrosos beneficis per a la salut. Segons la teoria de la xenohormesi, els animals detecten senyals químics a les fruites per preparar-se per a la situació que s'acosta. Els (poli)fenols podrien actuar com a senyals moleculars perquè aquests metabòlits secundaris es produeixen en resposta a l'estrès; per tant, les fruites mostren característiques fenòliques distintives segons condicions ambientals.

L‟objectiu d‟aquesta tesi doctoral va ser dilucidar els efectes saludables diferencials de característiques fenòliques específiques de la fruita en funció del fotoperíode de consum. En primer lloc, es van investigar els efectes metabòlics de nous extractes enriquits amb (poli)fenols de fruites estacional en rates F344 sanes exposades a diferents fotoperíodes. Alguns extractes van modular diversos marcadors bioquímics, com la glucosa a la sang i els nivells de lípids, de manera dependent del fotoperíode. En segon lloc, aquests extractes de fruites es van estudiar en rates F344 subjectes a proves metabòliques per imitar els trastorns metabòlics. Els extractes van restaurar l'homeòstasi alterada en rates F344 sotmeses a diferents reptes metabòlics, en funció de la composició dels extractes de fruita administrats i del fotoperíode en què es van consumir. Es va observar un estat inflamatori més alt en animals exposats a un fotoperíode curt que a un fotoperíode llarg, que es va contrarestar amb el consum d'extractes de fruita d'hivern.

Finalment, es van tastar dues cireres amb diferents característiques fenòliques en rates F344 sanes per comprendre la interacció entre els ritmes estacionals i l'adaptació perifèrica deguda pel consum de fruites. Els efectes neuroprotectors de l'hipocamp i els nivells circulants d'hormones tiroïdals van ser modulats per les cireres segons la composició específica de la fruita i el fotoperíode. En general, aquests resultats van mostrar que hi ha una interrelació complexa entre el consum de fruites riques en (poli)fenols i els ritmes estacionals, consumits ja sigui com a extractes de fruites enriquits amb fenols o com a fruites senceres, i en condicions saludables o d'estrès metabòlic. A més, l'època de l'any en què es consumeixen hi juga un paper fonamental

Abbreviations

AUC area under the curve

BDNF brain derived neurotrophic factor

Bmal1 aryl hydrocarbon receptor nuclear translocator-like protein1

BW body weight

CHGA Chromogranin A

Clock circadian locomotor output cycles kaput

CRP C reactive protein

DIO2 deiodinase 2

DIO3 deiodinase 3

DPPH 2,2-diphenyl-1-picrylhydrazyl

EYA3 Eye absent 3

G6pc glucose-6-phosphatase catalytic

Ghrh growth hormone-releasing hormone

Gk glucokinase

Glut2 glucose transporter 2

Gpx1 glutathione peroxidase 1

HDL high-density lipoprotein

HOMA homeostatic model assessment

HPA hypothalamic-pituitary-adrenal axis

HPG hypothalamic pituitary-gonadotropic

HPT hypothalamic-pituitary-thyroidal axis

IL-6 interleukin-6

IR insulin resistance

LD long day

LDL low-density lipoprotein

LPS lipopolysaccharide

MetS metabolic syndrome

Nampt nicotinamide phosphoribosyltransferase

NeuroD1 neuronal differentiation 1

Nrf nuclear factor erythroid-derived 2-like

OLTT oral lipid tolerance test

Pck1 Phosphoenolpyruvate carboxykinase 1

PT pars tuberalis

Raldh1 retinaldehyde dehydrogenase 1

SCN suprachiasmatic nucleus

SD short day

Sod1 superoxide dismutase type 1

Sst somatostatin

T3 triiodothyronine

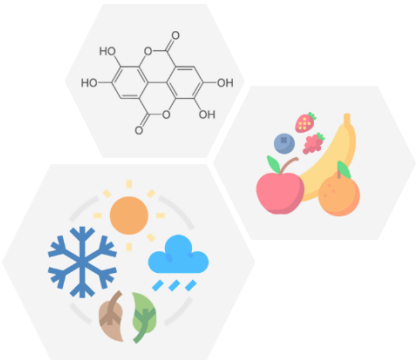
T4 thyroxine

TAGs triglycerides

TEAC Trolox equivalent antioxidant capacity

TNF-α tumor necrosis factor alpha

TSH thyroid-stimulating hormone



INTRODUCTION

1. Phenolic compounds

In the body, macronutrients (carbohydrates, fats, and proteins) are digested and immediately used or stored, while micronutrients such as, vitamins and minerals, are stored or provisionally retained and are fundamental for facilitating biochemical reactions [1]. A multitude of other compounds that are not stored in the body and are not directly involved in biochemical reactions but regulate cell functionality and protect against cellular stress, as for example phenolic compounds [2]. Phenolic compounds are secondary metabolites of plants, synthesised as a defence against UV radiation, pathogens attack, as well as for nutrition growth and reproduction [3], [4]. Approximately 8,000 different phenolic compounds have been described, which all have in common the presence of one or more aromatic ring coupled to a single or more hydroxyl groups linked to an aromatic ring [5]. Some compounds that do not share the structure of polyphenols are integrated in the group as “honorary”, such as stilbenes. For this reason, the nomenclature has been rewritten in (poly)phenols [6]. Phenolic compunds can be divided into different groups according to some characteristics such as the number of phenol rings, the number and disposition of their carbon atoms and structural elements that can bind the phenolic ring as sugars and organic acids. Hence, the most accepted classification divides phenolic compounds into flavonoids and non-flavonoids (Figure 1) [5].

Flavonoids: they have a general structure of 15-carbon skeleton made of two phenyl rings and a heterocyclic ring. Flavonoids are the most abundant and so another sub classification is required for this group:

i. Flavonols: represent the most ubiquitous flavonoids present in vegetables and fruits. An example is quercetin and kaempferol, commonly founds as glycosides with conjugation at 5, 7, 3’, 4’, and 5’ positions. Onions are rich in this subgroup of flavonoids.

ii. Flavones: less common, comprehend the hydrophobic flavonoids, such as apigenin and luteolin similar in the structure to flavonols but they lack oxygenation at C-3. They are present in the skin of fruits as in parsley and celery.
iii. Flavanones: differ for the absence of double bond and the presence of a chiral centre at C-2. They are abundant in citric fruits, such as hesperidin in orange and naringenin in grapefruit.
iv. Anthocyanins widely spread in fruits, are water soluble pigments which confer the characteristic colour red, blue, and purple.
v. Isoflavones, this class is similar in structure to oestrogens. Indeed, can bind its receptor and it is found exclusively in leguminous plants.
vi. Flavan-3-ols: their structure comprehends from monomers to oligomers. Catechins are present abundantly in red wine, green tea, and chocolate [7].

Non-flavonoids:

i. Lignans: polyphenols that derive from phenylalanine via dimerization of substituted cinnamic alcohols. They took largely part in human diet since they are present in wheat rice and fruits and are metabolized by the intestinal microbiota in enterodiol and enterolactone, two healthy compounds.
ii. Stilbenes: compared to other polyphenols they are less frequent in human diet. One example is resveratrol found in red wine and peanuts, which is associated to healthy effects.

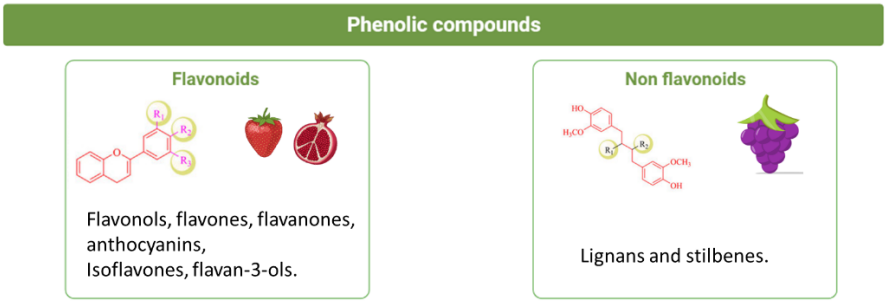


Figure 1. Classification of phenolic compounds. Flavonoids with different subclasses such as flavonols, flavones flavanones, anthocyanins, isoflavones, flavan-3ols Example of fruits rich in one of the classes is represented.

1.2. Dietary (poly)phenols and benefits

Healthy diets, such as Mediterranean diet, are rich in fruit consumption source of fibres, vitamins, and phenolic compounds. This latter group of bioactive compounds are largely present in the human diets, and exert different beneficial effects widely demonstrated by scientific community during last years as antihypertensive properties, protective in metabolic syndrome, improving insulin resistance, inflammation, and lipid metabolism [5]. The average of consumption of polyphenols with diets change among different geographical origin and culinary habits, Mediterranean diet has been estimated to guarantee around 1g/day through fruits, vegetables, wine, olieve, etc [8]. Some effects of polyphenols are generic (reducing properties) and shared among this large group of compounds and others are specific, for instance the interaction of isoflavones with estrogen receptors [9]. Polyphenols vary largely for the molecular weight, chemical structure, and glycosylation which determine the absorption through the intestine and their extraction. The gut barrier constitutes the first step of the polyphenols journey to enter in the bloodstream. They are recognized as xenobiotics by the organism and go through a biotransformation,since show very low bioavilability in their native form [10]. The phenolic compounds in the form of glycosides needs to

be de deglycosilated to increase its absorption. The main Phase-II reaction that occurs in the gut is glucorinidation, accompanied by methylation. Gut microbiota play an important role at colonic level in the metabolization of (poly)phenols and (poly)phenols modulate the microbiome composition, highlighting the bidirectional interaction among them [11]. The enterohepatic circulation allows (poly)phenols metabolites to reach the liver where are metabolized by several enzyme and subsequently they reach through the circulation the other organs. Thus, the (poly)phenols metabolites found in bloodstream and that exert their actions in the organs differ from native molecules. Moreover, depending on the target organ, they can also find other barriers to overcome. As in the case of brain the blood brain barrier (BBB) is highly selective and reduce the bioavailability of (poly)phenols [6].

The consumption of some (poly)phenols is associated to anti-hypertensive effect, by acting as free radical scavengers and modulate the activity of endothelial nitric oxide synthase expression [12], which play a major role in endothelial cells against vascular damage [13]. Moreover, several studies in clinical trials have showed that phenolic compounds consumption is associated to reduction in LDL cholesterol, hyperglycaemia, and reduction of body mass index as well as waist circumference, and improvement of adiponectin and leptin in serum of individuals diagnosed with metabolic syndrome [14]–[16].

The bioactivity of the phenolic compounds strictly depends on their bioavailability, which in turn is regulated by several internal and external factors such as concentration dose of treatment, sex and age [4] . Moreover, recently it has been pointed out that other factors that can regulate their bioavailability are circadian and seasonal rhythms [17] A growing field in the last decades, chrono-nutrition, focuses on the relationship between eating patterns and circadian rhythms [18]. Circadian rhythm is a 24-hour internal clock responsible for the coordination of many daily processes, as regulating the sleep/wake cycle, food intake, energy expenditure [19], [20]. Optimising metabolic health may be achieved by aligning food intake to times of day when circadian rhythms are optimal [21]. Studies that

prove the close relationship between diet and circadian clock, had shown how phenolic compounds can act as external cues in their modulation in the central circadian clock in the hypothalamus (Figure2) [22] and several peripheral organs [23], [24]. Moreover, the Nutrigenomics research group in the last 10 years studied the crosstalk between seasonal rhythms and phenolic rich fruits and extracts consumption, which is well explained in the Manuscript 1 included which is included at the end of this introduction.

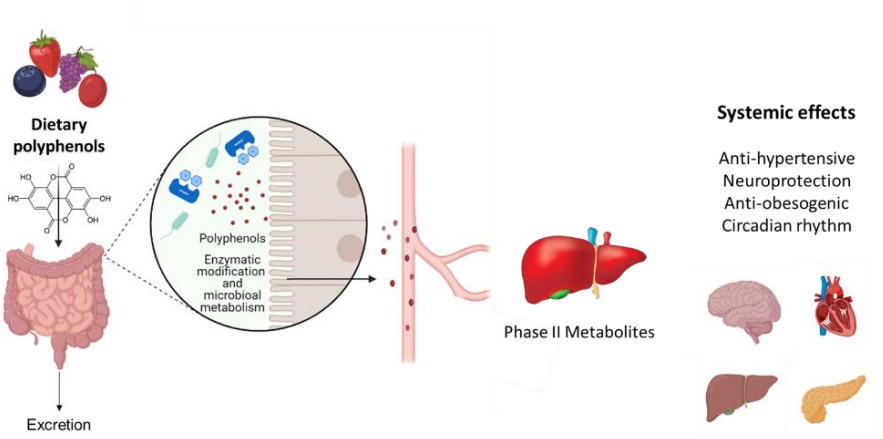


Figure 2. Dietary polyphenols journey in the body and their systemic effect.

1.2.1. Phenolic compounds in the management of dyslipidemia

Dyslipidemia defines the condition of imbalanced amount of lipids in the blood such as triglycerides (TAGs), total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), which are associated with cardiovascular risk and can be consequence of obesity, insulin resistance and metabolic syndrome [25]. In the gut dietary lipids are transported in the form of chylomicrons and its production is positively correlated with diets high in fats such as western diets as well as to atherosclerosis. Overproduction of intestinal chylomicrons promotes the atherogenic process associated with metabolic disorders [26]. Nutritional strategies accompanied by health lifestyle have been

proposed for the management of dyslipidemia, such as consumption of fruits and vegetables rich in (poly)phenols. Indeed, several studies have demonstrated that its consumption is associated with hypolipidemic state by interacting and inhibiting hepatic enzymes of lipogenesis such as Sterol regulatory element binding protein 1 and diacylglycerol O-acyltransferase 2 [27], or by interaction with microRNAs involved in lipid regulation [28]. A method to assess the intestinal absorption and whole-body lipid metabolism is the oral lipid tolerance test (OLTT). It is a quick and easy technique to perform however is not standardized, since several fat sources are used which differ for farty acid composition and could lead to different results depending on the amount of long, medium, and short chain fatty acids [29]. One of the most used is the lard oil. Once selected the fat source and oral gavage is performed in the animal and blood is collected in different time points to evaluate [30]. This metabolic challenge test can be used to assess the effects of dietary compounds on lipid management as it is described in Manuscript 4.

1.2.2. Inflammation and phenolic compounds

Among the properties attributed to the consumption of fruits rich in phenolic compounds are included the anti-inflammatory, immune-modulatory and antioxidant [31], [32]. Inflammation state is related to metabolic diseases such as diabetes type II [33], obesity [34], and to neurodegenerative disorders [35]. Inflammatory markers, circulating in the bloodstream, can be used as predictive biomarkers for cardiovascular risk such as C-reactive protein (CRP) [36] or other proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), associated to obesity and insulin resistance [37]. Indeed, the expansion of adipose tissue is correlated with stress in endoplasmatic reticulum, lipid peroxidation which induce the release of fatty acids and proinflammatory cytokines. White blood cells as macrophages M1 are proinflammatory and are the ones responsible for the release of inflammatory cytokines associated to the expansion of adipose tissue [38].

A widely used technique to provoke systemic inflammation is the lipopolysaccharide (LPS) induced inflammatory challenge in experimental animal models [39]. LPS is a bacterial toxin, part of the outer membrane of Gram-negative bacteria. An intraperitoneal injection of an appropriate dose leads to the production of pro-inflammatory cytokines and can be used to study how drugs or natural compounds can be effective to counteract the inflammatory action of LPS [40]. This type of challenge could be useful to select which natural compounds, for example, can be the more effective in prevent inflammation associated to metabolic disorders. Previous studies have shown that dietary (poly)phenols, such as resveratrol counteract the expression of pro-inflammatory cytokines due to obesity chronic inflammation [41]. Moreover, proanthocyanidins, a class of phenolic compounds, have been reported to be effective in counteracting inflammation status produced by LPS induced inflammatory challenge (Figure 3) in both nutritional and pharmacological dose [42]. Thus, this challenge has been used in this thesis for a similar purpose well described in the Manuscript 4.

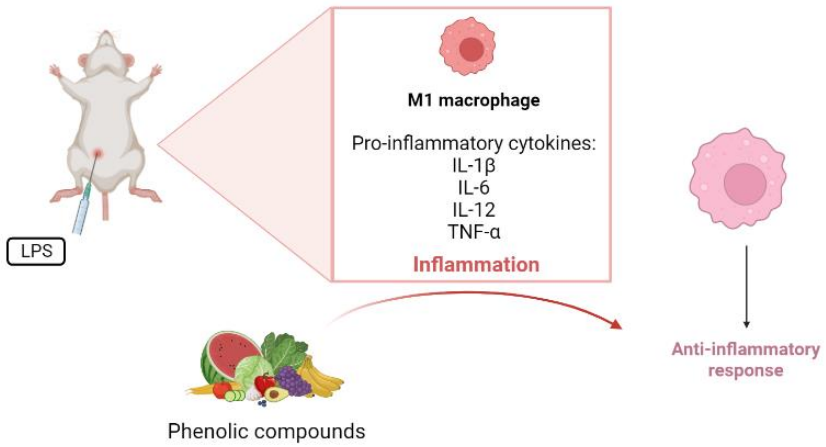


Figure 3. Lipopolysaccharide (LPS) induced inflammatory challenge enhance pro-inflammatory cytokines expression and can be counteracted by the supplementation of phenolic compounds that increase the anti-inflammatory response.

1.2.3. Neuroprotective role of fruits (poly)phenols

Sedentary habits coupled with a diet high in fats and sugars contributes not only to obesity and its comorbidities, but also to cognitive decline [43]. As a matter of fact, preclinical studies carried on rats showed correlation of consumption of high fat diet and impairment in hippocampal memory functions accompanied by a decreased expression in neurotrophic factors such as brain derived neurotrophic factor (BDNF) [44]. The hippocampus is an intricate brain structure tucked deep within the temporal lobe, plays an important role in learning and memory and is sensitive to a variety of stimuli that can damage this plastic and vulnerable structure [45], [46]. Among the external stimuli, nutrients play a key role in its modulation and functionality. However, before arriving in hippocampal region those stimuli need to be able to cross the blood brain barrier (BBB). The BBB is a dynamic and highly selective barrier of endothelial cells, which mainly prevents the enter of molecules from the blood to cross it and reach brain areas [47]. At the same time allows the passage of the molecules required such as nutrients and non-nutrients [48]. Among the non-nutrients, metabolites derived from (poly)phenols metabolism can cross the BBB and have been shown to exert neuroprotective effects [49], as well as have been recently proposed as therapeutics for neurodegenerative disease [50]. Fruits rich in (poly)phenols have been widely reported to enhance the neuroprotective signalling of neurotrophic factors [51], thanks to the ability of certain (poly)phenols derived metabolites, that can cross the blood brain barrier (BBB) [52]. These bioactive compounds can activate neurotrophins signalling, a family of growth factors involved in neuronal survival, development, and plasticity, such as nerve growth factor (NFG) or BDNF [53]. For instance, tart cherry exerted anti-neuro-inflammatory properties and suppressed neuronal apoptosis and stimulated pro-survival signalling cascades in microglial cells [54]. Specific phenolic compounds such as, genistein [55], quercetin [56], resveratrol [57] etc. have been reported to increase BDNF levels by activating ERK-

CREB pathway first, which in turn activates BDNF in *in vitro* models. Not only *in vitro* studies but also clinical trials showed that tart cherry consumption improve cognitive functions [58]. Other studies showed that blueberry, another fruit rich in (poly)phenols, showed an improvement of working memory and an upregulation of hippocampal *Bdnf* gene expression in aged rats, which play a key role in the improvement of neuronal plasticity [59]. Moreover, (poly)phenols activate nuclear factor erythroid-derived 2-like (*Nrf2*) pathway. NRF2 pathway activation allows neuronal cells to fight against oxidative stress and external negative stimuli. Indeed, this protein enters in the nucleus and functions as transcription factors on antioxidant responsive element (ARE), by increasing gene expression of genes encoding for antioxidant enzymes such as glutathione peroxidase (*Gpx)* and superoxide dismutase (*Sod*) that play a pivotal role in the protection of the cell from oxidative stress (Figure4) [60].

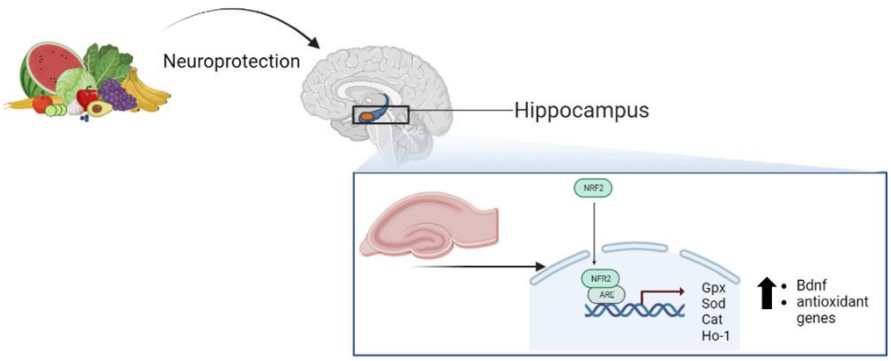


Figure 4. Fruits rich in phenolic compounds exert neuroprotective effect enhancing gene expression of brain derived neurotrophic factor (*Bdnf*) and genes encoding for antioxidant enzymes.

2. Fruits extracts and their impact on circular economy

About one-third of the food produced in the world per year for human consumption is lost or wasted [61]. In 2019, food losses were estimated to be 14 %, and they are

continuously increasing [62]. It is generally considered that fruits and vegetables suffer the most losses, even if some strategies to avoid post-harvest losses have been employed, such as check the maturity and improve the storage [63], or the use of an alternative to synthetic chemicals as the application of medicinal plant extracts in postharvest preservation of crops [64], more strategical actions are needed to reduce these losses. Circular economy advocates strategies to manage and valorise by-products to reduce pollution and promote a more sustainable economy [65]. It is possible to extract many bioactive molecules from fruit and vegetable by-products; among these vitamins and phenolic compounds are the most important, and their health promoting properties make them suitable as nutraceuticals [66]. Nutritional supplements have the benefit of a more controlled delivery and are designed to be more effective and be absorbed in the right place, as well as the bioactive compounds are more concentrated respect to the food of origin. Previous studies showed that when bioactive compounds, such as polyphenols, when are absorbed through the diet they are consumed with all matrices, and fibres and proteins are well known to be a limiting factor of their absorption [67]. On the other hand, phenolic compounds can interact with other nutrients, such as carbohydrates, which influence their digestion, by inhibiting glucose transporters and promoting hypoglycaemia [68]. Thus, probably if supplements of phenolic compounds are taken with or without food the effects can vary [2].

The production of extracts starting from fruits rich in polyphenols such as sweet cherry, has been already proposed as a strategy to improve its use and promote a sustainable way against fruit loss. In the study by Vilas-Boas et al. the extraction process allowed to obtain an extract with high antioxidant properties correlated to high phenolic content [69]. There are several extraction methods of phenolic compounds from different vegetal materials, which vary depending on the starting material and possible interfering matrices. The change of protocol's parameters, such as the extraction time, the temperature, and the solvents, will influence the

extraction yield [70]. The most conventional methods are the liquid-liquid and solid-liquid extraction. The main solvents used are ethanol, methanol, acetone used at different concentration and diluted with water. Since they are considered as possible risky for health, purification process, such as evaporation is necessary [71]. More modern processes involve ultrasound-assisted extraction, microwave-assisted extraction or supercritical fluid extraction [70]. Moreover, other types of extraction that are interesting from a sustainable point of view are enzyme-assisted extraction which allow the release of phenolic compounds by breaking cell walls and decrease the production of residual substances [72].

3. Seasonal fruits

Food consumption patterns can be made more sustainable by consuming seasonal food, based on the assumption that this would reduce the impact on the environment [73]. Among seasonal food, fruits are very interesting for their health benefits, attributed to their micronutrients and phenolic compounds. Each fruit presents a different phenolic profile depending on the activation of specific genes involved in phenolic compounds biosynthesis [74]. The expression rates of key genes involved in the synthesis of phenolic compounds can also vary among different varieties of the same fruit, so the concentration of different polyphenol representatives in fruit can also differ [75]. Moreover, among varieties of a fruits gene expression also change which determine a different composition. The genetic background of fruits plays a huge role, but they are also profoundly influenced by external factors like light exposure, temperature, soil content, all of which play a part in the formation of their phenolic hallmark. Moreover, it has been pointed out that abundance and composition in phenolic compounds can vary across the seasons, being environmental factors fundamental [76]. Among seasonal summer fruits apricots, plum, cherries and strawberry are widely consumed. Apricots (*Prunus armenica*) are source of several minerals, vitamins and phenolic compounds, the latter are found in the peel and the pulp. Apricots have both

flavonoids and non-flavonoids and chlorogenic and neochlorogenic acids are the most abundant [77], and their consumption is associated to anti-inflammatory action [78]. Strawberry (*Fragaria x ananassa*) is another highly consumed summer fruits, is rich in anthocyanins and ellagitannins [79] and its consumption showed several benefits in improving antioxidant biomarkers as well as dyslipidemia in humans [80]. Plum (*Prunus domestica*) is rich in his peel and pulp of phenolic compounds such as chlorogenic acids, caffeic acid and p-coumaric, its consumption is associated to different health benefits such as anti-lipidemic and cardioprotective [81]. Sweet cherries (Prunus avium L.) are widespread summer fruits and exist in both sour and sweet varieties. Anthocyanins are responsible for the colour, and their chemical structure differs between the two varieties. Indeed, sweet cherry contains mainly cyanidin-3-o-rutinoside, whereas sour cyanidin-3-glucosylrutinoside [82]. Moreover, the taste is different due to higher presence of sugars (fructose, sucrose, glucose, sorbitol and maltose) in sweet variety and less presence of malic acid respect to the sour cherries. It is a valuable source of vitamin A (3 µg/100 g of fresh weight (FW)), C (6-10 mg/100 g FW) and E (70 µg/100g FW) and minerals (potassium, calcium) [82] and phenolic compounds [83]. However, the composition in phenolic compounds change depending on the cultivar system and environmental conditions, thus the colour of the skin results more or less dark in proportion with the concentration of anthocyanins [84]. Sweet cherries are also good source of melatonin (22.4 ng/100 g) and serotonin (37.6 ng/100g) [85], [86]. The intake of sweet cherry is associated to several healthy benefits, it has been demonstrated in preclinical studies that sweet cherry consumption counteracts the detrimental effect of metabolic disorders induced by diet high in sugars [87]. Moreover, previous study of our group showed that depending on the season of consumption its effects vary. [23]. Specifically, sweet cherry when consumed out of its season increased hyperglycaemia in obese rats, respect to obese rats that did not consume it [88]. Additionally, specific geographical origins of sweet cherry showed to play an important role in its composition and effect depending on the

photoperiod of consumption. Consumption of local sweet cherry (from Spain) decreased hepatic lipid peroxidation markers in healthy rats but not non-local sweet cherry (from Chile) [89].

Among fruits typical of autumn-winter season are worthy to mention grapes, persimmon kaki, orange, and pomegranate. Red grape (*Vitis Vinifera L.*) is abundantly consumed in Mediterranean diet, and several studies have described that its intake led to many benefits for the health such as anti-obesity, cardioprotective, anti-diabetic etc. and among phenolic compounds present in peel, pulp and seeds, some characteristics are resveratrol, proanthocyanidins, and catechins [90]. Persimmon kaki (*Diospyros kaki*) is rich proanthocyanidins but mostly is characterized for the presence of other bioactive compounds, carotenoids. Although little is known about its benefits, it has been suggested to have anti-obesogenic properties [91].

Orange (*Citrus sinensis L.*) can be classified in two variety: sweet that is the most consumed and sour. Among the phenolic compounds, flavanones and are the most representatives, specifically hisperdin and narigenin [92]. Different studies reported that its consumption is correlated to health benefits such as hepatic and renal protection [93]

Pomegranate (*Punica granatum L.*) is a fruit rich in anthocyanins and others phenolic compounds that are poorly absorbed by gastrointestinal tract, until now there are few studies that correlates its consumption with moderation for cardiovascular risk factor by improving blood pressure [94].

4. Seasonal rhythm

On the Earth most of the environments show seasonal fluctuation in the climate, and as a mechanism of adaptation most vertebrates exhibit profound cyclical adaptations in behaviour, physiology, and morphology [95]. In essence, neuroendocrine processes control seasonality since the brain senses the environment and controls endocrine and autonomic functions [96].

The reason why seasons exist is due to the tilt in the Earth's axis relative to its plane of rotation around the sun. The axis is tilted around 23.5 degrees relative to its path around the sun [97]. As a result, light falls directly on its equator but strikes the North and South poles at angles, and as a result sunlight are spread on a greater area in winter respect to the summer [98]. As consequence all photosynthetic processes and food production are affected by changes in sunlight duration and radiation intensity across the annual cycle [99].

Photoperiodism is an anticipatory signal for season adaptation depending on short-day (SD, winter-like season) and long-day (LD, summer-like season) and the photoreception is a complex molecular pathway, which starts in the retina. Photoperiodism response is a complex neuroendocrine response, which shows differences among species. Light exposure stimulates the photoreceptors cones and rods of the retina with the support of intrinsically photosensitive retinal ganglion cells (ipRGCs), which are hosted in the ganglia cell layer. These photosensitive ganglion cells send the information via retinohypothalamic tract to the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is a brain region able to generate and activate neural and hormonal activities that drive body processes in 24 hours. It contains around 20.000 neurons, which form a neuronal network responsible for the regulation of both circadian (self-sustained rhythms with a period of 24 hours) and circannual rhythm (self-sustained rhythms with a period typically less than 1 year) [100].

The SCN rhythmicity ensures stable oscillation of sleep/wake cycles, food intake, activity, body temperature, and the output of many hormones, by ultimately regulating neuroendocrine axis as the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-thyroidal (HPT) axis, and the hypothalamic pituitary-gonadotropic (HPG) axis. In mammals, the circannual rhythms are influenced by the SCN and melatonin secreted from the pineal gland. Melatonin hormone is secreted during the dark and represents a fundamental seasonal indicator, ultimately regulating the function of many areas of the brain and body involved in seasonal

physiology. In the last few years, there has been increasing evidence on the crosstalk of biological rhythms and nutrition, and their effects on the regulation energy balance and metabolism. In this sense it has been described that phytochemicals, contained in plants could act as chemical cues and inform the animals that are consuming them about the environmental change [17], [23]. This mechanism allows the physiology to adapt the organism to adverse or favourable conditions and is known as Xenohormesis. Further details to discuss current knowledge on seasonal rhythms and their interactions with phytochemicals is deeply described in the review (Manuscript 1).

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Manuscript 1

Seasonal fruits as signals of circannual rhythms

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Abstract

Physiological and metabolic adaptations to the environment are enabled by annual changes in day length, which is a key cue for entraining seasonal rhythms in animals. Although research is still elucidating the underlying mechanisms of circannual rhythms, they likely rely on molecular seasonal markers and the remodelling of the pituitary-hypothalamic network. Metabolic parameters and gene expression have been shown to vary throughout the year according to season in several tissues. Moreover, the incidence of coronary heart disease, metabolic syndrome and seasonal affective disorders increases during winter, suggesting that health is prevalently endangered during the short-day-like season. One outstanding question is whether diet plays a role in regulating homeostasis depending on the photoperiod. In this sense "xenohormesis theory" proposes that phytochemicals are signals that can be recognized by heterotrophs, allowing them to prepare for adversity while conditions are still favourable. Phenolic compounds could be one of these signalling molecules because they are secondary plant metabolites produced in response to stress. However, one of the important changes in current dietary patterns is the intake of fruits outside of their natural season, which could provide misleading signals. Recent findings have shown that fruits rich in phenolic compounds have different metabolic effects, depending on the season of consumption. However, how the specific fruit phenolic hallmarks are involved in the regulation of metabolism has not been explored, which opens new insights for further research.

Keywords: seasonal rhythms, photoperiod, fruits, phenolic hallmark, metabolism.

1. Introduction

Biological rhythms are endogenous oscillations that cause repeated cycles of organism function with a nearly stable interval. These rhythms, circadian and circannual, are generated by the nocturnal duration of melatonin production, which varies depending on the day length [1], [2]. The daily and annual shift in the light-dark cycle is fundamental to allow animals living in latitudes other than the equator to adapt their metabolism and physiology to environmental changes, as survival strategy [3]. These rhythmic oscillations regulate various physiological processes, such as sleep, energy balance, and food intake, and allow organisms to synchronize their physiology in a daily and season-dependent manner [4], [5].

A broad range of studies described the molecular machinery of circadian rhythms and their functions in the organism. In this sense, the suprachiasmatic nucleus (SNC) in the hypothalamus hosts the circadian clock and synchronises the rhythms throughout the body [6]. Moreover, not only the light but other external cues such as nutrients can entrain circadian rhythms. The relationship between circadian rhythms and nutrition is known as chrononutrition. Lifestyle variations in modern societies characterized by different dietary patterns, including modifications in the frequency and timing of meals can alter circadian rhythms. This misalignment between external cues and the internal clock disrupts rhythms and has been related to severe long-term health problems [7]. In recent years, different studies have shown that phenolic-enriched extracts and phenolic-rich fruits are able to revert these disruptions [8]. However, the crosstalk between seasonal variations in physiology and metabolism and this class of compounds remains unclear.

Therefore, this review aims to shed light on the current knowledge of seasonal rhythms, seasonal variations in metabolism and physiology and the role of fruits rich in phenolic compounds in peripheral photoperiodic adaptation.

2. Mechanisms behind the biological rhythms

2.1. Circadian rhythms

Circadian rhythm is a self-sustained timing system responsible for the coordination of many daily processes such as regulating the sleep-wake cycle, food intake, energy expenditure, and metabolism [9], [10]. Aside from the master hypothalamic clock, several peripheral clocks are also present in all organs and tissues with essential functions, including the liver, muscles, heart, lungs, and kidneys [11]. Peripheral oscillators receive signals from the central clock via the autonomic nervous system or circulating hormones such as melatonin to maintain rhythmicity and ensure temporally synchronized physiology [12].

In mammals, the machinery of the circadian clock is an intracellular mechanism with the same molecular components in the SCN and peripheral cells. A transcription-translation autoregulatory feedback loop, which has a period of 24 h, is the molecular basis of this rhythmicity [13]. Figure 1 shows a representation of the molecular machinery. It is initiated by the Circadian locomotor output cycle kaput (CLOCK), which dimerizes with Brain and muscle Arnt-like protein-1 (BMAL1). The heterodimer CLOCK:BMAL1 activates the expression of the genes Period (*Per*) and Cryptochrome (*Cry*) upon binding to E-box (5'-CACGTG -3') and E-box-like promoter sequences. PER and CRY, in turn, after reaching the proper concentration in the cytoplasm, translocate to the nucleus and repress the activity of CLOCK:BMAL1, inhibiting their own expression and establishing a first negative feedback loop [14]. In addition, since CLOCK:BMAL1 activates the transcription of thousands of clock-controlled genes, the state of this loop determines the daily rates of expression of these genes, and thus the metabolic and functional rates of the cells. Other genes activated by CLOCK:BMAL1 are the Orphan nuclear hormone receptors reverse-erb alpha (*Rev-erbα*) and Retinoic acid-related orphan receptors (*Ror*), which constitute a second feedback loop that represses and activates, respectively, *Bmal1* gene expression [13]. All this molecular machinery coordinates

many processes, such as regulating the sleep/wake cycle, food intake, energy expenditure among others. It has been pointed out that mammalian circadian clock exhibits seasonal variations in light sensitivity, highlight an interplay between circadian and seasonal rhythms [15].

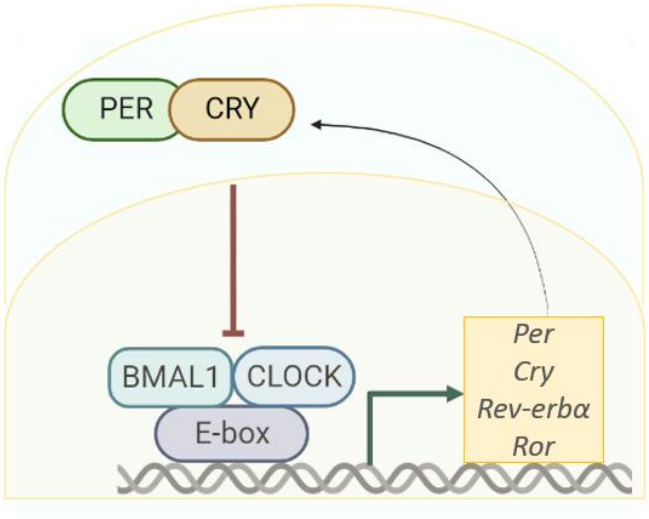

Figure 1. The molecular basis of circadian rhythms is the transcription-translation feedback loop (TTFL). The heterodimer CLOCK:BMAL1 (circadian locomotor output cycles kaput and aryl hydrocarbon receptor nuclear translocator-like protein1 respectively) functions as a transcription factor for genes encoding Period *(Per),* Cryptochrome *(Cry),* Nuclear receptor subfamily 1 group D *(Rev-Erbα),* and Retinoic acid-related orphan receptor *(Ror)*. PER and CRY proteins come back into the nucleus repressing their own transcription acting on CLOCK:BMAL1.

2.2. Circannual rhythms

Although recent studies have provided considerable information on the circadian core clock mechanism, little is known about the molecular mechanisms underlying seasonal rhythms. The survival of animals depends on their ability to adapt to environmental changes and anticipate and prepare themselves for changes in the environment. Mammals have developed intrinsic seasonal rhythms as an adaptation mechanism. These include hibernation, migration, sexual behaviour,

metabolic changes, weight gain and loss [16]. The seasonal program is scheduled based on external cues, such as light and food. Light, as for circadian time, plays a major role in setting and regulating the physiological adaptation to seasonal changes [17]. Organisms present different mechanisms in the setting and outcomes of circannual cycles; however, they share the main principle of integrating the function of the endogenous clock with signals from the environment [18]. For instance, hamsters show a huge decrease in body weight during a short photoperiod and regression in testis weight, which is only partially observed in Fischer 344 (F344) rats [19], [20].

In this regard, photoperiodism is an anticipatory signal for seasonal adaptation depending on the short day (winter-like season) and long day (summer-like season), and photoreception is a complex molecular pathway that starts in the retina [16], [21]. Light exposure stimulates photoreceptor cones and rods of the retina with the support of intrinsically photosensitive retinal ganglion cells, which are hosted in the ganglion cell layer [22]. These photosensitive ganglion cells send information via retinohypothalamic tract to the SCN of the hypothalamus. The SCN is a brain region capable of generating neural and hormonal activities that drive bodily processes within 24 h. It contains approximately 20,000 neurons that form a neuronal network responsible for the regulation of biological rhythms [6]. Globally, the SCN sends information to other hypothalamic nuclei and the pineal gland to modulate the production of hormones such as melatonin, that decreases during the light phase and increases during the dark phase to transmit information from the light/dark cycle [23]. Melatonin simultaneously regulates the circannual clock in approximately 1-year period and the circadian clock in a 24-h period, which is responsible for the synchronization of the circannual rhythms and plays a key role in the control of seasonal processes [24], [25]. The *pars tuberalis* (PT), which is located in the anterior lobe of the hypophysis, has specialized cells called thyrotrophs that contain melatonin receptors and are responsible for producing thyroid-stimulating hormone-beta (TSHβ). TSHβ binds to its receptor TSHR on the

membrane of specialized glial cells (tanycytes) in the hypothalamus, which in turn regulates the expression of enzyme type II iodothyronine deiodinase (DIO2) [24]. DIO2 converts inactive thyroxine (T4) into the active T3 form. However, DIO3 is dominant during SD, and this isoform catalyses the conversion of T4 to inactive metabolites such as reverse T3 or di-iodothyronine (T2) (Figure 2) [26]. All these mechanisms in animal models responsive to photoperiod, regulated by light/dark, result in higher availability of T3 during long photoperiods, which in turn increases food intake and decreases energy expenditure and fat oxidation [27], [28]. Local availability of thyroid hormone (TH) in the hypothalamus is fundamental for seasonal rhythm generation [26]. Thus, the hypothalamus is involved in the control of seasonal variations and its remodelling is a key component in modulating food intake and body weight changes.

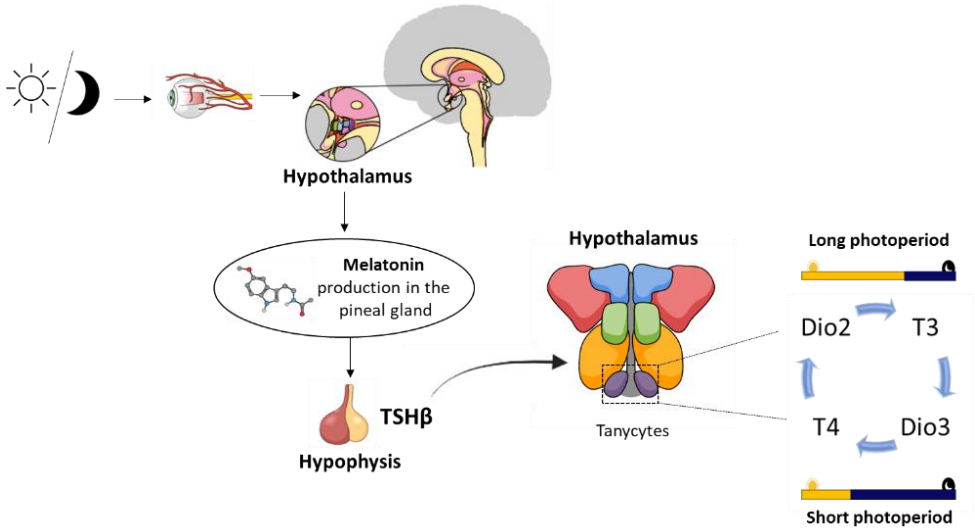

Figure 2. Dark signals active melatonin production in the pineal gland. Melatonin acts on the *pars tuberalis* of the hypophysis to regulate thyroid stimulating hormone beta (TSHβ) release, which in turn regulates thyroid hormone availability of the deiodinase enzymes DIO2 and DIO3 in hypothalamic tanycytes. Tanycytes express thyroid-stimulating hormone receptors and sense TSHβ signals from the hypophysis, the expression of which is upregulated by photoperiod and results in long photoperiod-induced *Dio2* mRNA expression and short photoperiod-induced *Dio3* mRNA expression. DIO2 converts the inactive form of thyroid hormone,

thyroxine (T4), to the active form triiodothyronine (T3), increasing the hypothalamic concentration of T3 under a long photoperiod.

2.2.1. Molecular markers of summer and winter

Photoperiod responsiveness to seasonal physiology and reproductive status may differ depending on the species studied. In this sense, Wood *et al.* 2015 [29] demonstrated the existence of a binary code in the PT, in which the proportion of cells expressing two different states embodies the circannual phase in sheep. These cells are so called “calendar cells” and define the circannual rhythm by expressing prevalently the protein Eyes absent 3 (EYA3) in the long day and Chromogranin A (CHGA) in the short day (Figure 3). EYA3 is a transcriptional coactivator that shows a peak phase during short day at the same time as nocturnal melatonin, which suppresses *Eya3* gene expression. Nevertheless, in the long day, the *Eya3* expression phase is coincident with light and the TSHβ promoter is subsequently coactivated in PT thyrotrophs, triggering the pathway that was previously described [28]. In addition, CHGA is a prohormone involved in the production of secretory granules in neuroendocrine tissues, and has inhibitory actions associated with PT dormancy during the short-day season. The PT changes its morphology and expresses these specific seasonal markers EYA3 or CHGA which constitute a binary code that defines the summer and winter phenotype, respectively [23], [29].

It has been proposed that EYA3 interacts and coordinates with circadian clock modulators rather than acting in isolation. Indeed, the E-box region in the *Eya3* gene promoter could be regulated by the transcription factors BMAL1 and CLOCK, which constitute core clock genes (Figure 2). Moreover, CRY, which acts together with PER in the inhibition of the CLOCK:BMAL1 heterodimer, rises due to the melatonin peak during the dark phase and at the same time supresses the transcription of *Eya3* [28] [39].

All these studies indicate a possible interaction between circadian and circannual rhythms, but more research is needed to evaluate their regulation by external cues.

It is noteworthy that circannual rhythmicity is not self-sustaining in peripheral organs but relies on the pineal gland to integrate photoperiodic signals and convert them into melatonin, which in turn regulates physiology at metabolism, body temperature, and immunity levels [30].

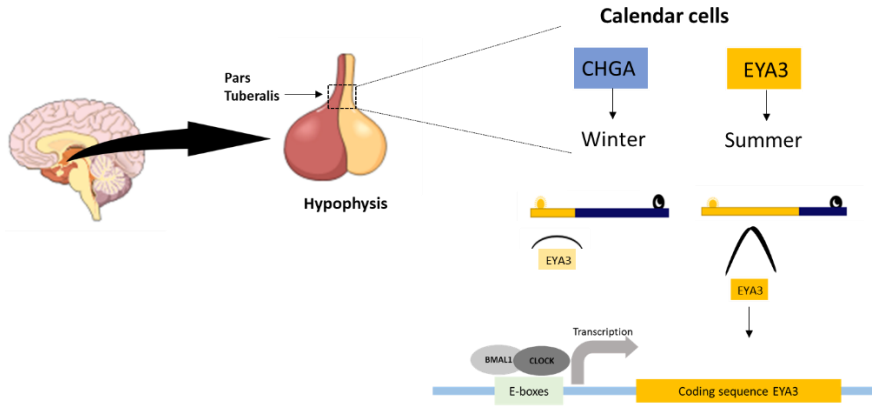

Figure 3. Molecular markers reside within the hypophysis. Eye absent 3 (EYA3) and Chromogranin A (CHGA) are a long and short photoperiod marker, respectively, expressed in the *pars tuberalis*. These molecular markers could interact with the molecular machinery of circadian rhythms, with the *Eya3* promoter being controlled by the heterodimer (circadian locomotor output cycles kaput and aryl hydrocarbon receptor nuclear translocator-like protein1 respectively) CLOCK:BMAL1.

3. Animal models responsive to photoperiod

Animals respond differently to seasonal physiology and reproductive status in response to photoperiod. Human studies present relevant limitations in the analysis of molecular mechanisms, such as those occurring at the central or peripheral organs. Thus, animal models are fundamental to study the underlying mechanisms of seasonal rhythms; however, it should be taken into account for the interpretation of the results that humans are diurnal, whereas some species such as rats are nocturnal.

A well-known model is the Fisher 344 (F344) rat strain, which is sensitive to photoperiod [31]. Moreover, the availability of genome data for microarray fabrication, enabling high-throughput transcriptome analysis of hypothalamic tissue in different photoperiods in this species, which is one of the main reasons that has been widely used despite its short life span [19]. Indeed, young F344 rats show dietary preferences due to the adaptation of energy metabolism to different photoperiods. In this sense, it seems to have a major role the carbohydrates content in the diet, since during long photoperiod F344 preferred a diet with more carbohydrates than the one with less, a preference that has not been observed during short photoperiod [32]. F344 rats seasonal physiology and reproductive status rely on hypothalamic control, which mainly involves TH and retinoic acid signalling [33], resulting in decreased body weight and food intake. Tanycytes markers of long-day physiology in F344 rats are *Dio2,* Ornithine Aminotransferase-Like 3 (*Oatp1c1),* and retinaldehyde dehydrogenase 1 (*Raldh1*) among others, which are downregulated in the short-day state [34], [35]. This adaptation is translated in F344 rats in a catabolic state during a short photoperiod, whereas a long photoperiod moves towards an anabolic state [36].

Among photoperiodic animals, Siberian hamsters have been quietly studied, showing several marked adaptations, such as the decrease in body weight up to 35% during short photoperiod [20]. Hamsters need reduced availability of T3 in the hypothalamus to adapt physiologically to cold conditions, enabling a state of hypometabolism to save energy [37]. Indeed, hypothalamic T3 contribute to torpor (long period of inactivity), whereas increased T3 serum concentrations do not inhibit torpor expression [37]. Moreover, Siberian hamsters show dietary preferences for specific food type depending on the photoperiod in which are exposed. Indeed, hamsters exposed to short photoperiod preferred diet with higher content in carbohydrates and protein compared to hamster housed in long photoperiod [20]. Additionally, short photoperiod could enhance immune cell-trafficking from the blood to the skin as an adaptive change for winter that included

increased glucocorticoid levels as well as circulating blood leukocytes, lymphocytes, T cells, and natural killer cell concentrations in this animal model [38].

Another model used to study photoperiod responsiveness, is the Soay sheep, which allow to obtain several tissues for the analysis and is mainly used because is a short-day breeding mammal and shows a physiologic switch between summer and winter [39]. Moreover, photoperiod is not only used by mammals to regulate seasonal responses, but also by birds such as Japanese quail [40], and lizards [41]. Despite the differences in metabolism and physiology, they all have in common the ability to respond to the annual change in day length.

4. Seasonal variations in humans

An extraordinary example of sensitivity to season is given by mammalian hibernators, which accumulate triglycerides in adipose tissue, gain body weight, and exhibit insulin insensitivity during autumn/winter. Despite humans are not hibernators it has been proposed that they share similar metabolic adaptation and seasonal adjustment [42]. Due to climate change and modern life habits, such as eating foods out of season or social jet-lag, it has become challenging to determine the seasonal rhythms of humans [43]. However prior research suggests that cardiovascular risk factor levels [44], mortality [45], leptin and prolactin levels [46] and insulin response [47] change depending on the season.

A seasonal gene expression pattern has also been suggested in some cells [48]. In fact, Dopico *et al*. 2015 [49] analysed expression level of more than 4,000 protein-coding mRNAs in immune system cells and adipose tissues from population that differed ethnically and geographically. In contrast to some housekeeping genes that did not exhibit seasonal expression patterns, the existence of extensive seasonal gene expression was confirmed, and those differences contribute to the knowledge of human physiology and health. In this study it was pointed out that European and Oceanian expression profiles follow an inverted pattern. For instance, the European profile showed an increase in interleukin-6 (IL-6) and C-reactive protein (CRP)

during winter, both pro-inflammatory proteins that are associated with cardiovascular mortality [49]. Moreover, *Bmal1*, previously mentioned as a clock core gene, showed seasonal variation together with 9 of the 16 clock genes analysed. Therefore, *Bmal1* presented seasonal expression pattern and its expression increased during the summer and resulted lower in the winter, and consequently it promoted an inflammation state in the winter months in humans [50]. According to these previous studies, gene expression is not only affected by circadian patterns, but also by seasonal rhythms showing different expression levels throughout the year. This evidence opens a new perspective for studying the molecular mechanism underlying the occurrence of seasonal diseases which could provide relevant treatments.

Seasonal fluctuations in metabolism have been observed in several studies in both animal models and humans. Human seasonal disorders in mood and behaviour have metabolic factors and the depletion of light exposure during winter contributes to shifts in appetite and body weight, making individuals more prone to weight gain during winter [51]. Moreover, an increase in body weight during winter is associated with the risk of developing seasonal affective disorder (SAD) in humans. Most cases of SAD are associated to short photoperiod exposure and patients affected by this disorder mainly present depression, lack of energy, weight gain and dietary preference for carbohydrates [52], [53]. The craving for carbohydrates is believed to be like a “self-medication” to balance the depletion of serotonin levels in the brain since people affected by SAD show lower levels of serotonin during the winter compared to summer. Additionally, it has been observed that diets deficient in tryptophan (Trp), a precursor of serotonin, are correlated with depression symptoms of SAD, and that their plasma levels vary seasonally. However, recent findings pointed that ingestion of specific proteins (soya protein, casein, gluten, α-lactalbumin) modulate the depression under the short photoperiod increasing the plasma Trp [54], [55]. Based on these results, specific nutritional patterns may be able to modulate seasonal disorders.

Winter-like responsiveness has been described for various metabolic parameters in humans. Studies conducted in humans showed seasonal variation in pre-prandial glucose, blood pressure, and LDL-cholesterol in diabetic patients showing increased levels in the winter compared to summer [56]. The aforementioned metabolic parameters are associated with metabolic syndrome (MetS). It is defined as a cluster of alterations characterized by visceral or intra-abdominal adiposity, insulin resistance (IR), glucose intolerance, hypertension, hypertriglyceridemia, and low HDL-cholesterol [57]. In order to diagnose MetS, three of these criteria must be met and the metabolic parameters associated with this syndrome are found predominantly during the winter, indicating that metabolic parameters change as the season changes [58]. The outcome is an overall disrupted homeostasis which is strictly connected not only to alteration in circadian clock [59], but also in seasonal oscillations.

Clinical studies have observed that insulin resistance has a seasonal pattern, with a higher incidence during the winter months in older people than in middle-aged people. It is worth mentioning that not only light but also temperature plays an important role in explaining these seasonal variations. Indeed, impairment of thermoregulation caused by aging and exposure to low temperatures may contribute to the increase in IR [60]. As a matter of fact, low temperatures are considered an environmental risk factor associated with coronary heart disease, and temperature decreases could explain the seasonal deaths due to coronary heart disease with a prevalence during the winter. Moreover, low temperatures cause vasoconstriction, resulting in an increase of blood pressure to force blood to narrowed veins and arteries and increase in cardiac output [61]. A study conducted on relationship between the occurrence of coronary heart disease and geography also revealed that the deficiency of vitamin D due to a lack of sunlight exposure could favour the synthesis of cholesterol instead of Vitamin D starting from the squalene common precursor [62]. This trend was confirmed by a later study in which was reported that vitamin D deficiency led to a reduction of the vitamin D

receptor (VDR) activity with a consequent increase in the sterol regulatory element-binding protein 2 (SREBP2) activity and the 3-hydroxy-3-methylgluyaryl-coenzyme A reductase (HMGCR) expression. All these changes provoked an enhanced cholesterol biosynthesis rate [63]. In the light of the reported evidence of seasonal environmental factors in cardiac health, it is conceivable that a good lifestyle starting from the diet could ameliorate cardiac health and reduce the number of deaths during winter. In addition to high concentrations of micronutrients, healthy diets characterized by the presence of fruits and vegetables are rich in other bioactive compounds, such as phenolic compounds, which have beneficial effects on cardiovascular and metabolic diseases [64].

Considering this evidence, it is reasonable to conclude that metabolism is sensitive to seasonal variation and these overall data suggest that health is prevalently endangered during winter compared to summer in humans.

5. Effect of seasonal fruit consumption in different light/dark schedules

In light of this evidence, metabolic processes fluctuate throughout the year, and these changes are not only influenced by environmental factors such as daylight duration, but also by dietary patterns. Diets rich in fruits and vegetables have been associated with several benefits, not only for their micronutrients but also because they are rich in phenolic compounds. Moreover, phenolic compounds have been reported to play a role in the modulation of circadian rhythms [8]. However, little is known about the role of phenolic compounds in modulating seasonal rhythms. The composition of macro-, micronutrients and non-nutrients (bioactive compounds) of fruits and vegetables depends on the cultivar and environmental conditions (humidity, temperature, light and internal circadian rhythms of the plant), which in turn changes the concentration and type of secondary metabolites, such as phenolic compounds [65]. Thus, the phenolic profile of plants reflects the

season environment [66], [67]. Phytochemicals interactions with mammals found its explanation in the xenohormesis theory (from Greek *xeno*, strange and *hormesis*, health benefits due to biological stress) according to which there is a coevolution between plants and animals. Plants produce different types of phytochemicals depending on the environmental conditions and animals that consume them can use chemical signals to adapt their metabolism and physiology [68]. A variety of phytochemicals as (poly)phenols, are largely present in human diet exerting beneficial effects on physiology thanks to antioxidant, anti-inflammatory properties, or antihypertensive effects between others [69].

Our research group has carried out studies to determine the effect of chronic exposure to different photoperiods on metabolic and physiological responses and whether the consumption of different phenol-rich fruits out of their harvesting season could provoke a misalignment of metabolism in normo-weight F344 rats and detrimental effects on rats with MetS [70].

The research focused on the effects of seasonal fruits intake on central controllers of seasonality depending on its consumption in or out of season. The effects of two different fruits, the cherry typical of spring summer season and the red grape typical of autumn-winter fruit were evaluated in the key site of circannual clock, the PT and hypothalamus. Interestingly only the red grape but not the cherry produces changes in the modulation on somatostatinergic and dopaminergic systems during short photoperiod. Whereas during long photoperiod red grape causes significant changes in seasonal molecular markers EYA3 and CHGA. Remarkably, the hypothalamic-pituitary-thyroid axis, which is implicated in the modulation of seasonal changes, results modulated by the consumption of red-grape [71].

Moreover (poly)phenol-rich fruit play a role in peripheral response to photoperiod adaptation. As a matter of fact, it has been shown that glucose and lipid metabolism are modulated by cherry consumption in a photoperiod dependent manner in F344 rats Indeed, normo-weight rats, exposed chronically to a short photoperiod, and

supplemented with a spring/summer fruit, the sweet cherry, have decreased phosphorylated levels of Akt2 in skeletal muscle, a protein that plays a pivotal role in insulin signal transduction. Moreover, genes involved in insulin signalling and glucose metabolism showed decreased level as *Insulin receptor substrate 1* (*Irs1*) in the skeletal muscle and *Glucose transporter 2* (*Glut2*) in liver, suggesting insulin insensitivity [70]. Moreover, sweet cherry intake produced higher level of fatty acids transport and beta oxidation gene in normo-weight rats, whereas in MetS rats increases the whole-body fat utilization as energy substrate, by enhancing the detrimental effects caused by a cafeteria diet in F344 rats with MetS when is consumed out of season [70]. Cafeteria diet is a hypercaloric and well-known diet capable to induce metabolic syndrome in animal models, increasing glucose levels, insulin resistance, dyslipidemia and hypertension [72]. Additionally, it has been demonstrated that sweet cherry consumption during long photoperiod could stimulate the lipogenic enzymes genes expression, reduce non-esterified fatty acids levels in serum, atherogenic index and enhance insulin sensitivity [73], highlight the great impact of photoperiod on the effects elicited by cherries consumption.

These studies are not limited to cherry and grape but also to other seasonal fruits such as orange, which displayed different effects depending on the photoperiod of consumption. The orange intake showed to impact the adipose tissue resulting in fat accumulation when consumed in the short photoperiod in healthy F344 rats [74]. Moreover, the consumption of orange during long photoperiod exacerbated the effects induced by cafeteria diet such as dyslipidemia and IR, increasing fatty acid synthesis in white adipose tissue [75].

Differential effects depending on photoperiod were reported for other plants, such as tomatoes are a relevant source of natural antioxidants (phenolics such as flavonoids). A preventive effect against cardiovascular risk was observed when tomatoes were administered in long photoperiod to F344 rats [76]. Moreover, their consumption in season prevented oxidative stress by improving the antioxidant status in healthy F344 rats [77].

Phenolic compounds vary in content and concentration among different fruits but can also vary in the same fruit variety depending on the ripeness of the fruit and geographical origin, which affect both dietary components and polyphenol content. Moreover, polyphenol content is influenced by soil nitrogen, as well as by the type of cultivar system, such as conventional or organic, along with environmental factors [78].

In this sense the bioavailability of phenolic compounds from grapes cultivated in different conditions is strongly modulated by the photoperiod [79]. The bioavailability of phenolic compounds metabolites of the red grapes changes depending on the cultivar systems (organic or conventional) and the photoperiod of consumption in F344 rats. Indeed, grapes consumed out of season and conventionally cultivated showed different bioavailability compared to the organic grape group [79]. It is plausible to believe that the conventional cultivation can provide erroneous chemical cues that would not be the same harvested in a natural condition, resulting in a wrong information in the consuming animals, disrupting their physiology.

Not only fruits but also proanthocyanidins extracts bioavailability have been shown to be influenced by photoperiod exposure depending on the microbiota. Different nutritional patterns are responsible for the variations in individual microbiomes [80] and variability of microbiota composition changes according to the season. A higher bioavailability of proanthocyanidins metabolites is observed under short photoperiod compared to long photoperiod [81].

Overall, these results provide evidence that fruits rich in phenolic compounds have an influence on the modulation of seasonal rhythms in F344 rats. These effects are not limited to the central level, but also occur in peripheral affecting various physiological processes (Figure 4).

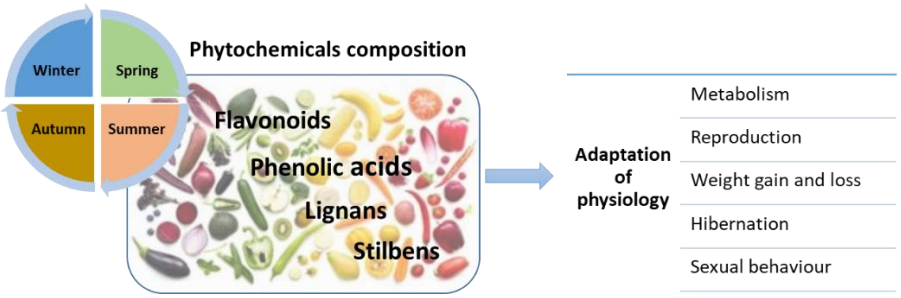

Figure 4. Consumption of seasonal fruits could cause a differential metabolic response in the organism after ingestion due to their specific composition of phenolic hallmark, thus influencing the adaptation of physiology differently.

6. Conclusions and future perspectives

Overall, these preliminary findings are promising for further investigation of nutritional strategies to benefit health. Polyphenol-rich fruit consumption effects change depending on the photoperiod in the animal model studied. Considering that in modern society with a global economy, it is possible to consume food outside their natural season and phenolic compound composition changes, human physiology could be affected in a different manner depending on the type of fruit and the season of consumption. In light of this, more studies are needed to elucidate the effect of seasonal fruit consumption in humans. This would allow to establish how the environment affects (poly)phenol content to further investigate the molecular mechanisms that connect seasonal rhythms with the consumption of fruits rich in polyphenols. Furthermore, eating seasonal food could be not only a healthy dietary behaviour but also more sustainable for the environment, avoiding trade from far away.

7. References

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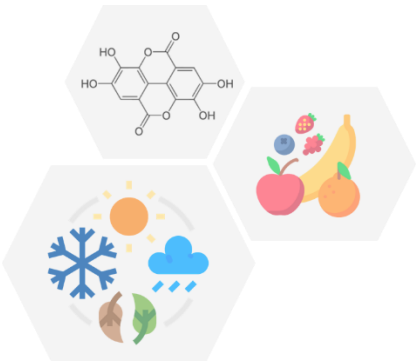


HYPOTHESIS AND OBJECTIVES

Hypothesis and objectives

Physiological and metabolic adaptations to external conditions are eased by annual changes in day length, which is a key cue for entraining seasonal rhythms. Although the underlying mechanisms of circannual rhythms are still being elucidated, they likely involve molecular markers and the remodelling of the pituitary-hypothalamus network. Several tissues display differences in gene expression patterns between winter and summer, and certain metabolic and mental disorders have different incidences, depending on the season. However, there are still outstanding questions regarding how nutritional patterns can modulate these seasonal changes by providing favourable or adverse signals.

In this regard, it has been postulated that phytochemicals contained in fruits and vegetables, such as phenolic compounds, can act as molecular signals in the body. Therefore, specific phytochemical hallmarks would cause distinctive metabolic, physiological, and behavioural responses, allowing the organism to adapt to environmental changes. Moreover, findings from our group showed that the consumption of phenolic-rich fruits has a photoperiod-dependent effect on metabolism. This could be explained by the de-synchrony between the phytochemicals signals and the consumer's photoperiod. Hence, the physiological and molecular changes elicited by seasonal fruit consumption could be partially explained by the modulation of the mammalian clock system. Recent research has shown that phenolic compounds modulate the mammalian clock system, reinforcing the idea that seasonal fruits, with their specific phenolic hallmarks, could act as signals of seasonal rhythms (**Manuscript 1**).

Therefore, **we hypothesized that specific phenolic hallmarks cause different responses in the organism depending on the photoperiod of consumption.**

Thus, the main objective of this PhD thesis was **to determine the differential effects of phenolic-enriched fruit extracts and cherries on the organism in Fischer 344 (F344) rats exposed to different photoperiods**. The substrain F344/IcoCrl was used

as an animal model of photoperiod responsiveness, which is reflected in changes in its physiology and reproductive status according to day length.

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

- **To assess the metabolic effects of short-term administration of fruit extracts enriched in phenolic compounds on healthy F344 rats exposed to different photoperiods** (**Chapter 1**).

  Previous results from our group have shown that phenolic-rich fruit consumption in healthy F344 rats exerts a photoperiod-dependent effect on metabolism. Nevertheless, the use of phenolic-enriched extracts with higher levels of phenolic compounds than the whole fruit could provide more information about the photoperiod-dependent effects of specific phenolic hallmarks. However, the metabolic effects of fruit extract intake in healthy rats subjected to different photoperiods have not been studied. Therefore, in this chapter, extracts with specific phenolic hallmarks obtained from seasonal fruits are used to test their photoperiod-dependent effects on metabolism.

Thus, to assess this objective two goals were proposed:

  - To obtain phenolic-enriched extracts from seasonal fruits and study the effects of their different phenolic hallmarks on serum biochemical biomarkers in F344 rats exposed to different photoperiods **(Manuscript 2)**.
  - To investigate the effects of the obtained phenolic-enriched fruit extracts on the serum metabolome profile of F344 rats exposed to different photoperiods **(Manuscript 3)**.

- **To evaluate the photoperiod-dependent effects of phenolic-enriched fruit extracts on obesity-related metabolic alterations (Chapter 2)**.

  Metabolic challenge tests were performed in rats exposed to photoperiods to determine the effects of phenolic-enriched fruit extracts on blood

markers. These tests provoke a temporarily disturbed body's homeostasis and in response the system strives to restore it. Consequently, quantification of the stress response reaction will provide information about the effect of phenolic extract on the metabolic alterations.

Thus, to assess this objective, the following goal was proposed:

- To evaluate the photoperiod-dependent effects of phenolic-enriched fruit extracts on blood lipid markers in F344 rats after an oral lipid tolerance test **(Manuscript 4)**.
- To investigate the photoperiod-dependent effects of phenolic-enriched fruit extracts on blood inflammatory markers in F344 rats after lipopolysaccharide (LPS) challenge **(Manuscript 4)**.

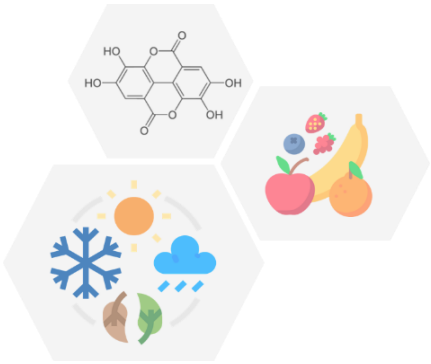
- **To study the impact of seasonal consumption of whole fruits with different phenolic hallmarks on cerebral and peripheral responses (Chapter 3)**.

  Fruit extracts are a good alternative for adequate intake of phenolic compounds; however, the effects of seasonal consumption of fresh fruits should also be considered to establish dietary recommendations. Previous studies of the group have investigated the effects of sweet cherries on the hepatic and adipose tissues in healthy animals. However, the crosstalk between the neuroendocrine response elicited by the photoperiod and the consumption of sweet cherries has not been fully elucidated. Therefore, cherries with different phenolic compositions were used in this study to test the photoperiod-dependent effectiveness of their specific phenolic hallmarks on central and peripheral tissues. To assess this objective, two goals were proposed:

  - To investigate the effects of sweet cherries with different phenolic hallmarks on the hippocampus and hypothalamus in a photoperiod-dependent manner in F344 rats **(Manuscript 5)**.

- To study the impact of seasonal consumption of sweet cherries with different phenolic hallmarks on thyroid hormone, an important seasonal marker that links neuroendocrine response to peripheral tissue, and its effect on hepatic metabolism in F344 rats **(Manuscript 6)**.

The research performed in this thesis has been supported by the grant number PID2020-113739RB-I00 funded by MCIN/AEI /10.13039/501100011033. This thesis has been carried out at the Nutrigenomics Research Group of the Universitat Rovira i Virgili with predoctoral fellowship from Universitat Rovira i Virgili—Martí i Franquès (Grant number: 2019PMF-PIPF-19). An international research stay was carried at the University of Bradford (UK) from 5th September 2022 to 3rd February 2023 in neuroendocrinology research group under the supervision of Dr. Gisela Helfer and has been supported by Research visit grant of British Society for Neuroendocrinology and by Aurora traineeship grant by Universitat Rovira I Virgili.



EXPERIMENTAL DESIGNS

Experimental designs

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

Here are showed different experimental design and complying with European legislation for experimental animal use, the principle of 3Rs was used and specifically the animals used in Manuscript 2 were used in Manuscript 3 and 4. A total of 144 male Fischer 344 rats, 8 weeks of age and fed a standard chow diet and tap water *ad libitum* throughout the experiments.

1. To assess metabolic effects of seasonal fruit extracts with specific phenolic hallmarks in F344 rats exposed to different photoperiods.

Animals were randomly housed into two groups (n = 72, per group) and subjected to different photoperiods, short photoperiod with 6 h light (L6, which would simulate the winter season of day length), or long photoperiod with 18 h light (L18, which would simulate the summer season of day length), in which lights were turned on at 7:00 a.m. After 4 weeks of adaptation to both photoperiods, rats were daily administered 100 mg/kg body weight (BW) of each extract for two weeks. A group supplemented with 1 mL of tap water (C) was added as a control for each photoperiod. Supplementation was performed by voluntarily liking a syringe between 8:00 a.m. and 9:00 a.m. After 15 days, the rats were deprived of food for 12 h and administered either the extracts or C. Blood was collected by to measure glucose, TAGs, cholesterol, and insulin (Manuscript 2).

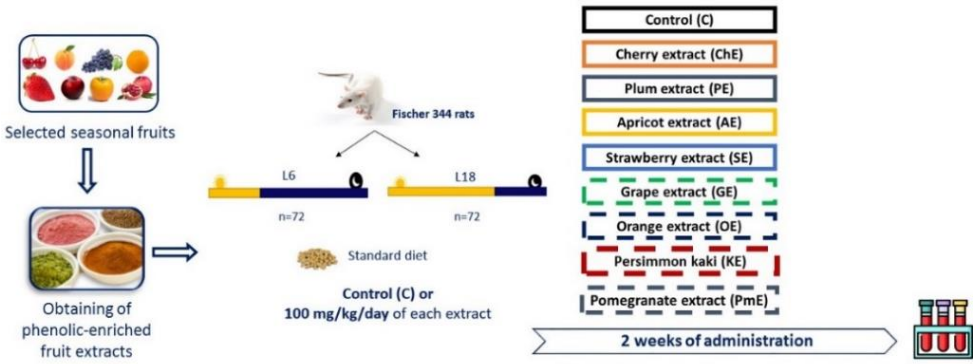


Figure 1 Experimental design. Eight seasonal fruit extracts were obtained and characterized and further administered at 100 mg/kg/day for 2 weeks to Fischer 344 rats exposed to short photoperiod (6 h of light, 18 of dark) and long photoperiod (18 h of light, 6 of dark).

Animals were fed *ad libitum* STD diet and water for a total of 4 days as washout. To investigate more deeply metabolome profile after 15 days of supplementation of fruits extracts, blood was collected from saphenous vein and analysed with gas chromatography coupled to Quadrupole Time-of-Flight Mass Spectrometry (Manuscript 3).

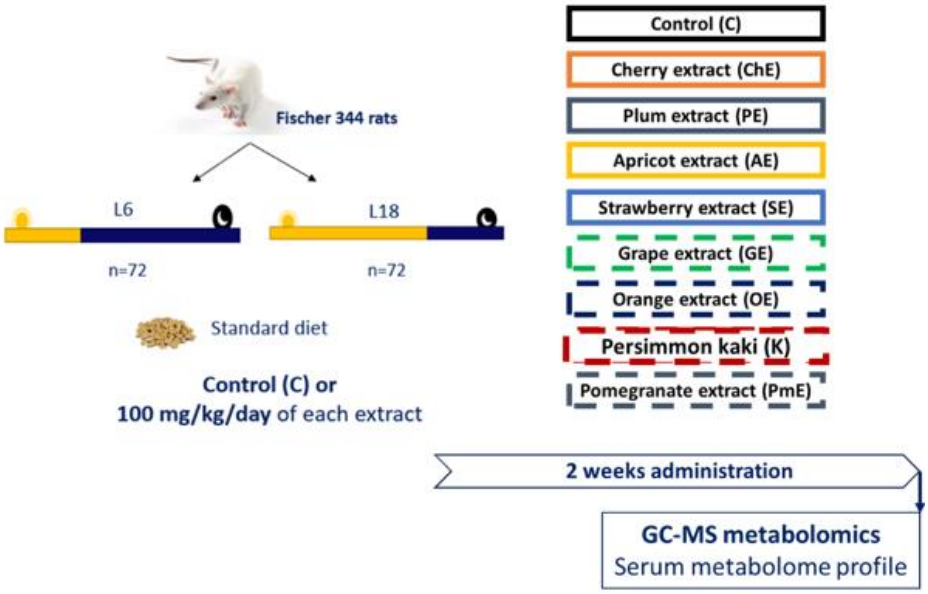


Figure 2. Rats were supplemented with eight seasonal fruit extracts and exposed to short and long photoperiod for 2 weeks and blood was collected at the end to analyse metabolome serum profile.

2. To assess the most effective fruit extract in the management of metabolic alterations related to obesity depending on the photoperiod.

Animals adapted to short and long photoperiod after 2 weeks of supplementation of eight fruit extracts were temporarily submitted to metabolic alterations. Animals were administered either extracts or control (tap water), after 3 h were orally gavage with lard oil (2.5 ml/Kg), to perform an oral lipid tolerance test (OLTT). Then blood from cut tail was collected to determine triglycerides at different time points: 0, 30, 60, 120, 240 min. After OLTT rats followed 4 days of wash-out, in which were not supplemented with fruit extracts and only STD-diet and water *ad libitum* were provided. After, rats were administered fruit extracts for 15 days. 3 hours after last extracts or water (C) were supplemented, 0.5 mg/kg of body weight of LPS was intraperitoneally injected. After 4 hours, rats were sacrificed by decapitation and blood was used to look at inflammatory markers (Manuscript 4).

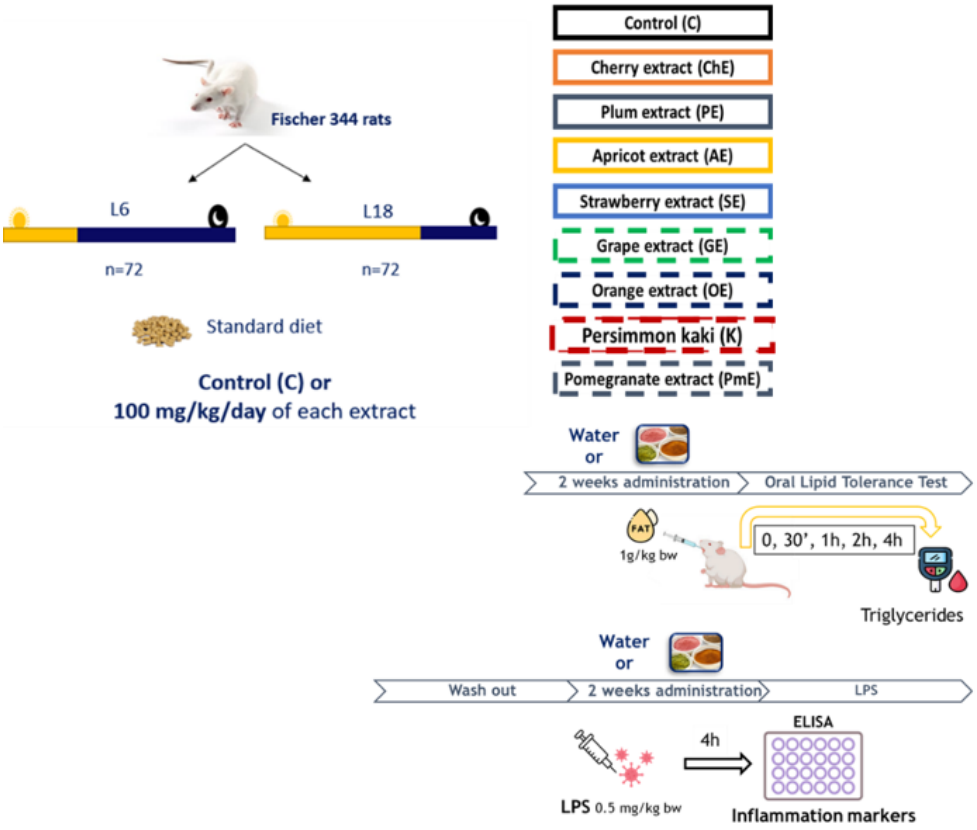


Figure 3. Rats exposed to short and long photoperiods consumed 8 seasonal extracts for 2 weeks and oral lipid tolerance test to assess triglycerides management and LPS-induced inflammation challenge to assess anti-inflammatory properties of fruits extracts were performed.

3. To study the impact of seasonal consumption of sweet cherries with different phenolic hallmarks on cerebral and peripheral responses.

Animals divided into three photoperiods: L18 (long; 18 h light/6 h dark), L12 (intermediate; 12 h light/12 h dark), and L6 (short; 6 h light/18 h dark), fed a standard chow diet and tap water ad libitum. After a 4-weeks photoperiod adaptation, rats housed in each photoperiod were subdivided into three groups (n= 8 per group) and supplemented daily for 7 weeks with 100 mg Ch1 dry weight

(dw)/kg BW (Ch1 group) and 100 mg Ch2 dw/kg BW (Ch2 group). Control group (C group) was administered 42.4 mg/kg BW of a mixture of glucose and fructose (1:1, w/w; vehicle), to simulate the sugar content contained in cherries.

To study the effects of sweet cherries with different phenolic hallmarks on hippocampus and hypothalamus in a photoperiod dependent manner in F344 rats, brain was collected at the end of the experiment and blood was used to look at photoperiod sensitive hormones (Manuscript 5).

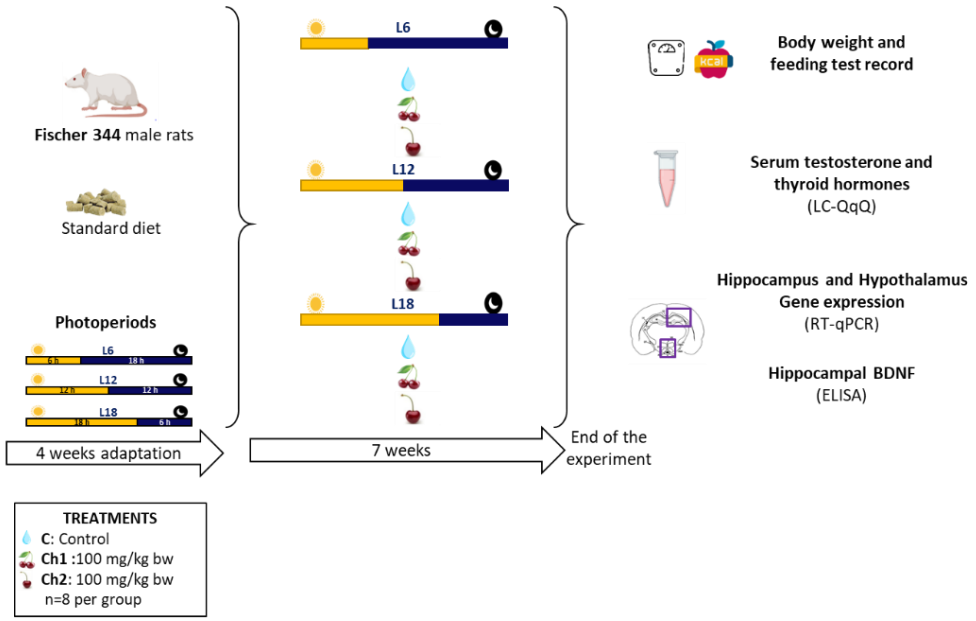


Figure 4. Fischer 344 rats exposed to different photoperiods were supplemented with cherries with different composition and from different geographical origin for 7 weeks. At the end of the experiment brain and blood was collected.

To investigate the impact of seasonal consumption of sweet cherries with different phenolic hallmarks on thyroid hormones and hepatic metabolism in F344 rats, liver was collected at the end of experiment (Manuscript 6).

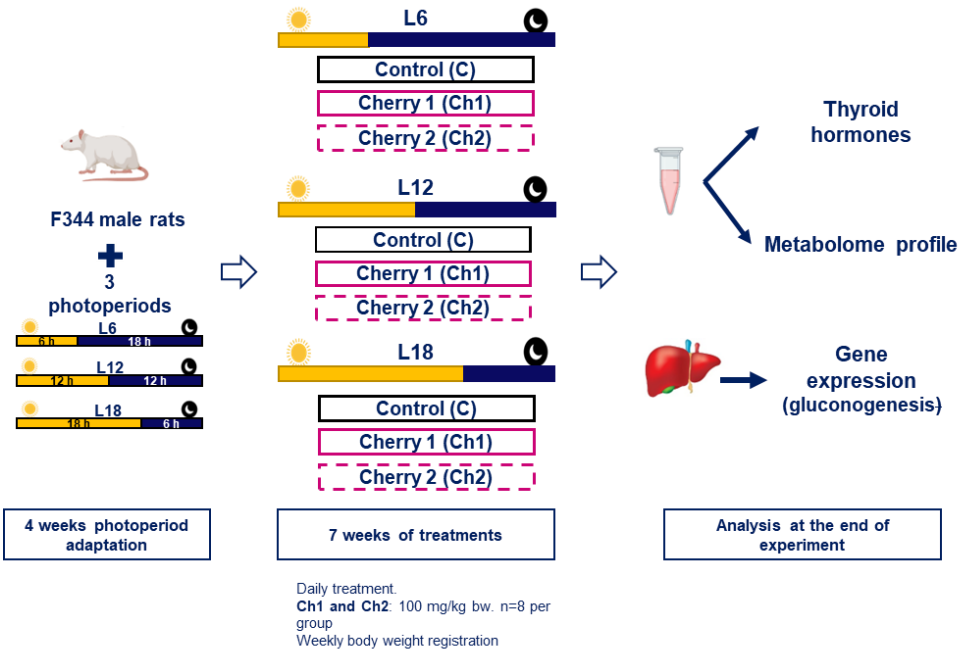
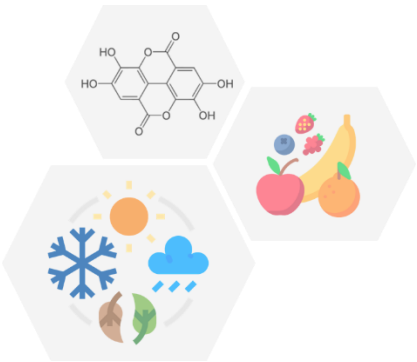


Figure 5. Fischer 344 rats exposed to different photoperiods were supplemented with cherries with different composition and from different geographical origin for 7 weeks. At the end of the experiment liver and blood was collected.



RESULTS

Chapter 1

To assess the metabolic effects of short-term administration of fruit extracts enriched in phenolic compounds on healthy F344 rats exposed to different photoperiods.

Manuscript 2

Photoperiod-dependent effects on serum biochemical markers of phenolic-enriched fruit extracts

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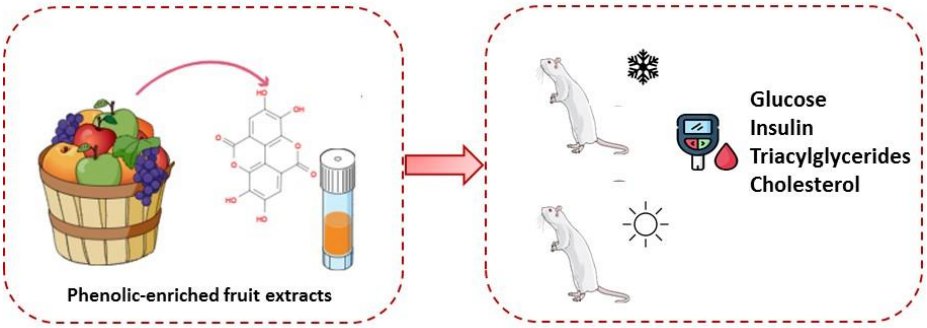
Graphical abstract



Abstract

The importance of eating seasonal fruit has recently received attention as a sustainable habit. Fruits are rich in bioactive compounds such as (poly)phenols, and their intake is associated with many health benefits, although recent studies have suggested that the photoperiod in which they are consumed influences their beneficial properties. However, fruit loss and waste are critical issues for modern society, and obtaining functional fruit extracts enriched in bioactive compounds can reduce fruit loss. Therefore, the aim of this study was to obtain eight phenolic-enriched extracts from seasonal fruits, four from winter (persimmon kaki, grape, orange, and pomegranate) and four from summer fruits (cherry, plum, apricot, and strawberry), enriched in phenolic content with respect to whole fruits, and to evaluate their effects on serum biochemical parameters depending on the photoperiod of consumption. Eight ethanolic extracts were obtained and characterized, and their potential beneficial effects were studied in Fischer 344 rats exposed to a short photoperiod (6 h light, L6) and a long photoperiod (18 h light, L18), which simulated winter and summer, respectively. The results showed phenolic enrichment in the extracts compared with the whole fruit. In addition, the effects of fruit extracts on biochemical markers depended on the photoperiod in which they were administered and were mainly detected when the animals were exposed to L6. Moreover, blood cholesterol and glucose levels decreased when animals consumed extracts obtained from winter fruits, indicating a correlation between the photoperiod of consumption and seasonality of the fruits.

Keywords: photoperiod, (poly)phenols, seasonal fruits, seasonality.

1. Introduction

The consumption of diets rich in fruits and vegetables has been linked to health benefits such as the reduction of high blood pressure, blood triacylglyceride (TAG), and cholesterol levels [1]. Nevertheless, between 4-7 % in the United States and Europe, and more than 10 % in other countries, such as Africa and Latin America, fruit production is lost [2]. Moreover, these losses have been established to be higher on farms, reaching values up to six times higher than the off-farm losses [3]. Although there are strategies to reduce these losses, including prolonging the half-life of these fruits during harvesting, storage, or transportation [4], [5], and to increase the consumption of more seasonal foods associated with health benefits [6], more strategies are needed. Thus, these unused fruits could be utilized to obtain extracts rich in bioactive compounds, which would allow their valorisation into value-added products and contribute to the farm circular economy.

Although fruits are rich in many bioactive molecules, (poly)phenols are one of the main compounds responsible for their beneficial effects [7]. Phenolic compounds are secondary plant metabolites produced in response to stress factors [8]. They comprise a wide range of structures, with more than 8,000 different compounds that contain at least one aromatic ring containing one or more hydroxyl groups [9]. Flavonoids are the major phenolic compounds consumed (60 %) and are subdivided into anthocyanidins, flavanols, flavanones, flavones, flavonols, and isoflavones [10]. Several studies have reported that (poly)phenols can exert a wide range of effects, including reduction of dyslipidaemia and insulin resistance [11]. Nevertheless, phenolic-induced health effects vary depending on the specific (poly)phenols consumed, dose, bioaccessibility, or bioavailability [12]. In this regard, the profile and quantity of phenolic compounds in a specific fruit or vegetable are affected by plant type and variety, as well as by environmental, harvesting, storage, and transport conditions [13]. In fact, consumption of sweet cherries from two different

geographical origins showed different phenolic compositions and exerted different effects on liver and serum antioxidant markers in Fisher 344 (F344) rats [14].

In this context, xenohormesis theory postulates that phenolic compound intake can act as a signal molecule in animals, informing of external conditions, and allowing them to adapt to environmental changes through variations in physiological, behavioural, and metabolic processes [15]. Additionally, it has been suggested that (poly)phenols can help in the adaptation of physiology to seasonal rhythms [16], [17]. Recent studies by our research group have shown that the beneficial effects of fruits vary depending on the photoperiod during which they are consumed [14], [16]. For instance, the intake of sweet cherries increased serum insulin levels and the HOMA index only in F344 rats exposed to a short photoperiod (6 h light, L6) [18]. In contrast, animals exposed to a long photoperiod (18 h of light, L18) showed decreased and increased serum levels of non-esterified fatty acids and high-density lipoprotein cholesterol, respectively, compared with the levels observed in the control group exposed to the same photoperiod. However, no effects were observed when the same cherries were consumed by animals exposed to L6 and an intermediate photoperiod (12 h light, L12) [18]. Other studies have shown that the effects of a grape seed phenolic-enriched extract on the liver and adipose tissue depended on the photoperiod at which they were consumed [19], [20]. Considering these antecedents, the aim of this study was to obtain phenolic-enriched extracts from different seasonal fruits and evaluate their effects on the biochemical parameters of glucose and lipid metabolism in F344 rats exposed to different photoperiods.

2. Materials and Methods

2.1. Obtaining of fruit extracts

Ripe summer (cherry (*Prunus avium L.*), apricot (*Prunus armeniaca*), strawberry (*Fragaria vesca* L.), plum (*Prunus domestica*)) and winter fruits (grape (*Vitis vinifera*), orange (*Citrus sinensis L.*), persimmon (*Diospyros kaki*), and pomegranate

(*Punica granatum*) were acquired during the summer and winter seasons, respectively, from local producers. Pedicels were manually removed, and whole fruits, including the skin, seed, and pulp, were mechanically triturated and mixed to obtain homogenates of strawberry and grapes. For cherry, apricot, plum and persimmon seed were removed before triturated and for orange and pomegranate skin was removed before being triturated. The homogenates were stored at -20 °C until the extraction process.

For extraction, the fruit homogenates were mixed with ethanol and formic acid (1 %) under the conditions listed in Table 1. These conditions were selected based on previous optimization of extraction methods for phenolic compounds, and plum and strawberry were extracted using the optimized method for cherries [21] persimmon kaki was extracted following the optimized method for apricots [22], pomegranate following the optimized method for grapes [23], and orange was extracted using its own optimized method [24]. The mixtures were vigorously stirred in a Max400 orbital shaker (Thermo Fisher Scientific, Madrid, Spain) at 250 rpm at the times and temperatures specified in Table 1. After extraction, the mixtures were cooled to room temperature and centrifuged (7,871 *x g*, 4 °C, 10 min). Ethanol was removed from the supernatants using a Hei-VAP rotary evaporator (Heidolph, Schwabach, Germany) at 40 °C, and the obtained samples were freeze-dried and stored at –20 ºC until further use. The yield of the extraction process was calculated by dividing the weight of each dried extract by the weight of the used dried fruit and was expressed as a percentage (g wt/100 g wt) (Table S1). Yields ranged between 28.85 and 86.57 % with the highest values in grape, cherry, and orange and the lowest in apricot and plum.

2.2. Proximate composition of fruits and extracts

Ash, protein, fat, and soluble and insoluble dietary fibre contents were determined in the extracts according to the official methods of analysis of the Association of Official Analytical Chemists (AOAC) [25]. The ash content was calculated as the

inorganic residue remaining after the water and organic matter were removed by heating (550 °C for 24 h). Protein content was quantified using the Kjeldahl method with conversion factor of 6.25 [25], [26]. Soluble and insoluble fibre contents were determined by treating the sample with heat-stable α-amylase, proteases from *Bacillus licheniformis*, and amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, Madrid, Spain), and subsequent precipitation of fibre with ethanol according to the AOAC protocol [25]. The total lipid content of the samples was determined using the Folch method [27]. For that, samples were prepared according to the method described in Hewavitharana *et al.* 2020 [28] as follows: lipids were extracted from the samples (5 % w/v) using chloroform:methanol (2:1 v/v), and the lower phase was recovered after the addition of a saline solution.

Total polyphenol, anthocyanin, and flavanol content was analysed following the methodology described by Iglesias-Carres et al. 2019 [29] with modifications. In this regard, protein precipitation with 1.48 % HCl was carried out in the samples to avoid the interference of amino acids in the determination of total polyphenols using the Folin-Ciocalteu method. The results are expressed as mean ± SD and expressed as mg of gallic acid equivalents (GAE)/g of dried product, catechin equivalents (CatE)/g of dried product, and cyanidin-3-O-rutinoside equivalents (Cy3RE)/g of dried product for total polyphenol, flavanol and anthocyanin content, respectively. All analyses were performed in triplicate. The proximate composition of the fruits was also determined and are shown in Table S2.

2.3. Determination of antioxidant activity

The antioxidant activity of the extracts was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity, as described by Mau *et al*. 2001 [30]. Different sample concentrations (0.02-1 mg/mL) were tested against DPPH (76 µM), and the absorbance at 517 nm was recorded after of 15 min incubation at room temperature. The IC_{50} was calculated using the linear correlation obtained

with increasing sample concentrations and is referred to as Trolox equivalents (TE). Results were expressed as Trolox equivalent antioxidant capacity (TEAC).

2.4. Experimental procedure in rats

A total of 144 male F344 rats, 8 weeks of age, with body weight around 250 g were purchased from Charles River Laboratories (L'Arblese, Cedex, France) and fed a standard chow diet (AO4, Panlab, Barcelona, Spain) and tap water *ad libitum* throughout the experiment.

Animals were adapted for 1 week and randomly housed into two groups (n = 72 per group) and subjected to L6 or L18 photoperiods, simulating winter and summer seasons, respectively, in which lights were turned on at 7:00 a.m. After 4 weeks of adaptation to both photoperiods, rats were administered 100 mg/kg body weight (BW) daily of each extract for two weeks (n=8 per group). A group supplemented with 1 mL of tap water was used as the control (C) for each photoperiod. Supplementation was performed by voluntarily liking a syringe between 8:00 a.m. and 9:00 a.m. Rats were weighed, once a week to monitor the welfare of the animals. After 2 weeks, the rats were deprived of food for 12 h and administered either the extracts or water. Blood was collected from a cut tail 3 h after treatment to measure the blood levels of glucose, TAGs, and cholesterol. For insulin quantification, blood was collected from the saphenous vein 3 h after treatment administration and centrifuged (3,000 x g for 15 min at room temperature), and the serum was stored at -80 °C until use. The experimental design is illustrated in Figure 1. All procedures were conducted in accordance with the guidelines established by the Animal Ethics Committee of the Universitat Rovira i Virgili (Tarragona, Spain) and were approved by the Generalitat de Catalunya (permission number 11610).

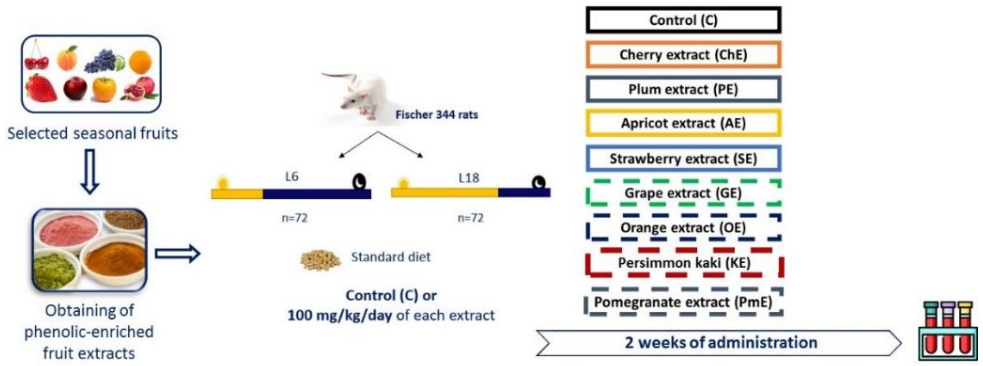


Figure 1. Experimental design used in this study.

2.5. Biochemical analysis

Blood glucose levels were measured using a glucometer (glucomen A. Menarini Diagnostics). The total blood TAG and cholesterol levels were measured using an Accutrend Plus instrument (Roche Diagnostics, Barcelona, Spain). Blood was centrifuged, and serum was used to quantify insulin with an ELISA Kit rat/mouse Insulin 96 Well plate assay (EMD Millipore Corporation, St Louis, Missouri, USA). Samples were assayed in duplicate, as indicated by the manufacturer's protocol. The HOMA index was calculated as follows: HOMA= fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5.

2.6. Statistical analysis

Two- and one-way analyses of variance (ANOVA) were used to determine differences among groups using the Post-hoc Tukey test. Two-way ANOVA is indicated as P, photoperiod effect; E, extract effect; PxE, photoperiod effect, and extract. * indicate significant differences between groups ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively). The Student's t-test was used to estimate significant differences ($p < 0.05$) produced by photoperiod in each treatment. The Statistical Product and Service Solutions (SPSS) software (SPSS Inc., Chicago, IL, USA) was used

for the statistical analysis. Outliers were identified using SPSS and removed before statistical analysis.

3. Results

3.1. Proximate composition of fruit and fruit extracts

No differences were found in ash, protein, lipid, and fibre contents between extracts (Table 2), while protein contents were different among them, ranging from 0.18 to 1.03 %, with the highest values found in orange extract and the lowest in grape extract. Total polyphenol content of the extracts ranged between 4.40 and 54.79 mg GAE/g of dried extract, with the highest values found in the strawberry, cherry, and orange extracts and the lowest in the persimmon kaki extract (Table 3). Total flavanols in the extracts ranged between 0.09 and 1.92 mg CatE/g of dried extract, with the highest values found in the grape extract>strawberry extract>plum extract. Finally, total anthocyanidin content ranged from 0.01 to 0.3 mg Cy3RE/g of dried extract. The highest values in anthocyanins were observed in the plum extract, whereas the content in anthocyanins was not detected in apricot, persimmon kaki, and pomegranate extracts. All the extracts showed higher total polyphenol content per dry mass than the original fruits (Tables S2 and 3). Specifically, it is worth highlighting that the total polyphenol content was three times higher in persimmon kaki and orange extract, and twice in strawberry extract, in comparison with the amount found in their respective whole fruits. Moreover, the extraction process significantly concentrated the flavanol content in all the extracts. Extracts from cherry, plum, and orange were also enriched in anthocyanidins.

The antioxidant activity of the extracts ranged between 10.61 and 174.90 µmol TE/g of dried extract (Table 3). Pomegranate, grape, and strawberry extracts exhibited the highest antioxidant capacities, while persimmon kaki extract showed the lowest activity.

3.2. Effects of fruit extracts on blood lipid profile

Exposure to different photoperiods had a significant effect on blood TAG levels, which increased in animals under L6 respect to L18 ($p < 0.05$, two-way ANOVA) (Figures 2A and B). Moreover, L6-exposed animals showed higher TAG levels when only control groups were compared. In addition, blood TAGs significantly decreased in F344 rats exposed to the L6 photoperiod and administered cherry and apricot extracts compared with the control L6-exposed rats (Figure 2A). No differences in this parameter were observed for other extracts. In the L18 photoperiod, none of the extracts reduced TAG levels in the treated animals compared with the L18-C group. In contrast, pomegranate extract significantly increased TAGs in L18-exposed rats (Figure 2B), with no significant differences between this group and L6-exposed rats administered water and pomegranate extract (Figure 2A). In addition, a photoperiod-dependent differential effect was observed in animals consuming strawberry and persimmon kaki extracts, with higher TAG values found in the L6 photoperiod for both extracts (Figures 2A and B).

Cholesterol levels decreased when rats consumed three of the four winter-fruit extracts (grape, orange, and pomegranate extracts) compared to the C group during the L6 photoperiod, and no changes were observed in the cholesterol levels of animals administered the other winter-fruit extract (persimmon kaki extract) (Figure 2C). However, none of the extracts decreased cholesterol levels compared to C group in the animals were exposed to the L18 photoperiod (Figure 2D). When L6 and L18 were compared, an interaction between the photoperiod and extract consumption was observed (two-way ANOVA, P and E, $p<0.05$). A differential effect was observed in L6- and L18-exposed rats after intake of plum ($p<0.05$), strawberry ($p<0.05$), orange ($p<0.05$), and pomegranate ($p<0.05$) extracts, with lower levels in the L18 photoperiod (Figures 2C and D).

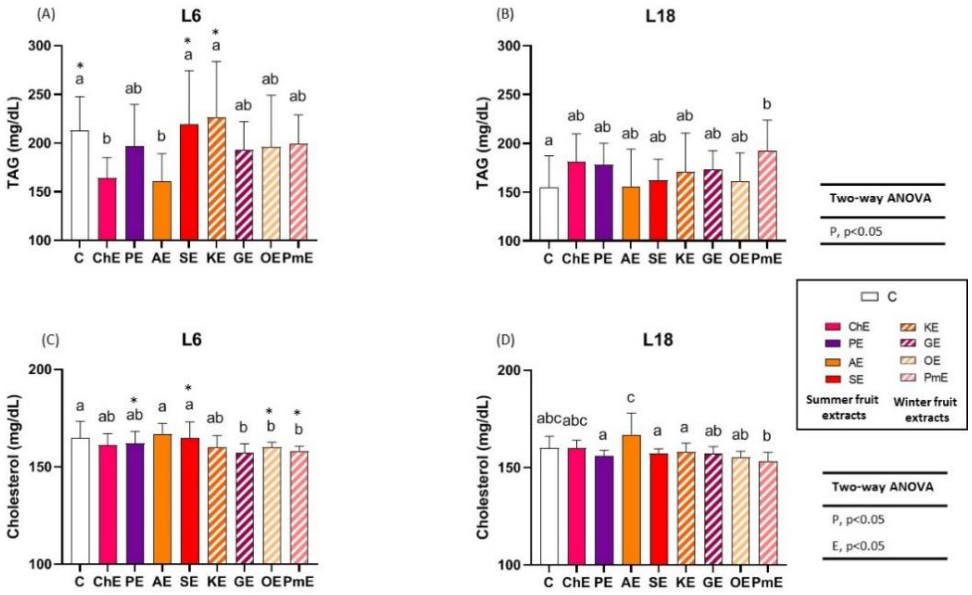


Figure 2. Blood total triacylglyceride (TAG) (A and B), and cholesterol levels (C and D) of Fisher 344 rats exposed to L6 (6 h light) (A and C) and L18 (18 h light) (B and D) photoperiods, and administered water (C) or 100 mg/kg body weight of cherry (ChE), plum (PE), apricot (AE), strawberry (SE), persimmon kaki (KE), grape (GE), orange (OE) and pomegranate (PmE) extracts (n=8 per group, mean ±standard deviation). Different letters above the bars indicate significant differences among groups in a specific photoperiod (one-way ANOVA; post-hoc Tukey). Differences found by two-way ANOVA ($p<0.05$, post-hoc Tuckey-HSD) was indicated as P, photoperiod effect; E, extract effect; PxE, interaction between photoperiod and extract effect. * indicates significant differences of a specific treatment between photoperiods (Student's t-test, $p<0.05$).

3.3. Effects of fruit extracts on blood glucose and insulin levels

A significant effect of photoperiod was observed on glucose levels in control L6- and L18-exposed animals (Figures 3A and B). Fasting blood glucose levels were affected by photoperiod exposure, the type of extract consumed and an interaction between both factors were found (two-way ANOVA P, E, PxE $p<0.05$). Most winter-fruit extracts (grape, orange, and pomegranate extracts), except for persimmon kaki extract, were able to reduce blood glucose levels in animals exposed to the L6

photoperiod in comparison to the C group; however, only the summer-fruit extract obtained from strawberry reduced glucose levels in the L6 photoperiod (Figure 3A). No effects on fasting blood glucose levels were observed in L18-exposed animals consuming the different fruit extracts compared with those in the L18-C group (Figure 3B). Moreover, L6-exposed animals consuming all extracts showed higher blood glucose levels than L18-exposed animals consuming the same extract (Figures 3A and B).

Regarding insulin levels, an interaction between photoperiod and fruit extract consumption was observed (two-way ANOVA, P, E, PxE $p<0.05$). Blood insulin levels were higher in animals exposed to the L6 photoperiod and administered apricot, strawberry, persimmon, grape, orange, and pomegranate extracts than in the L6-C group (Figures 3C). Regarding to L18-exposed animals, cherry, grape, orange, and pomegranate extracts increased insulin levels respect to L18-C group (Figure 3D). Photoperiod effects were observed in control animals and rats consuming cherry, plum, and persimmon extracts, with higher values found in L18-exposed animals than in L6-exposed animals, except for persimmon extract, which showed the opposite effect (Figures 3C and D). Overall, HOMA index showed similar pattern to insulin levels (Figure 3E and F). Nevertheless, in this case, the photoperiod effect was only observed in control animals and in rats administered cherry and persimmon extracts (Figures 3E and F). As it was observed for insulin levels, rats that consumed pomegranate extract showed the highest HOMA value in L18 photoperiod.

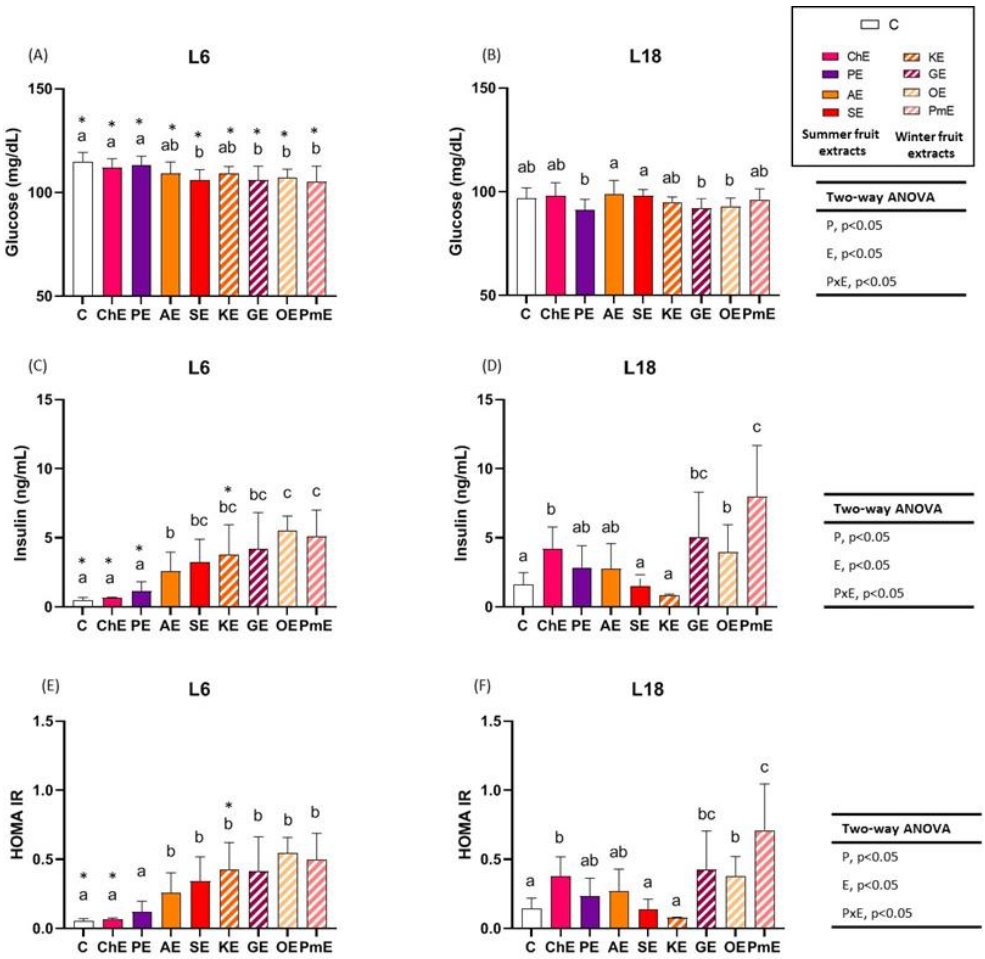


Figure 3. Blood glucose levels (A and B) and insulin levels (C and D), and HOMA index (E-F) of Fisher 344 rats exposed to L6 (6 h light) (A, C and E) and L18 (18 h light) (B, D and F) photoperiods, and administered water (C) or 100 mg/kg body weight of cherry (ChE), plum (PE), apricot (AE), strawberry (SE), persimmon kaki (KE), grape (GE), orange (OE) and pomegranate (PmE) extracts (n=8 per group, mean ± standard deviation). Different letters above the bars indicate significant differences among groups in a specific photoperiod (one-way ANOVA; post-hoc Tukey, $p<0.05$). Differences found by two-way ANOVA ($p<0.05$, post-hoc Tuckey-HSD) was indicated as P, photoperiod effect; E, extract effect; PxE, interaction between photoperiod and extract effect. * indicates significant differences of a specific treatment between photoperiods (Student's t-test, $p<0.05$).

4. Discussion

According to the Food and Agriculture Organization of the United States, 14 % of food was lost after harvest in 2019 [31]. The highest losses are fruits and vegetables [3], and strategies to avoid these losses are required. In this regard, fresh fruits represent a useful source for obtaining extracts enriched in bioactive compounds that can exert relevant health benefits [32]. Phenolic compounds are among the most fruit-bioactive compounds and have been shown to exert beneficial effects [33]. However, recent studies show that these effects can differ depending on the photoperiod during which they are consumed [16]–[18]. Therefore, the purpose of this study was to obtain phenolic-enriched extracts from seasonal fruits and evaluate whether their effects on the modulation of biochemical markers in healthy F344 rats were photoperiod-dependent.

Phenolic compounds are soluble in organic solvents, and extraction with ethanol, methanol, acetone, or their combination with water is the most used method [34]. This extraction process breaks down plant cell walls, and polymeric complexes are freed into lower-molecular-weight compounds, which show higher bioavailability [35]. Ethanol was chosen for the generation of the fruit extracts, as it is generally recognized as a safe (GRAS) substance that can be used by the food industry [36]. In addition, ethanol extraction is widely used to prepare functional extracts [37]. Our results showed that ethanol-based extractions at low temperatures and in short times allowed us to obtain phenol-enriched extracts from all fruits. Differences in fruit variety, pre-and post-harvest conditions, fruit parts used to obtain the extract and extraction conditions can explain the variations in phenolic content between extracts [37], [38]. Interestingly, the extraction conditions used in this study are easily replicable on an industrial scale, which could facilitate the potential transfer of this methodology to the food industry to produce phenol-enriched fruit extracts for use as functional ingredients. Similar values in total (poly)phenol content of fruit extracts have been reported by strawberry extracts

(1.3 to 2.7 mg GAE/g dried extract) and by pomegranate juice and extracts obtained from pomegranate seeds and peels (12.0 to 276 mg GAE/g dried extract) [39]. However, higher values of total phenolics were reported for other fruit extracts, but they were obtained using a higher extraction time than in the present study (2 days vs 20-100 min, respectively) [37]. Spigno *et al.*, 2007 also reported an increase in the total polyphenol content of extracts from red grapes when extraction time was increased from 1 to 20 h in both 45 and 60 °C, with the highest values at 60 °C [38].

One of the most important activities of phenolic compounds is their antioxidant capacity [40]. Fresh fruits, such as strawberries [41], grapes [42], plums [43], and pomegranate [44], have shown high antioxidant activity, which is correlated with the abundance of phenolic compounds. All eight extracts showed DPPH scavenging capacity, with pomegranate extract being the most active. Other studies have also reported high antioxidant activity measured by the β-carotene bleaching test of aqueous ethanolic extracts (80 %, 25 °C, 48 h) of peel, seed, and juice from different varieties of pomegranate [45]. In addition to pomegranate, a high antioxidant capacity has been reported for other fruit extracts, such as an aqueous ethanol cherry extract (70 %, room temperature, 2 h), measured as nitric oxide scavenging activity [46].

Along with their antioxidant properties, phenolic compounds have been shown to play a key role in the regulation of several metabolic processes, acting on protein regulation and gene expression, and their effects can differ depending on the photoperiod of consumption [16]. In this present study, two summer-fruit extracts (cherry and apricot extracts) decreased the TAG levels respect to the control-L6 exposed rats and the winter-fruit extracts (grape, orange and pomegranate extracts) showed hypocholesterolemic activity in short photoperiod. Previous studies have reported that the consumption of some whole fruits by healthy animals produced an improvement in blood lipids. For instance, rats consuming sun-dried apricot for 120 days switched towards a hypotriglyceridaemia state, and

increased blood total cholesterol and high-density lipoprotein cholesterol (HDL-C) levels in a gender-dependent manner [47]. But, to the best of our knowledge, this is the first time that phenol-enriched fruit extracts exhibit different effects depending on the photoperiod.

The effects of phenol-enriched fruit extracts on lipid profile were not only dependent on the photoperiod, but different properties were also observed for the different extracts. It is known that different fruits have different phenolic compositions and activities when ingested [48]. Interestingly, fruits from the same variety have also shown distinctive phenolic hallmarks, depending on the cultivar system, geographical origin, and other factors, and display different effects depending on the fruit variety and photoperiod [14]. As a matter of fact, sweet cherry consumption for 7 weeks affected blood TAG levels in F344 rats in a photoperiod-dependent manner, increasing or not their levels in the L6 photoperiod, depending on the phenolic composition of the cherry. These differential effects between cherries of the same variety were attributed to their different (poly)phenol signatures [49]. However, in this study, none of the cherries produced changes in total cholesterol levels in comparison with untreated animals, as observed in the present study. Similarly, Gibert-Ramos *et al.* 2019 observed that two oranges of different origins showed distinct fat accumulation in F344 rats exposed to L6 and L18 photoperiods, which was attributed to the different concentrations of phenolic compounds present in each orange [50]. Furthermore, no differences were observed in TAG and total cholesterol levels in F344 rats after intake of any of the oranges for 10 weeks in comparison with the control group [50]. This is not in agreement with our results, in which orange extract reduced total cholesterol levels when consumed in short photoperiod. These discrepancies could be attributed to differences in the phenolic hallmarks and levels of total (poly)phenols between whole fruits and extracts. For instance, in the present study, (poly)phenol concentrations in cherry and orange extracts were 1.4 and 3 times higher, respectively, than those quantified in the corresponding whole fruits). In

addition, the bioavailability of phenolic compounds present in the extract can vary with respect to the whole fruit, as they are released from the cellular matrix [51].

Regarding the effects of the extracts on blood glucose levels, cherry, grape, orange, and pomegranate extracts decreased this parameter in L6-exposed F344 rats. Phenolic compounds have been shown to control glycemia, which was attributed to the inhibition of salivary and pancreatic enzymes during sugar digestion [52]. In addition, another study showed that pomegranate and grape extracts were able to modulate carbohydrate digestive enzyme activity, including the *in vitro* inhibition of α-amylase and α-glucosidase [53]. Photoperiod effects on blood glucose levels were also observed in F344 rats exposed to different photoperiods and consuming the enriched-phenolic grape seed extract GSPE for 9 weeks, although and unlike our study, GSPE increased its concentration in the L6 and L18 photoperiods compared to their respective control animals and treated and untreated L12-exposed animals [19]. In healthy animals, insulin is released by the pancreatic islets after carbohydrate meals to remove glucose from the bloodstream. When elevated levels of insulin are produced owing to the intake of high-glycaemic index foods, an insulin-resistant phenotype can develop [54]. In the present study, all animal groups were considered insulin-sensitive, with a HOMA index below 1, although most of the extracts tended to increase the HOMA index under fasting conditions (close to 1). Moreover, a differential effect on insulin and HOMA index was observed after the intake of cherry, plum, and persimmon extracts (plum only for insulin levels) when they were consumed in different photoperiods. Cherry and plum extracts tended to increase the effect of the photoperiod. However, pomegranate extract consumed in L6-photoperiod increased the insulin levels, and the HOMA index was higher than that shown by the L18-C group and did not produce any effects when consumed in the L18 photoperiod. These findings are in concordance with those of previous studies, in which serum insulin levels and the HOMA index were higher in F344 rats exposed to a short photoperiod (L6) and consuming sweet cherries for 7 weeks than in control animals, whereas no effects

were observed in animals under the L18 photoperiod [18]. However, in the present study, cherry extracts increased serum insulin levels only during the L18 photoperiod, which could be due to the different profiles and amounts of phenolic compounds between the whole cherry and the cherry extract used in this study.

5. Conclusions

In this study, it was shown that fruits can be used to produce extracts enriched in phenolic compounds with beneficial effects that can be easily replicated at an industrial scale. Moreover, the extracts were capable of modulating blood fasting lipid, glucose, and insulin levels in a photoperiod-dependent manner. Fruit extracts produced greater effects when administered under a short photoperiod than under a long photoperiod. The pomegranate extract stood out for its in vitro antioxidant activity and caused a clear differential effect depending on photoperiod. The extract presented cholesterol-lowering and fasting glucose-lowering properties in L6, whereas it increased TAG levels in L18, highlighting the importance of seasonal consumption of this extract. Other extracts from winter fruits presented similar patterns, such as grape and orange extracts, which decreased blood cholesterol and glucose levels only when administered in season (in L6). In addition, plum extract could help maintain fasting glucose levels administered during the L18 photoperiod and did not alter the HOMA index and TAG and cholesterol levels. However, cherry, and apricot extracts decreased TAG levels only when administered at L6. To the best of our knowledge, this is the first time that fruit extracts have been shown to exhibit photoperiod-dependent effects. These results suggest that the season of consumption should be considered in the study of the beneficial effects of phenolic-enriched extracts and functional ingredient design. Nevertheless, further studies are needed to investigate whether these fruit extracts could be useful in the prevention of hyperlipidaemia or hyperglycaemia, and to identify the most effective extracts for each photoperiod.

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8. Conflict of Interest

The authors declare no conflict of interest.

9. Author Contributions

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

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Tables

Table 1. Extraction conditions used for each fruit.

Fruit	Solvent to solute (mL/g)	Ethanol (%)	Time (min)	Temperature (°C)
Cherry	20	72	20	55
Plum	20	72	30	55
Apricot	20	72	30	38
Strawberry	40	72	30	55
Persimmon kaki	20	72	30	38
Grape	80	65	100	72
Orange	30	70	40	55
Pomegranate	80	65	100	72

Table 2. Proximate composition of the dried extracts.

Fruit extract	Protein (%)	Fat (%)	Insoluble fibers (%)	Soluble fib (%)	Ash (%)
Cherry	0.85 ± 0.02[a]	4.19 ± 2.59	2.13 ± 0.96	1.95 ± 0.67	1.02 ± 0.27
Plum	0.42 ± 0.01[b]	6.27 ± 0.87	2.59 ± 0.98	1.22 ± 1.14	0.90 ± 0.31
Apricot	0.81 ± 0.01[a]	8.41 ± 2.92	1.81 ± 0.04	1.00 ± 1.29	1.85 ± 0.18
Strawberry	0.75 ± 0.01[c]	8.47 ± 0.29	2.24 ± 0.70	1.14 ± 1.19	1.61 ± 0.10
Persimmon kaki	0.33 ± 0.01[b]	8.75 ± 3.41	1.69 ± 0.15	1.04 ± 1.29	0.89 ± 0.11
Grape	0.18 ± 0.02[e]	9.82 ± 0.25	9.21 ± 3.39	2.49 ± 1.12	1.16 ± 0.01
Orange	1.03 ± 0.02[d]	6.45 ± 1.23	2.17 ± 0.61	1.13 ± 0.74	0.76 ± 0.07
Pomegranate	0.48 ± 0.08[b]	2.46 ± 0.30	2.41 ± 0.12	2.95 ± 1.48	1.07 ± 0.02

Values are expressed as the mean ± standard deviation (n=3). Different letters indicate significant differences between groups for each compound ($p \leq 0.05$; one-way ANOVA, post-hoc Tukey test).

Table 3. Total polyphenol, flavanol, and anthocyanidin contents of the dried fruit extracts and their antioxidant activity

Fruit extract	Total polyphenols (mg GAE/g dw)	Total flavanols (mg CatE/g dw)	Total anthocyanidins (mg Cy3RE/g dw)	Antioxidant activity (µmol TE/g)
Cherry	14.50 ± 0.64^{a}	0.30 ± 0.00 ^{a}	0.21 ± 0.01^{a}	34.79 ± 5.13^{a}
Plum	19.26 ± 0.02^{b}	1.7 ± 0.10^{b}	0.29 ± 0.00^{b}	56.50 ± 5.47^{b}
Apricot	9.19 ± 0.02^{a}	0.09 ± 0.00^{c}	n.d.	26.72 ± 4.14^{a}
Strawberry	54.79 ± 0.28^{d}	1.88 ± 0.03^{b}	0.18 ± 0.01 ^{a}	81.78 ± 4.10^{c}
Persimmon	4.40 ± 0.19^{e}	0.10 ± 0.00^{c}	n.d.	10.61 ± 3.14^{d}
Grape	17.44 ± 0.52^{b}	1.92 ± 0.12^{b}	0.01 ± 0.00^{c}	107.62 ± 8.61^{e}
Orange	12.48 ± 0.39^{a}	0.18 ± 0.01^{a}	0.03 ± 0.00^{d}	26.11 ± 1.26^{a}
Pomegranate	12.85 ± 0.41^{a}	0.25 ± 0.00^{a}	n.d.	174.90 ± 7.29^{f}

Values are expressed as the mean ± standard deviation (n=3). Different letters indicate significant differences between groups in each column ($p<0.05$; one-way ANOVA, post-hoc Tukey test). CatE, catechin equivalents; Cy3RE, cyanidin-3-O-rutinoside equivalents; GAE, gallic acid equivalents; dw, dried weight; n.d, not detected; TE, Trolox equivalents.

Supplementary material

Table S1. Yield of the extract process

Fruit	**Yield** (%)
Cherry	83.27 ± 5.09^{ab}
Plum	33.84 ± 0.94^{d}
Apricot	31.26 ± 3.41^{d}
Strawberry	64.66 ± 2.63^{bc}
Persimmon kaki	55.40 ± 2.55^{c}
Grape	86.57 ± 4.05^{a}
Orange	80.07 ± 2.89^{ab}
Pomegranate	73.43 ± 0.77^{b}

Yield of the extraction process was calculated by dividing the weight of each dried extract by the weight of the used dried fruit. Different letters indicate significant differences between groups (one-way ANOVA, $p<0.05$, post-hoc Tukey test)

Table S2. Proximate composition of the dried fruits.

Fruits	Protein (%)	Fat (%)	Insoluble fiber (%)	Soluble fibers (%)	Ash (%)	Total polyphenols (mg GAE/g dw)	Total flavanols (mg CatE/g dw)	Total anthocyanidins (mg Cy3RE/g dw)
Cherry	6.77 ± 0.04 ^{a}	0.66 ± 0.01 ^{a}	2.66 ± 0.51 ^{a}	2.54 ± 1.89	5.48 ± 1.37	10.17 ± 1.03 ^{a}	0.00 ± 0.00	0.13 ± 0.01 ^{a}
Plum	4.38 ± 0.22 ^{b}	1.00 ± 0.09 ^{b}	4.36 ± 0.95 ^{a}	6.10 ± 1.56	9.03 ± 3.43	12.97 ± 1.86 ^{a}	0.01 ± 0.00	0.05 ± 0.01 ^{b}
Apricot	6.14 ± 0.07 ^{a}	0.74 ± 0.00 ^{ab}	10.22 ± 1.29 ^{b}	6.77 ± 1.75	9.38 ± 2.36	6.26 ± 0.28 ^{b}	n.d.	0.01 ± 0.00 ^{c}
Strawberry	8.93 ± 0.09 ^{c}	0.59 ± 0.00 ^{a}	15.35 ± 1.88 ^{b}	6.17 ± 3.22	4.93 ± 0.25	22.92 ± 1.43 ^{c}	0.01 ± 0.00	0.30 ±0.03 ^{a}
Persimmon kaki	4.20 ± 0.14 ^{b}	1.11 ± 0.20 ^{a}	26.81 ± 1.43 ^{c}	4.17 ± 1.19	2.23± 0.02	1.20 ± 0.03 ^{d}	0.00 ± 0.00	0.03 ± 0.00 ^{b}
Grape	2.91 ± 0.99 ^{b}	3.09 ± 0.22 ^{c}	23.26 ± 3.92 ^{c}	1.83 ± 0.81	10.05 ±5.49	10.10 ± 1.68 ^{a}	0.02 ± 0.00	0.08 ± 0.01^{b}
Orange	6.95 ± 2.47 ^{a}	2.12 ± 0.04 ^{c}	19.61 ± 5.41 ^{c}	15.38 ± 11.23	3.95 ± 1.47	3.62 ± 0.03 ^{e}	0.00 ± 0.00	0.02 ± 0.00 ^{c}
Pomegranate	7.09 ± 0.89 ^{a}	2.65 ± 0.35 ^{c}	13.57 ± 0.18 ^{b}	4.17 ± 1.29	2.86 ± 1.65	9.79 ± 0.08 ^{a}	0.00 ± 0.00	0.16 ± 0.01^{a}

Values are expressed as mean ± standard deviation (n=3). Different letters indicate significant differences between groups (one-way ANOVA, $p<0.05$, post-hoc Tukey test). CatE, catechin equivalents; Cy3RE, cyanidin-3-O-rutinoside equivalents; GAE, gallic acid equivalents; dw, dried weight; n.d, not detected.

Manuscript 3

Seasonal fruit extracts regulate metabolome serum profile depending on photoperiod

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Abstract

Fruit rich diets are associated with health benefits. There is growing evidence that phenolic compounds contained in fruits not only exert health benefits but also act as signals in the regulation of biological rhythms. However, the metabolic response when extracts from fruits of different seasons are consumed under different light/dark schedules remains to be elucidated. A useful tool for analysing the metabolic response to certain supplements widely used nowadays is the metabolomic approach. Our goal was to evaluate the effects of seasonal fruit extracts from eight fruits on the metabolome serum profile in Fischer 344 rats exposed to short (6h of light, 18h of dark) and long photoperiod (18 h of light, 6 h of dark), which simulate winter and summer seasons, respectively. Winter-fruit extracts acted differently on the metabolome serum profile depending on whether they were consumed during short or long photoperiods.

Keywords: fruits, extracts, phenolic compound, photoperiod, metabolome.

1. Introduction

Plants produce phenolic compounds in response to stressful environmental factors, such as UV radiation and temperature [1]. Their consumption through diets rich in fruits and vegetables has been associated with the prevention of chronic metabolic diseases such as hypertension, diabetes, and obesity [2], [3]. However, their effects vary depending on the administered doses, phenolic profile, and concentration, which in turn affects their bioavailability and active phenolic derived metabolites. Moreover, recent studies have shown that fruits rich in (poly)phenols, as well as grape seed proanthocyanidin extract (GSPE), exerted different metabolic outputs depending on the photoperiod of consumption [4]. In this regard, orange consumption was associated with fat accumulation in the adipose tissue of healthy rats under a short photoperiod [5]. Moreover, sweet cherry consumption increased the cell area of adipocytes [6] as well as blood glycaemic levels in obese rats when consumed during a short photoperiod but not during a long photoperiod [7]. In addition, GSPE affected hepatic metabolism and modulated gut microbiota composition in a photoperiod-dependent manner [8] [9]. However, the photoperiod effects of different fruit extracts on metabolic responses in Fisher 344 (F344) rats have not yet been investigated.

Current metabolomic profiling approaches offer an alternative tool for evaluating intermediate molecules and products of metabolism [11]. Indeed, metabolomic profiles indicate the whole metabolic response to dietary patterns, taking into account genetic variations and the intervention of the gut microbiota in the physiological response to diet [12]. The present study focused on evaluating the effects of eight different seasonal-fruit extracts on the metabolome serum profile of F344 rats exposed to a short photoperiod (6 h light, 18 h dark), which simulates the winter light schedule, and a long photoperiod (18 h light, 6 h dark), which simulates the summer light schedule.

2. Materials and Methods

2.1. Fruit extracts and reactives

Ripe summer (cherry (*Prunus avium L.*), apricot (*Prunus armeniaca*), strawberry (*Fragaria vesca* L.), plum (*Prunus domestica*)) and winter fruits (grape (*Vitis vinifera*), orange (*Citrus sinensis L.*), persimmon (*Diospyros kaki*), and pomegranate (*Punica granatum*) were acquired during the summer and winter seasons, respectively, from local producers. Pedicels were manually removed, and whole fruits, including the skin, seed, and pulp, were mechanically triturated and mixed to obtain homogenates of strawberry and grapes. For cherry, apricot, plum and persimmon seed were removed before triturated and for orange and pomegranate skin was removed before being triturated. The homogenates were stored at -20 °C until the extraction process. Then, the fruit homogenates were mixed with ethanol and formic acid (1 %) under the conditions listed in Table S1. The mixtures were vigorously stirred in a Max400 orbital shaker (Thermo Fisher Scientific, Madrid, Spain) at 250 rpm at the times and temperatures specified in Table S1. After extraction, the mixtures were cooled to room temperature and centrifuged (7,871 *x g*, 4 °C, 10 min). Ethanol was removed from the supernatants using a Hei-VAP rotary evaporator (Heidolph, Schwabach, Germany) at 40°C, and the obtained samples were freeze-dried and stored at –20 °C until further use.

The internal standards succinic acid (D4, 98%), myristic acid (D27, 98%), norvaline, adonitrol, glycerol (13C3, 99%), D-glucose (13C6, 99%) were provided by Sigma Aldrich. (4-Hydroxyphenyl-2,3,5,6-) acetic-2,2-acid (D6, 98%) was purchased from CDN Isotopes (Leacock, Canada). Alpha-ketoglutaric acid (D6, 98%) and the metabolomics amino acid mix standard containing glycine (13C2, 99%; 15N, 99%), L-Alanine (13C3, 99%; 15N, 99%), L-Arginine·HCl (13C6, 99%; 15N4, 99%), L-Aspartic Acid (13C4, 99%; 15N, 99%), L-Cystine (13C6, 99%; 15N2, 99%),L-Glutamic Acid (13C5, 99%; 15N, 99%), L-Histidine·HCl·H2O (13C6, 97-99%; 15N3, 97-99%), L-Isoleucine (13C6, 99%; 15N, 99%), L-Leucine (13C6, 99%; 15N, 99%), L-Lysine·2HCl

(13C6, 99%; 15N2, 99%), L-Methionine (13C5, 99%; 15N, 99%), L-Phenylalanine (13C9, 99%; 15N, 99%), L-Proline (13C5, 99%; 15N, 99%), L-Serine (13C3, 99%; 15N, 99%), L-Threonine (13C4, 97-99%; 15N, 97-99%), L-Tyrosine (13C9, 99%; 15N, 99%), L-Valine (13C5, 99%; 15N, 99%)) were obtained from Cambridge Isotope Laboratories. All reagents were analytical grade.

2.2. Animal experiment

Eight-week-old male F344 rats (n=144) were purchased from Charles River Laboratories (L'Arblese, Cedex, France) and fed a standard chow diet (AO4, Panlab, Barcelona, Spain) and tap water ad libitum during the experiment. Animals were housed in two groups (n = 72 each) in two different photoperiods, short photoperiod of 6h light and 18h dark (L6) or long photoperiod of 18 h light and 6 dark (L18), for both lights turned on at 7:00 a.m. During the 4 weeks of photoperiod adaptation, the rats were trained to lick a syringe with tap water. After acclimatisation, rats were administered for two weeks for 100 mg/kg of body weight (BW) per day of each extract (cherry, plum, apricot, strawberry, persimmon kaki, orange, grape, and pomegranate extracts). A group supplemented with tap water (C) was used as a control for each photoperiod. Supplementation was performed by voluntarily liking a syringe between 8:00 and 9:00 a.m. Body weight and food intake were recorded once a week. After 15 days of supplementation, blood was collected from the saphenous vein in non-heparinized tubes and centrifuged (3,000 *×g* for 15 min at room temperature) to obtain serum. It was stored at -80 °C until use. The experimental design is illustrated in Figure 1. All procedures were conducted in accordance with the guidelines established by the Animal Ethics Committee of the Universitat Rovira i Virgili (Tarragona, Spain) and were approved by the Generalitat de Catalunya (permission number 11610).

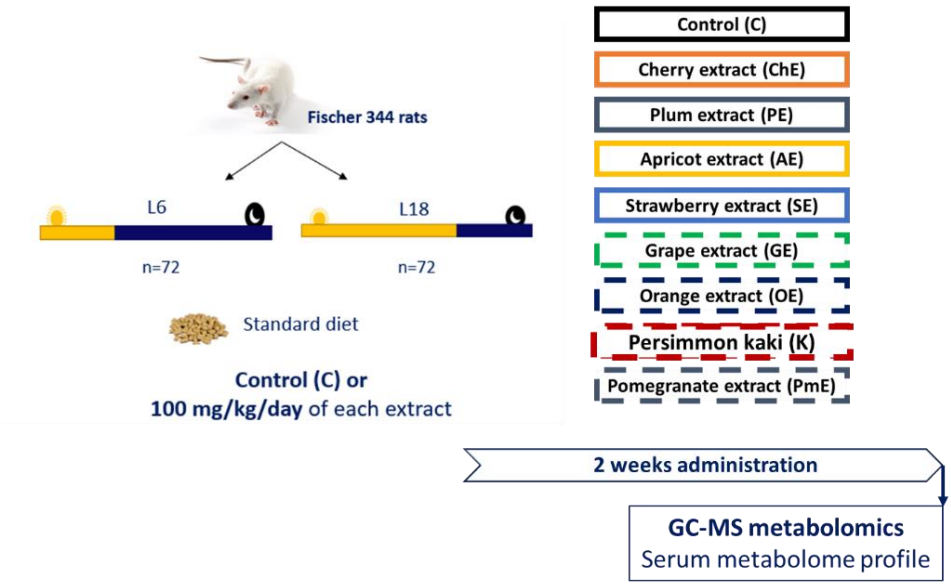

Figure 1. Experimental design. Fischer 344 rats were exposed to a short photoperiod (L6, 6 h light and 18h dark) and a long photoperiod (L18, 18 h light and 6 h dark) for six weeks. The last two weeks, the standard diet was supplemented with water (control, C) and 100 mg/kg body weight of four summer-fruit (solid line) or four winter-fruit extracts (dashed lines). On the final day, blood from the saphenous vein was collected and serum was analysed using gas chromatography coupled to quadrupole time-of-flight mass spectrometry (GC-qTOF) to study the metabolome profile of animals.

2.3. Metabolomics analysis

A protein precipitation extraction was carried by adding eight volumes of methanol:water (8:2) containing internal standard mixture to serum samples. Then, the samples were mixed and incubated at 4 °C for 10 min, centrifuged at 15,000 rpm and supernatant was evaporated to dryness before compound derivatization (methoximation and silylation). The derivatized compounds were analysed by gas chromatography coupled to quadrupole time-of-flight mass spectrometry (GC-qTOF, model 7200 of Agilent, USA).

Identification of metabolites was accomplished using commercial standards and by matching their EI mass spectrum and retention time to metabolomic Fiehn library

(Agilent), which includes more than 1.400 metabolites. After putative identification of metabolites, these were semi-quantified in terms of internal standard response ratio. The multivariate analysis was performed using MetaboAnalyst software 5.0 available online, for partial least squares discriminant analysis (PLS-DA), variable importance projection (VIP) score. All metabolites values are in Table S2 of supplementary material section. To validate the model, permutation testing was performed allowing 1,000 permutations. The significance of the models ranged from $p<0.01$ and $p < 0.001$, verifying the validity of the models.

2.4. Statistical analysis

Body weight and food intake are represented as mean ± standard deviation and analysed to two-way analysis of variance (ANOVA) to determine photoperiod (P) and extract (E) effect, or interaction between the two factors (PxE) (Post-hoc Tuckey), using the Statistical Product and Service Solutions (SPSS) software (SPSS Inc., Chicago, IL, USA). Differences were considered with ($p< 0.05$). Outliers were identified using SPSS and removed before the statistical analysis.

3. Results

3.1. Body weight and food intake

Final body weight after 2 weeks of supplementation with water (C) or fruit extracts are shown in Table 1. The two-way ANOVA revealed a photoperiod effect ($p<0.01$), with a decrease in this parameter in animals exposed to short photoperiod with respect to animals exposed to the long photoperiod. No differences were observed between the groups exposed to the same photoperiod. Regarding food intake, no differences between extract consumption or photoperiod were observed (Table 1).

Experimental groups	Body weight		Food intake	
	L6	L18	L6	L18
Control	318.9 ± 18.9	333.75 ± 13.6	16.18 ± 0.6	17.4 ± 1.3
Cherry	317.1 ± 16.3	344.9 ± 11.1	17.15 ± 1.2	18.7 ± 1.8
Plum	315.5 ± 19.2	333.2 ± 19.8	16.4 ± 1.3	18.4 ± 1.8
Apricot	312.9 ± 14.5	339.2 ± 24.5	17.2 ± 1.1	16.9 ± 0.7
Strawberry	312.0 ± 15.7	337.1 ± 23.1	17.3 ± 1.7	18.8 ± 1.2
Persimmon kaki	312.6 ± 25.5	336.6 ± 13.8	17.2 ± 1.1	17.3 ± 1.7
Grape	310.1 ± 14.3	350.4 ± 22.9	16.9 ± 0.7	19.0 ± 1.3
Orange	320.5 ± 21.6	338.5 ± 11.4	17.5 ± 2.0	18.0 ± 0.3
Pomegranate	320.5 ± 14.7	346.5 ± 25.8	17.9 ± 1.9	18.8 ± 1.7
2-way ANOVA	**P (p<0.01)**		n.s.	

Table 1. Final body weight and food intake of Fischer 344 rats exposed to L6 (6 h light/ 18 h dark) and L18 (18 h light/ 6 h dark) photoperiods for 6 weeks and administered water (control) or extracts (100 mg/kg body weight) the last two weeks. Values are expressed as mean ± standard deviation (n = 8 per group). P indicates significant differences in photoperiod (two-way ANOVA, 2wA, $p<0.05$), n.s. indicate non-significant differences. No significant differences among groups per photoperiod were observed (one-way-ANOVA, Post-hoc Tuckey, $p<0.05$).

3.2. Multivariate analyses PLS-DA

59 serum metabolites were identified by GC-qTOF and analysed with PLS-DA model. PLS-DA showed clear clustering of the control groups depending on photoperiod (Figure 2A). The separation between both control groups was due to several metabolites with VIP scores equal to or higher than 1. Oxoproline, citric acid, and taurine levels were found higher in the L18-C group than in the L6-C group, whereas

phosphoric acid, palmitic acid, and lactic acid levels were higher in the L6-C group than in the L18-C group (Figure 2B).

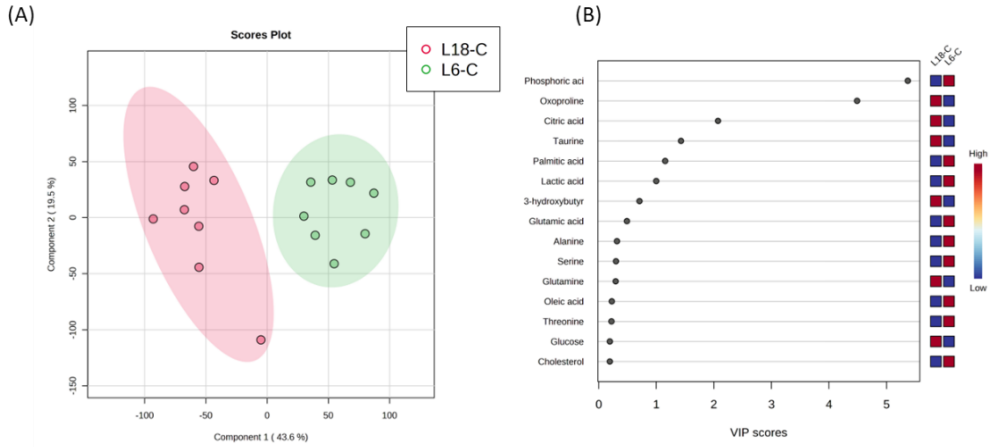

Figure 2. (A) Scores plot of partial least squares-discriminant analysis (PLS-DA) based on metabolomics data between serum samples of Fisher 344 rats exposed to the short photoperiod (L6, 6h light) and the long photoperiod (L18, 18 h light) for six weeks and administered water (control, C) the last two weeks (n=8 per group). Shaded circles represent 95% confidence intervals, while colored dots illustrate individual samples. The axes are labelled by the first and second components with the percentages of variance of the data explained by that component in parentheses. (B) Variable importance in projection (VIP) scores of serum metabolomics data of serum samples of Fisher 344 rats exposed to the short photoperiod (L6, 6h light) and the long photoperiod (L18, 18 h light) for six weeks and administered water (control, C). The colored legend on the right indicates the relative abundance of variables, with red and blue indicating high and low values, respectively.

Figure 3A shows the PLS-DA obtained based on the serum metabolomics of F344 rats exposed to both photoperiods and administered the eight fruit extracts or water (C). PLS-DA analysis showed a different pattern between the consumption of winter-fruit extracts in the L6 and in L18 photoperiods (Figure 3A and B). In the L6 photoperiod, animals consuming persimmon kaki extract had the highest separation, followed by animals consuming grape extract compared to the control and other extracts (Figure 3A). When the same winter-fruit extracts were

consumed under long photoperiod grape and orange extracts were completely separated by the L18 control group (Figure 3C). However, this evident separation of both groups was not observed when animals were administered extracts during the L18 photoperiod. Regarding the effects of the summer-fruit extracts, the separation observed for these treated groups was quite similar between both photoperiods (Figure 3B and D). Moreover, apricot extract-treated animals were separated from their respective control groups under both L6 and L18 photoperiods. The responsible metabolic compounds of the observed group separation in the L6 photoperiod based on the VIP score (equal or higher than 1) were glucose, taurine, phosphoric acid, oxoproline, palmitic acid, lactic acid, glutamine, 3-hydroxybutyric acid (Figure 4A). In the case of the L18 photoperiod, the VIP score was high for oxoproline, taurine, glucose, palmitic acid, and glutamine (Figure 4C). The VIP score that determined the separation under L6 was higher for phosphoric acid, oxoproline, citric acid and lactic acid; under L18 the VIP score equal to or greater than one was for phosphoric acid, oxoproline, citric acid, glutamine, palmitic acid, lactic acid, 3-hydroxybutiric acid, and taurine (Figure 4 B and D).

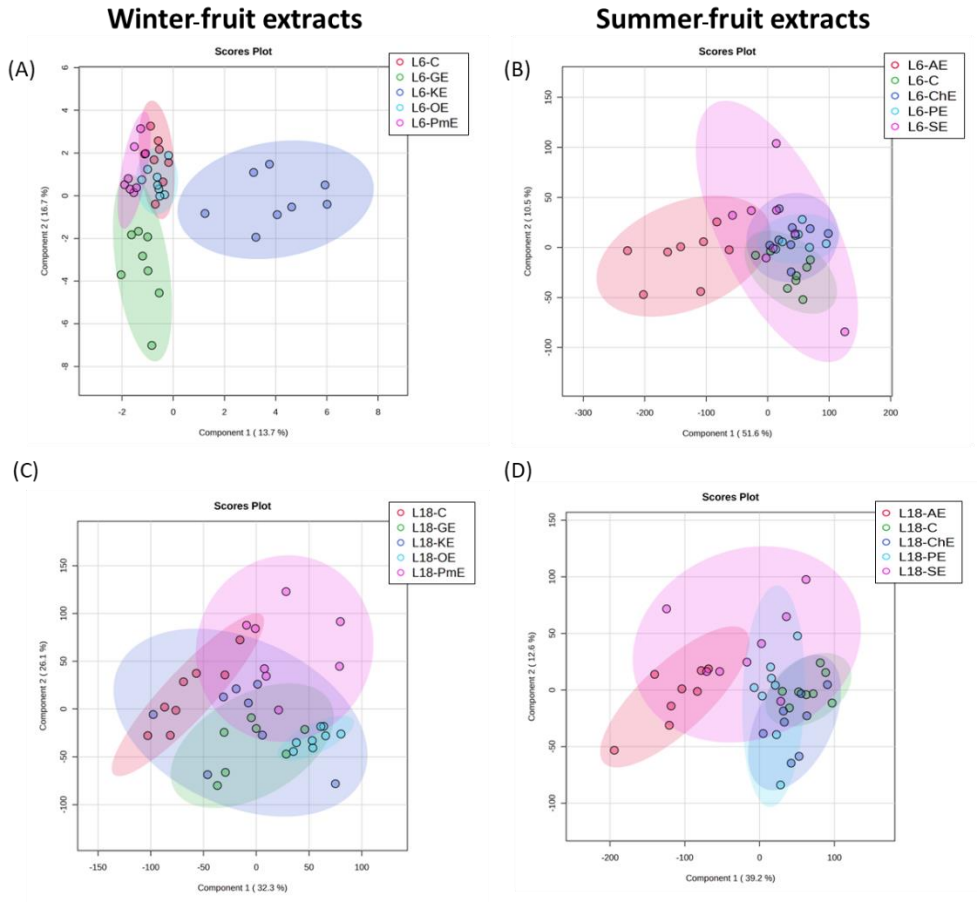

Figure 3. Scores plot of partial least squares-discriminant analysis (PLS-DA) based on metabolomics data between serum samples of Fisher 344 rats exposed to the short photoperiod (L6, 6h light, figure A and B) and the long photoperiod (L18, 18 h light, figure C and D) for six weeks and administered water (control, C) or extracts (E, 100 mg/kg body weight) obtained from four winter-fruits (persimmon kaki (K), grape (G), orange (O), and pomegranate (Pm), Figure A and C) and four summer fruits (cherry (Ch), plum (P), apricot (A), strawberry (S), figure B and D) the last two weeks (n=8 per group). Shaded circles represent 95% confidence intervals, while colored dots illustrate individual samples. The axes are labelled by the first and second components with the percentages of variance of the data explained by that component in parentheses.

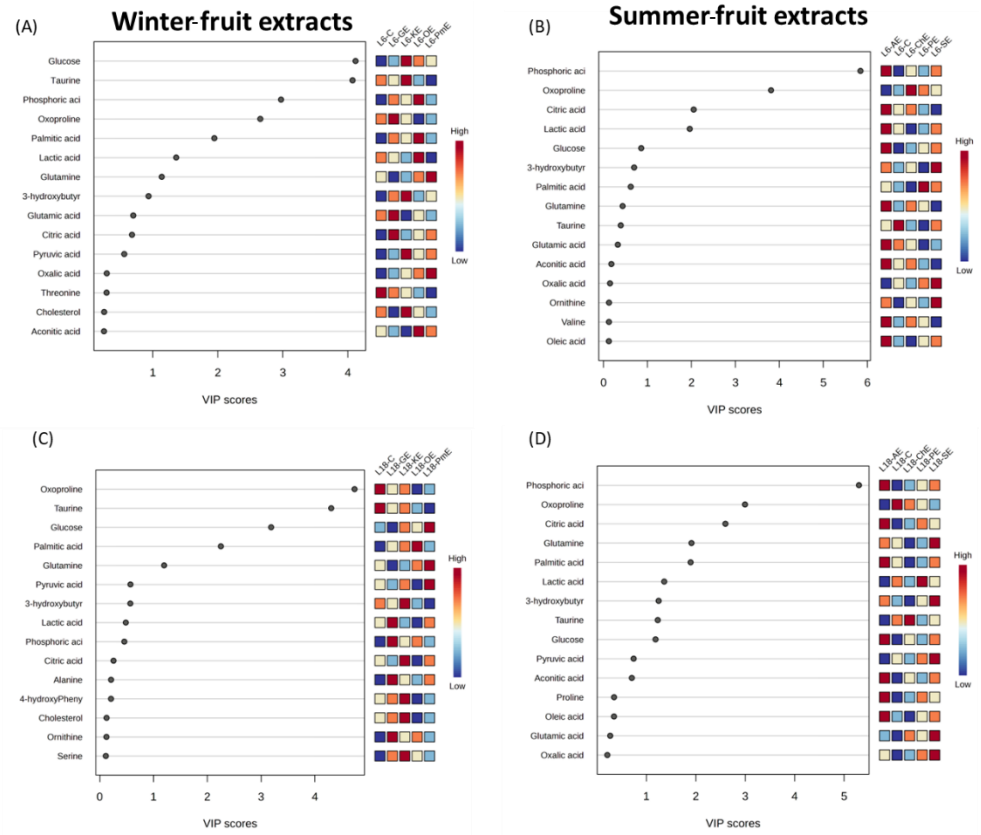

Figure 4. Variable importance in projection (VIP) scores of component 1 of the PLS-DA identifies the top 15 discriminating parameters in descending order of importance from the serum metabolomics data of serum samples of Fisher 344 rats exposed to the short photoperiod (L6, 6h light, figure A and B) and the long photoperiod (L18, 18 h light, figure C and D) for six weeks and administered water (control, C) or extracts (E, 100 mg/kg body weight) obtained from four winter-fruits (persimmon kaki (K), grape (G), orange (O), and pomegranate (Pm), Figure A and C) and four summer fruits (cherry (Ch), plum (P), apricot (A), strawberry (S), figure B and D) the last two weeks (n=8 per group). The colored legend on the right indicates the relative abundance of variables, with red and blue indicating high and low values, respectively.

To examine possible differences among the clusters depending on the photoperiods where the same extract was consumed PLS-DA analysis was performed by comparing control groups of each photoperiod with groups of each extract consumed in both photoperiods. Among the winter-fruit extracts, kaki extract when consumed during L6 share almost the same metabolome profile of L6-C, whereas when consumed during L18 tends towards L6 (Figure 5A). The grape extract consumed under L6 or L18 shared some features with L6-C group, whereas L18-C behaved in a different manner (Figure 5B). Orange extract consumption does not seem to influence the organization of clusters towards a photoperiod, as all four groups are separated from each other (Figure 5C). Pomegranate extract when administered during L6 followed the same pattern as L6-C, however when administered in L18, it shared features with both photoperiods (Figure 5D). Among the summer-fruit extracts the serum profile of the animals that consumed cherry extract showed the same behaviour as that of the L18-C group and resulted clearly separated from the L6 photoperiod (Figure 6A). Plum extract did not show a specific pattern towards photoperiod, sharing metabolite profiles with all three other groups (Figure 6B). Animals that consumed apricot extracts did not show any specific pattern towards a photoperiod, as they clearly clustered in a different manner (Figure 6C). Animals that consumed strawberry tended to have the same metabolome profile as the control depending on whether they consumed it under L6 or L18 (Figure 6D) as it was shown for cherry.

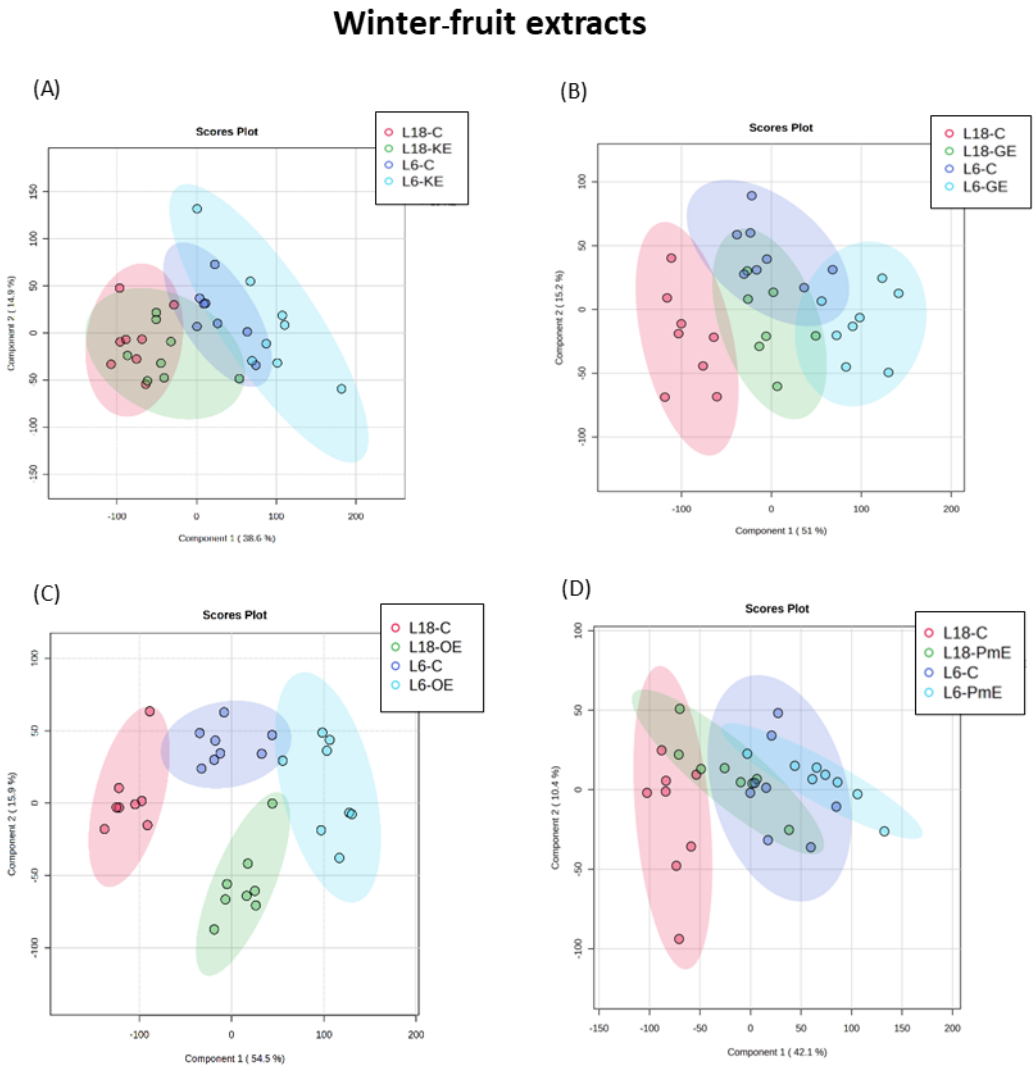

Figure 5. Scores plot of partial least squares-discriminant analysis (PLS-DA) based on metabolomics data between serum samples of Fisher 344 rats exposed to the short photoperiod (L6, 6h light) and the long photoperiod (L18, 18 h light) for six weeks and administered water (control, C) or extracts (E, 100 mg/kg body weight) obtained from four winter-fruits (persimmon kaki (K, figure A), grape (G, figure B), orange (O, figure C), and pomegranate (Pm, figure D) the last two weeks (n=8 per group). Shaded circles represent 95% confidence intervals, while colored dots illustrate individual samples. The axes are labelled by the first and second components with the percentages of variance of the data explained by that component in parentheses.

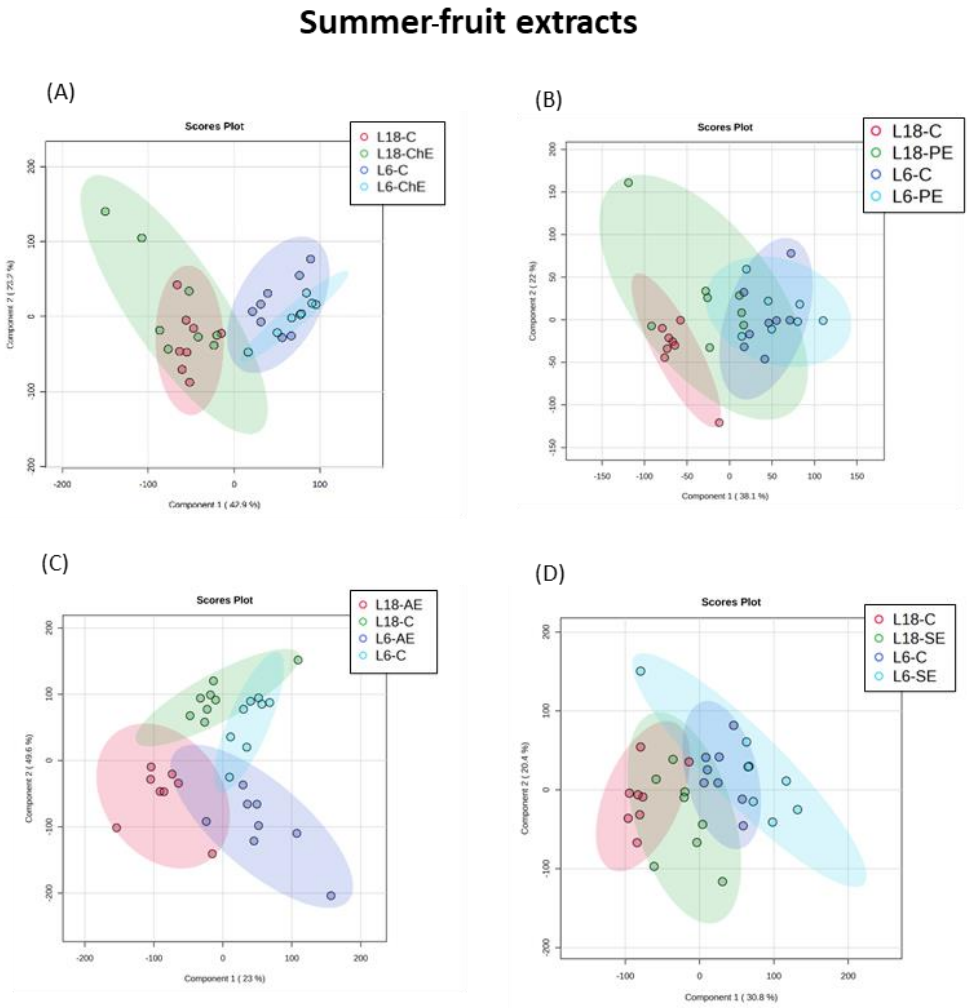

Figure 6. Scores plot of partial least squares-discriminant analysis (PLS-DA) based on metabolomics data between serum samples of Fisher 344 rats exposed to the short photoperiod (L6, 6h light) and the long photoperiod (L18, 18 h light) for six weeks and administered water (control, C) or extracts (E, 100 mg/kg body weight) obtained from four summer-fruits (cherry (Ch, figure A), plum (P, figure B), apricot (A, figure C), and strawberry (S, figure D) the last two weeks (n=8 per group). Shaded circles represent 95% confidence intervals, while colored dots illustrate individual samples. The axes are labelled by the first and second components with the percentages of variance of the data explained by that component in parentheses.

4. Discussion

The aim of this study was to investigate the changes in the metabolome profile of blood serum in rats supplemented with four winter-fruit extracts and four summer-fruit extracts, depending on the photoperiod of administration. More than 50 serum metabolites, including amino acids, organic acids, and glycosides were analysed by GC-MS in response to two weeks of administration of fruit extracts, depending on the photoperiod of exposure. F344 rats responded to photoperiod exposure as confirmed by lower body weight during short photoperiod compared to animals exposed to long photoperiod, as has been previously shown [13], suggesting that this is a suitable animal model to study parameters influenced by photoperiod.

Overall, exposure to different photoperiods modulated metabolome profile of F344 rats. Among the parameters, lactic acid was higher in the L6 photoperiod suggesting that glycolysis is favoured under this photoperiod, which is not in agreement with previous studies in which lactic acid was higher in L18 [14], [15]. However, the weeks of exposure to photoperiods were more respect to this present study which could explain why a different behaviour is observed. As rats are nocturnal animals, glycolysis might be favoured under the L6 photoperiod because animals are in the active phase, which explains these results and is in agreement with photoperiod responsiveness [16]. Moreover, phosphoric acid increased in animals exposed to short photoperiod, which could be related to lower body weight. This is the first time that has been shown that this marker has a photoperiod dependent effect, however previous studies in humans have shown that phosphorus supplementation is inversely correlated with body weight in overweight individuals [17].

The increase in oxoproline levels, an intermediate metabolite of glutathione (GSH) metabolism [18], was increased only in animals exposed to long photoperiod (not in short photoperiod) and not supplemented with any of the extracts, suggesting

that long photoperiod exposure leads to a reduced availability of amino acids necessary for GSH synthesis. Indeed, glutathione is the most important low-molecular-weight antioxidant synthesized in cells, and previous studies have shown that an increase in oxoproline in the urine is a suitable indicator of decreased availability of cysteine, glycine, and methionine for GSH synthesis [19], [20]. The oxoproline levels decreased during long photoperiod in the groups supplemented with strawberry, apricot, and orange extracts. Our previous studies showed a high antioxidant in vitro activity of strawberry extract, and clinical studies showed that as fresh fruit, its consumption is correlated with an increase in antioxidant biomarkers [21], which could explain the results obtained in this study. In contrast, very little information is available in the literature regarding the effects of apricot on GSH metabolism, and no correlation has been found between its consumption and the modulation of GSH in clinical trials [22].

Moreover, taurine metabolism is one of the pathways most affected. Taurine is a semiessential amino acid that can be either synthetised by cysteine or acquired by food [23]. It has been associated with positive cardiovascular effects, such as regulating endothelial function [24] and neuroprotective actions by reducing oxidative stress through the inhibition of oxidase enzyme [25]. In this study, taurine levels are associated with consumption of persimmon kaki extract during short photoperiod but not during long photoperiod, and with cherry extract consumption only during long photoperiod suggesting that not only the composition of the extract is important but also the photoperiod administration. Previous studies have suggested that cherry consumption as a fresh fruit can elicit neuroprotective effects by reducing neuroinflammation in F344 rats [26]. Further studies are needed to elucidate the possible neuroprotective role and cardiovascular benefits of cherry extract consumption during a long photoperiod.

Despite the hour of starvation, in several groups of animals, glucose resulted in one of the metabolites that mostly influenced the groups separation, which could be attributed to the presence of carbohydrates in the extracts (Manuscript1).

However, all extracts showed carbohydrate content, thus the regulation of its levels in the blood, accompanied by other metabolites such as citric acid or lactic acid could not only suggests that the glycolysis/gluconeogenesis pathway is affected but could also indicate that this pathway regulation could be part of the synergic activities of other components of the extracts such as phenolic compounds. Indeed, diets rich in (poly)phenols are associated with beneficial effects on glucose metabolism [27] thanks to their ability of inhibit glucose transporters [28]. It has been previously described that the different phenolic compounds and food matrix interactions could cause a synergistic effect or an antagonistic effect depending on the circumstances [29], here it has been demonstrated that photoperiod could also influence their metabolic outcomes.

This study offers a preliminary approach to understand that seasonal fruit extracts not only cause changes in the metabolome profile depending on their composition but also that the same fruit extract can cause different effects depending on the photoperiod of consumption.

A limitation of the study could be that in the pathways detected few metabolites are present; however according to our literature research, this is the first study that used a metabolomic approach to look at possible metabolic differences in rats exposed to different photoperiod and supplemented with seasonal fruit extracts, thus more research is needed to shed light on this first screening approach. Moreover, this study reinforces the idea that peripheral adaptations to photoperiod need to be better understood to design nutraceuticals that could be used in one specific season and allow the adaptation of metabolic changes.

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7. Conflict of Interest

The authors declare no conflict of interest.

8. Author Contributions

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

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Supplementary material

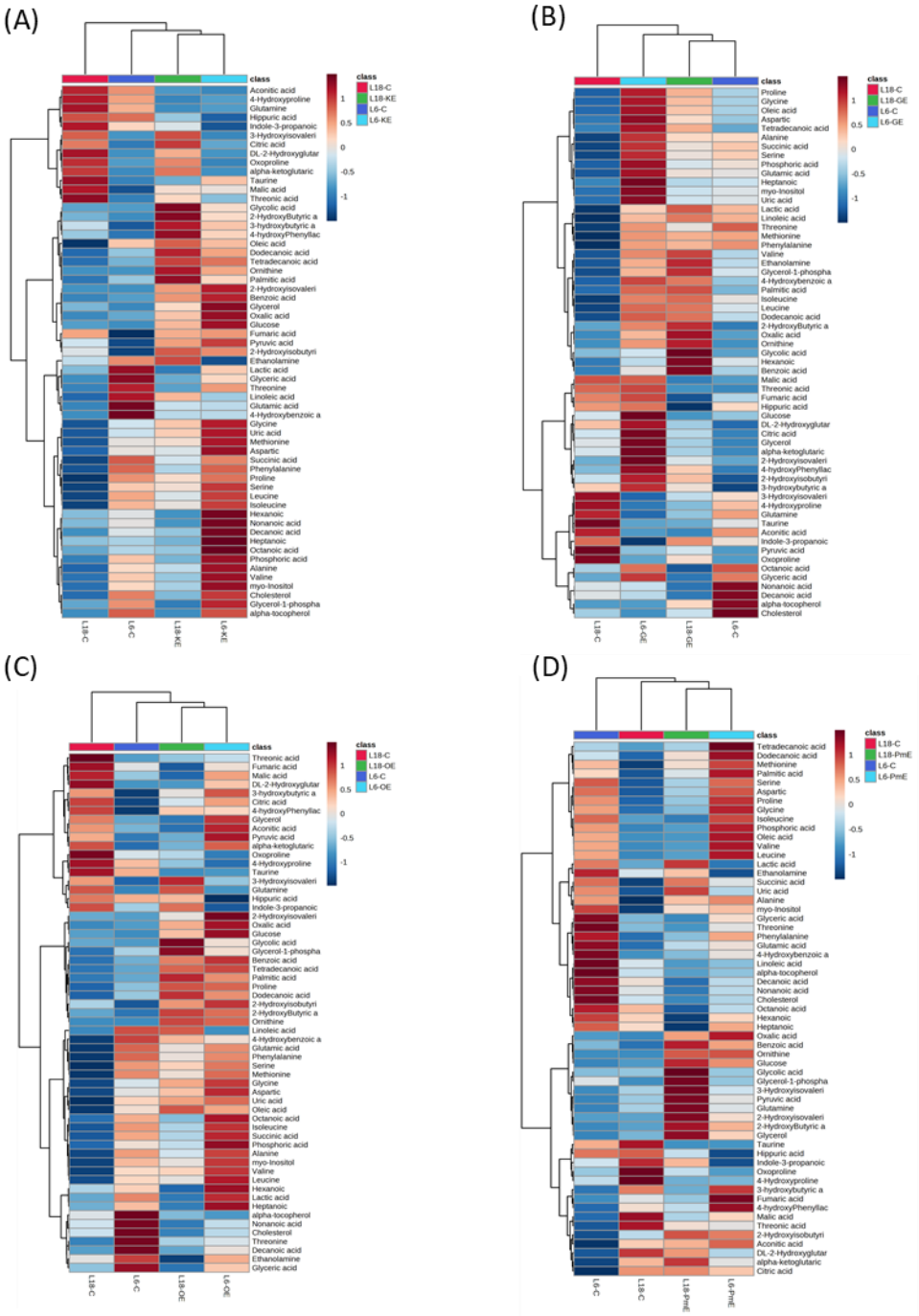

Figure S1 Heatmaps of abundance of serum metabolites of winter-fruit extracts (A, B, C, D).

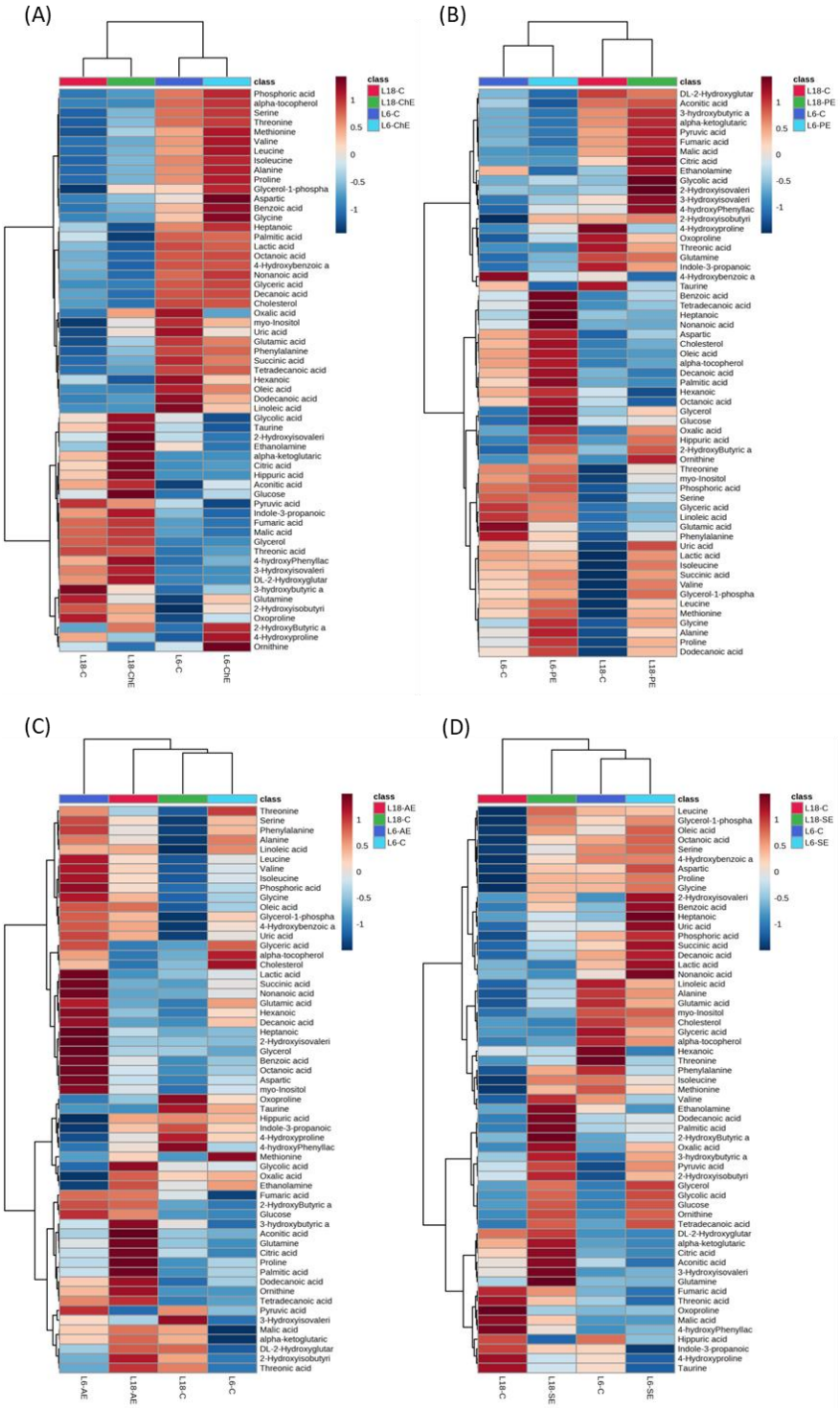

Figure S2 Heatmaps of abundance of serum metabolites of summer-fruit extracts (A, B, C, D).

Table S1. Extraction conditions used for each fruit.

Fruit	Solvent to solute ratio (mL/g)	Ethanol (%)	Formic acid (%)	Time (min)	Temperature (°C)
Cherry	20	72	1	20	55
Plum	20	72	1	30	55
Apricot	20	72	1	30	38
Strawberry	40	72	1	30	55
Persimmon kaki	20	72	1	30	38
Grape	80	65	1	100	72
Orange	30	70	1	40	55
Pomegranate	80	65	1	100	72

Table S2. Serum metabolites samples of Fisher 344 rats exposed to the short photoperiod and administered water (control, C) or fruit extracts (E, 100 mg/kg body weight) (n=8 per group, mean ± standard deviation) identified by GC-qTOF.

	L6								
Serum metabolites	C	ChE	PE	AE	SE	KE	GE	OE	PmE
Piruvic acid	15.2 ± 3.9	13.2 ± 1.4	14.4 ±2.6	18.7 ± 4.7	19.6 ± 8.1	20.2 ± 6.1	15.5 ± 3.7	19.0 ± 5.3	19.7 ± 3.4
Lactic acid	164.5 ± 20.1	163.7 ±15.2	163.7 ±17.5	197.9 ±15.0	178.2 ± 36.3	156.4 ± 37.5	163.4 ±17.5	169.4 ±23.0	146.3 ± 10.0
Succinic acid	0.25 ±0.05	0.24 ± 0.05	0.26 ± 0.02	0.36 ± 0.2	0.24 ± 0.05	0.24 ± 0.5	0.27 ±0.05	0.26 ± 0.05	0.22 ±0.02
Fumaric acid	2.6 ± 0.8	2.5 ± 0.5	2.3 ±0.7	3.8 ± 1.2	2.4 ± 0.7	3.3 ± 0.9	3.5 ± 1.3	2.7 ± 0.8	4.7 ± 1.5
Malic acid	1.6 ± 0.6	1.5 ± 0.3	1.6 ± 0.5	2.4 ± 0.9	1.6 ± 0.5	2.1 ± 0.6	2.8 ± 0.6	2.2 ± 0.6	2.1 ± 0.5
a-ketgluacid	3.7 ± 1.0	3.7 ± 0.7	3.3 ± 0.9	4.9 ± 1.1	3.5 ± 0.9	3.7 ± 1.4	7.8 ± 1.9	5.0 ± 0.9	4.7 ± 1.1
Citric acid	116.0 ± 29.5	118.5 ±13.2	114.5 ± 32.5	148.6 ± 26.8	107.5 ± 25.8	121.4 ± 29.0	207.6 ± 37.9	140.5 ±22.9	141.9 ± 26.9
Glucose	127.0 14.5	129.2 ± 20.0	134.7 ± 15.5	175.9 ±21.1	154.2 ± 27.6	181. 2± 21.7	142.5 ±15.7	173.3 ± 17.4	157.6 ± 21.3
Alanine	34.4 ± 2.4	35.8 ± 2.0	38.4 ± 2.8	38.6 ± 2.9	33.5 ± 4.8	38.2 ± 3.8	37.1 ± 3.7	35.5 ± 2.6	34.6 ± 2.2
Valine	18.9 ± 1.1	19.5 ± 2.4	19.2 ± 1.5	20.7 ± 1.1	18.0 ± 4.0	20.1 ± 2.0	20.5 ± 1.8	19.8 ± 1.0	19.7 ± 0.7
Leucine	14.6 ± 1.1	15.5 ± 1.6	15.2 ± 1.5	16.8 ± 1.4	14.5 ± 3.9	15.2 ± 2.8	16.2 ± 1.6	16.0 ± 0.8	15.7 ± 0.8
Isoleucine	10.3 ± 1.0	10.7 ± 1.4	10.3 ± 1.1	12.2 ± 0.7	9.8 ± 2.7	10.8± 2.1	11.1 ± 1.2	10.7 ± 0.7	10.5± 0.5
Proline	11.1 ± 0.7	11.4 ± 1.3	12.0 ± 1.9	11.2 ± 3.0	11.2 ± 3.6	11.1 ± 2.1	13.0 ± 1.9	13.3 ± 2.1	11.3 ± 3.5
Glycine	7.9 ± 0.2*	8.1 ± 0.1	8.3 ± 0.1	8.4 ± 0.4	8.0 ± 0.5	8.2 ± 0.3	8.4 ± 0.2	8.2 ± 0.2	8.0 ± 0.2
Serine	17.5 ± 2.9 *	18.1 ± 1.5	17.2 ± 1.9	18.9 ± 1.1	17.8 ± 3.0	19.4 ± 4.5	19.7 ± 1.6	17.8 ± 1.7	17.3 ± 1.4
Threonine	15.1 ± 1.6	15.5 ± 1.1	15.3 ± 1.8	15.5 ± 1.2	15.5 ± 1.2	14.2 ± 2.7	14.7 ± 2.7	13.0 ± 1.1	12.7 ± 0.5
Methionine	6.4 ± 0.2	6.6 ± 0.3	6.6 ± 0.3	6.4 ± 0.2	6.2 ± 0.9	7.0 ± 0.5	6.4 ± 0.4	6.4 ± 0.3	6.6 ± 0.2
Aspartic	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.3	1.0 ± 0.2	0.7 ± 0.1	0.8 ± 0.4	1.1 ± 0.2	0.8 ± 0.1	0.6 ± 0.2
Phenylalanin	10.4 ± 1.2	10.3 ± 0.8	9.7 ± 0.9	11.1 ± 0.5	10.4 ± 0.6	10.4 ± 0.7	10.4 ± 0.8	10.3 ± 0.4	9.8 ± 0.3
Glutamine	21.3 ± 3.2	24.1 ± 3.2	22.3 ± 3.5	34.0 ± 21.2	20.5 ± 13.3	14.7 ± 6.1	9.0 ± 3.0	24.3 ± 5.5	25.6 ± 18.5
Ornithine	4.1 ± 1.0	4.8 ± 0.7	4.7 ± 0.7	5.9 ± 2.8	6.1 ± 1.8	5.5 ± 1.7	6.1 ± 1.8	6.7 ± 1.4	6.1 ± 1.8
Glutamic ac	14.9 ± 4.2	13.9 ± 1.8	17.9 ± 2.2	17.9 ± 2.2	13.0 ± 2.8	11.0 ± 0.8	22.4 ± 3.1	14.3 ± 1.9	11.1 ± 2.2
Hexanoic ac	0.48 ± 0.07	0.46 ± 0.05	0.52 ± 0.10	0.52 ± 0.15	0.42 ± 0.15	0.59 ± 0.10	0.47 ± 0.05	0.55 ± 0.15	0.44 ± 0.15
Heptanoic ac	0.13 ± 0.01	0.13 ± 0.02	0.26 ± 0.07	0.30 ± 0.1	0.23 ± 0.08	0.26 ± 0.05	0.15 ± 0.03	0.14 ± 0.01	0.12 ± 0.01
Glycolic ac	0.14 ± 0.01	0.13 ± 0.01	0.15 ±0.01	0.14 ± 0.01	0.17 ±0.04	0.17 ± 0.03	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
2-hydroxybut	1.29 ± 0.22	1.55 ± 0.56	1.42 ± 0.30	1.57 ± 0.77	1.46 ± 0.24	1.46 ± 0.28	1.54 ± 0.51	1.52 ± 0.27	1.64 ± 0.27
Oxalic ac	10.12 ± 1.62	9.98 ± 1.26	11.12 ± 2.08	9.59 ± 0.63	11.12 ± 1.86	11.51 ± 1.84	11.35 ± 1.02	12.50 ± 1.23	12.81 ± 1.18
3 hydroxybut	27.25 ± 5.75	30.05 ±5.78	27.34 ± 4.90	27.34 ± 4.90	51.11 ±19.29	43.13 ±14.13	42.82 ± 9.98	39.20 ± 7.32	39.52 ± 9.35
Benzoic acid	0.76 ± 0.21	0.96 ± 0.16	1.38 ± 0.46	2.54 ± 0.52	2.01 ± 0.57	3.89 ± 1.68	1.18 ± 0.79	1.86 ± 0.73	1.43 ± 0.49
Octanoic acid	0.36 ± 0.02	0.36 ± 0.05	0.42 ± 0.03	0.50 ± 0.13	0.36 ± 0.08	0.53 ± 0.07	0.35 ± 0.03	0.37 ± 0.03	0.29 ± 0.02
Glycerol	3.55 ± 0.59	3.56 ± 0.67	4.34 ± 0.64	5.06 ± 1.88	5.06 ± 1.33	4.88 ± 1.35	4.09 ± 1.12	3.79 ± 0.55	4.21 ± 1.24
Glyceric ac	2.18 ± 1.44	2.23 ± 0.85	2.02 ± 1.17	2.25 ± 1.78	1.80 ± 0.55	1.67 ± 0.60	2.40 ± 0.63	1.63 ± 0.52	1.55 ± 0.55
Nonaoic ac	0.91 ± 0.06	0.96 ± 0.13	1.78 ± 0.55	1.61 ± 0.25	1.54 ± 0.30	1.68 ± 0.40	0.60 ± 0.09	0.61± 0.06	0.59 ± 0.12
Decanoic ac	0.22 ± 0.02	0.24 ± 0.04	0.27 ± 0.03	0.26 ± 0.04	0.24 ± 0.04	0.33 ± 0.05	0.16 ± 0.01	0.19 ± 0.02	0.19 ± 0.02
Dodecanoic	0.49 ± 0.05	0.48 ± 0.03	0.52 ± 0.07	0.54 ± 0.09	0.51 ± 0.13	0.57 ± 0.08	0.54 ± 0.08	0.57 ± 0.11	0.57 ± 0.06
Tetradecanoi	4.46 ± 0.86	4.45 ± 0.88	5.01 ± 1.14	5.92 ± 1.92	5.99 ± 2.55	5.99 ± 2.55	5.21 ± 1.46	5.69 ± 1.35	5.62 ± 0.88
Palmitic acid	248.1 ± 20.0	247.3 ± 16.4	270.3 ± 10.2	283.3 ± 10.2	255.5 ± 33.6	267.87 ± 46.8	292.7 ± 36.3	313.0 ± 42.5	259.7 ± 28.1
Linoleic ac	5.58 ± 0.91	4.84 ± 0.94	5.31 ± 1.33	5.31 ± 1.33	5.11 ± 2.54	5.53 ± 1.58	5.53 ± 1.58	4.11 ± 1.33	3.69 ± 0.71
Oleic ac	17.02 ± 3.90	16.24 ± 4.6	18.55 ± 4.67	22.10 ± 9.32	18.84 ± 10.45	17.12 ± 6.61	24.17 ± 7.84	17.88 ± 4.83	18.88 ± 3.47
Cholesterol	9.87 ± 2.47	9.80 ± 1.8	11.64 ± 1.98	8.50 ± 3.29	9.37 ± 2.35	11.73 ± 5.11	6.41 ± 1.74	6.90 ± 1.27	6.81 ± 1.26
Aconitic acid	3.82 ± 0.54	4.38 ± 0.81	3.20 ± 0.91	5.90 ± 3.76	1.84 ± 0.83	2.53 ± 0.91	2.53 ± 0.91	5.06 ± 3.04	4.05 ± 3.52

Etohamine	0.62 ± 0.05	0.57 ± 0.05	0.58 ± 0.08	0.59 ± 0.07	0.59 ± 0.10	0.56 ± 0.10	0.64 ± 0.07	0.61 ± 0.09	0.57 ± 0.05
Phosphoric a	199.3 ± 39.7	216.3 ± 28.5	204.2 ± 29.2	353.1 ± 53.2	259.05 ± 43.5	259.9 ± 69.6	275.5 ± 34.4	279.4 ± 25.7	230.5 ± 39.5
Oxoproline	160.8 ± 15.9	190.4 ± 27.1	187.2 ± 35.4	97.8 ± 24.2	163.22 ± 39.1	155.2 ± 28.8	162.5 ± 21.2	124.0 ± 13.2	150.4 ± 39.1
Indole3propanoic	1.68 ± 0.28	1.55 ± 0.28	1.73 ± 0.49	1.13 ± 0.39	1.20 ± 0.3	1.34 ± 0.32	1.21 ± 0.32	1.53 ± 0.25	1.48 ± 0.28
Myoinositol	1.26 ± 0.43	1.16 ± 0.33	1.31 ± 0.36	1.83 ± 0.50	1.24 ± 0.28	1.54 ± 0.36	1.73 ± 0.34	1.37 ± 0.31	1.17 ± 0.45
Vit E	0.11 ± 0.02	0.12 ± 0.02	0.13 ± 0.02	0.10 ± 0.04	0.10 ± 0.03	0.11 ± 0.06	0.07 ± 0.02	0.05 ± 0.01	0.06 ±0.01

Table S3. Serum metabolites samples of Fisher 344 rats exposed to the long photoperiod (L18, 18 h light) and administered water (control, C) or fruit extracts (E, 100 mg/kg body weight), (n=8 per group, mean ± standard deviation) identified by GC-qTOF.

	L18								
Serum metabolites	C	ChE	PE	AE	SE	KE	GE	OE	PmE
Piruvic acid	17.7 ± 3.4	16.9 ± 2.4	19.1 ±2.9	15.7 ± 2.0	19.2 ± 2.0	19.2 ± 2.0	15.9 ± 4.0	15.8 ± 3.2	25.0 ± 3.4
Lactic acid	149.9 ± 14.0	143.3 ±17.8	165.0 ±24.0	138.7 ±21.9	144.1 ± 25.3	146.5 ±19.7	168.4 ±25.6	146.3 ± 15.5	167.9 ±3 1.1
Succinic acid	0.19 ± 0.02	0.2 ±0.02	0.25 ± 0.03	0.2 ± 0.04	0.23 ± 0.05	0.22 ± 0.03	0.23 ± 0.03	0.23 ±0.04	0.25 ± 0.08
Fumaric acid	3.3 ± 0.9	3.4 ± 0.9	3.7 ± 1.7	3.8 ± 0.8	3.1 ± 1.1	3.3 ± 0.9	1.8 ± 0.4	2.1 ± 0.7	3.4 ± 0.7
Malic acid	2.5 ± 0.7	2.6 ± 0.7	2.9 ± 0.9	2.6 ± 0.5	2.4 ± 0.9	2.4 ± 0.5	1.5 0.4	1.3 ± 0.4	2.3 ± 0.5
a-ketgluacid	5.0 ± 0.8	6.1 ± 1.8	5.6 ± 1.7	5.3 ± 0.6	5.9 ± 1.6	4.8 ± 1.4	4.4 ± 1.1	4.2 ± 0.9	5.7 ± 1.7
Citric acid	164.6 ± 22.2	180.3 ± 45.8	183.3 ± 50.2	226.4 ±33.3	183.2 ± 35.7	151.6 ± 32.0	137.9 ± 26.1	136.1 ± 24.2	146.5 ± 23.9
Glucose	129.8 ±10.7	134.4 ± 17.6	130.4 ±12.9	165.5 ± 24.0	153.9 ± 36.1	158.7 ±14.1	127.0 ± 24.5	158.7 ± 22.5	164.0 ± 15.8
Alanine	29.8 ± 1.8	31.3 ± 2.9	35.2 ± 1.2	33.0 ± 2.5	31.7 ± 3.1	33.0 ± 2.2	34.2 ± 2.4	32.6 ± 2.3	34.3 ± 3.1
Valine	17.3 ± 0.8	17.7 ± 2.1	19.3 ± 1.3	19.3 ± 1.1	19.3 ± 4.1	18.0 ± 1.5	20.7 ± 1.5	18.6 ± 2.3	17.7 ± 1.6
Leucine	12.4 ± 0.7	13.0 ± 1.6	14.6 ± 1.1	14.9 ± 0.6	15.1 ± 3.0	13.9 ± 1.3	15.8 ± 1.1	13.8 ± 1.9	13.0 ± 1.6
Isoleucine	10.5 ± 0.5	8.8 ± 0.4	10.6 ± 0.7	10.7 ± 0.8	10.3 ± 2.1	10.0 ± 0.8	10.9 ± 0.9	9.2 ± 1.4	8.8 ± 1.1
Proline	10.0 ± 1.4	10.3 ± 1.9	11.5 ± 1.8	15.1 ± 2.1	11.1 ± 3.6	10.8 ± 1.9	12.0 ± 1.9	14.0 ± 1.9	9.7 ± 3.7
Glycine	7.7 ± 0.1	7.7 ± 0.4	8.2 ± 0.2	8.2 ± 0.1	7.9 ± 0.4	8.2 ± 0.2	8.2 ± 0.2	8.0 ± 0.2	7.7 ± 0.1
Serine	13.1 ± 0.7	14.5 ± 1.5	15.3 ± 1.0	16.9 ± 0.7	16.1 ± 1.3	16.9 ± 1.0	16.7 ± 1.0	16.6 ± 1.4	15.0 ± 1.2
Threonine	11.9 ± 0.5	13.0 ± 1.8	14.2 ± 0.6	13.1 ± 1.0	12.7 ± 1.3	12.2 ± 0.8	13.7 ± 1.1	12.4 ± 0.9	11.6 ± 0.9
Methionine	5.8 ± 0.3	6.1 ± 0.5	6.5 ± 0.1	6.1 ± 0.3	6.2 ± 0.7	6.4 ± 0.2	6.3 ± 0.2	6.2 ± 0.3	6.3 ± 0.4
Aspartic	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
Phenylalanin	8.5 ± 0.4	9.1 ± 1.1	9.4 ± 0.6	10.0 ± 0.5	9.9 ± 1.2	9.4 ± 0.8	10.2 ± 0.4	9.7 ± 0.5	8.9 ± 0.5
Glutamine	25.6 ± 2.9	23.6 ± 4.9	25.2 ± 2.2	53.9 ± 24.2	74.9 ± 38.8	14.2 ± 5.1	14.6 ± 4.2	30.0 ± 13.4	36.8 ± 16.6
Ornithine	4.1 ± 0.9	3.8 ± 0.6	4.9 ± 0.9	7.2 ± 2.1	8.4 ± 2.0	6.2 ± 0.9	7.1 ± 1.6	6.5 ± 0.9	6.1 ± 1.2
Glutamic ac	7.8 ± 0.8	10.6 ± 2.2	9.9 ± 1.9	9.3 ± 1.4	10.8 ± 1.9	10.0 ± 2.8	14.9 ± 2.1	12.2 ± 2.1	10.3 ± 1.7
Hexanoic ac	0.44 ± 0.06	0.42 ± 0.04	0.38 ± 0.09	0.46 ± 0.19	0.44 ± 0.27	0.42 ± 0.09	0.52 ± 0.27	0.39 ± 0.11	0.37 ± 0.14
Heptanoic ac	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.15 ± 0.04	0.15 ± 0.07	0.11 ± 0.03	0.13 ± 0.03	0.12 ± 0.02	0.10 ± 0.01
Glycolic ac	0.15 ± 0.02	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.03	0.17 ± 0.02	0.21 ± 0.02	0.17 ± 0.01	0.19 ± 0.03	0.20 ± 0.02
2-hydroxybut	1.33 ± 0.17	1.51 ± 0.19	1.42 ± 0.15	1.55 ± 0.39	1.66 ± 0.20	1.66 ± 0.20	1.59 ± 0.3	1.57 ± 0.23	1.9 ± 0.31
Oxalic ac	9.96 ± 2.16	10.06 ± 1.43	10.85 ± 2.67	10.46 ± 1.93	12.02 ± 1.90	12.15 ± 1.80	12.30 ± 1.94	12.44 ± 0.72	10.86 ± 1.08
3 hydroxybut	37.57 ±6.66	32.31 ±12.91	41.46 ± 9.15	50.82 ±11.35	60.74 ±17.36	51.99 ± 12.43	36.06 ±12.8	32.76 ±10.20	30.99 ± 4.69
Benzoic acid	0.48 ± 0.15	0.81 ± 0.20	0.71 ± 0.26	1.07 ± 0.37	1.31 ± 0.47	2.97 ± 1.34	1.77 ± 1.22	1.40 ± 0.69	1.94 ± 0.88
Octanoic acid	0.32 ± 0.03	0.31 ± 0.02	0.26 ± 0.03	0.26 ± 0.03	0.35 ± 0.20	0.34 ± 0.08	0.31 ± 0.03	0.33 ± 0.05	0.24 ± 0.03
Glycerol	3.74 ± 0.69	3.76 ± 0.87	3.99 ± 0.73	3.77 ± 0.88	4.88 ± 0.95	4.18 ± 0.78	3.76 ± 1.28	3.39 ± 0.75	5.03 ± 1.28
Glyceric ac	1.20 ± 0.51	1.13 ± 0.27	1.42 ± 0.62	1.06 ± 0.57	1.16 ± 0.54	1.29 ± 0.55	0.96 ± 0.29	0.90 ± 0.25	1.05 ± 0.31
Nonaoic ac	0.63 ± 0.08	0.55 ± 0.06	0.62 ± 0.17	0.61 ± 0.17	0.56 ± 0.14	0.50 ± 0.14	0.45 ± 0.05	0.46 ± 0.10	0.46 ± 0.09
Decanoic ac	0.17 ± 0.02	0.16 ± 0.02	0.15 ± 0.04	0.18 ± 0.04	0.19 ± 0.11	0.19 ± 0.05	0.14 ± 0.02	0.17 ± 0.02	0.12 ± 0.02
Dodecanoic	0.45 ± 0.03	0.44 ± 0.03	0.50 ± 0.07	0.60 ±0.09	0.63 ± 0.25	0.61 ± 0.10	0.54 ± 0.07	0.59 ± 0.08	0.51 ± 0.08
Tetradecanoi	4.20 ± 0.60	4.28 ± 0.92	4.32 ± 0.68	6.37 ± 1.28	5.94 ± 1.24	5.34 ± 1.31	5.46 ± 1.45	5.48 ± 1.32	4.54 ± 1.51
Palmitic acid	231.2 ± 15.4	212.0 ± 9.5	253.4 ± 14.9	311.1 ± 28.3	295.4 ± 75.8	300.6 ± 63.3	301.1 ± 32.0	328.4 ± 37.3	242.9 ± 39.2
Linoleic ac	4.07 ± 0.80	4.07 ± 0.80	4.41 ± 1.13	5.46 ± 1.25	4.57 ± 1.76	5.06 ± 1.02	5.86 ± 1.43	5.06 ± 1.31	3.59 ± 1.38
Oleic ac	13.71 ± 3.10	13.60 ± 3.46	13.60 ± 3.46	21.67 ± 5.9	18.26 ± 5.86	18.03 ± 5.00	19.81 ± 5.91	17.60 ± 5.05	14.52 ± 6.45
Cholesterol	7.07 ± 1.21	6.60 ± 1.31	7.73 ±1.91	6.28 ± 3.05	6.79 ± 2.14	7.93 ± 1.10	7.46 ± 1.94	5.47 ±1.18	5.81 ± 1.29
Aconitic acid	4.67 ± 0.90	4.95 ± 1.34	4.77 ± 1.55	14.82 ± 7.63	8.06 ± 4.08*	1.54 ± 0.51	1.56 ± 0.41	4.24 ± 2.17	4.32 ± 1.92

Etohamine	0.59 ± 0.06	0.68 ± 0.08	0.64 ± 0.08	0.64 ± 0.11	0.66 ± 0.06	0.63 ± 0.05	0.64 ± 0.11	0.58 ± 0.08	0.59 ± 0.05
Phosphoric a	121.0 ± 25.8	131.4 ±17.4	154.9 ± 26.4	239.9 ± 61.7	172.6 ± 44.3	143.2 ± 32.9	186.2 ± 21.2	172.6 ± 30.4	131.7 ± 42.1
Oxoproline	226.3 ± 34.9	207.0 ± 40.1	201.9 ± 22.5	125.7 ± 21.4	166.3 ± 43.8	211.4 ± 31.2	185.8 ± 15.3	144.1 ± 28.1	177.3 ± 31.4
Indole3propanoic	1.91 ± 0.14	2.03 ± 0.37	1.84 ± 0.39	1.71 ± 0.41	1.69 ± 0.69	1.61 ± 0.28	1.84 ± 0.32	1.86 ± 0.47	1.80 ± 0.36
Myoinositol	0.93 ± 0.13	1.11 ± 0.21	1.16 ± 0.28	1.28 ± 0.28	1.00 ± 0.24	1.21 ± 0.24	1.20 ± 0.27	1.20 ± 0.27	1.12 ± 0.30
Vit E	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.06 ± 0.03	0.07 ± 0.03	0.07 ± 0.01	0.08 ± 0.03	0.05 ± 0.01	0.05 ±0.01

Chapter 2

To evaluate the photoperiod-dependent effects of phenolic-enriched fruit extracts on obesity-related ´ metabolic alterations

Manuscript 4

New phenolic-enriched fruit extracts with photoperiod-dependent effects for the prevention of metabolic disorders.

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Abstract

Fruits are rich in bioactive compounds such as (poly)phenols, and the obtaining of extracts enriched in these compounds can reduce fruit loss. Phenolic compounds have demonstrated beneficial properties in the prevention of metabolic diseases, including hypolipidemic and anti-inflammatory effects. Moreover, there is growing evidence that their activity depends on the season in which they are consumed. Therefore, this study aimed to investigate the photoperiod-dependent effects of seasonal fruit extracts on lipid metabolism and inflammation. Male Fischer 344 rats were housed in short (L6, 6h light) and long (L18, 18h light) photoperiods and orally supplemented with 100 mg/kg body weight of each extract or water (control) for 2 weeks. Oral lipid tolerance test (OLTT) and lipopolysaccharide (LPS)-induced inflammatory challenge were performed to temporarily induce a degree of disturbance in homeostasis and to evaluate the benefits of each extract depending on photoperiod consumption. Results from the OLTT showed differences in the area under the curve depending on the fruit phenolic extract administered and the photoperiod of consumption. Additionally, the inflammatory markers interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) were significantly higher in the short photoperiod and winter-fruit extracts were the most efficient in lowering pro-inflammatory cytokines. Specific fruit extracts may be effective in preventing or treating metabolic disorders by restoring disturbed homeostasis, and their effects depend on photoperiod.

Keywords: dyslipidaemia, inflammation, LPS, OLTT, (poly)phenols, seasonality.

1. Introduction

Non-communicable diseases are a significant public health problem that cause 74 million deaths annually, corresponding to 74 % of global deaths [1]. Their incidence is increased by various metabolic factors, such as obesity, raised blood pressure, and high blood glucose and lipid levels, as well as behavioural factors including sedentary lifestyle, alcohol consumption, tobacco consumption, and unhealthy diets [2], [3]. Features related to obesity, such as high waist circumference, dyslipidemia, insulin resistance and hypertension are some of the criteria used to diagnose metabolic syndrome (MetS). Moreover, circulating inflammatory cytokines are associated with the expansion of adipose tissue in obese individuals and are considered useful biomarkers for predicting non-communicable diseases [4].

Changes in lifestyle and dietary patterns through acquisition of healthier choices may prevent weight gain and alleviate non-communicable disease comorbidities [5], such as exercising or increasing the consumption of vegetables and fruits [6]. Low consumption of plant-based foods has been linked to an increased risk of several diseases including obesity, diabetes, and cardiovascular diseases [7]. These foods contain various bioactive compounds, including fatty acids, carotenoids, sterols, organosulfur molecules, alkaloids, and phenolic compounds [8]. The consumption of phenolic compounds has been associated with improvements in glucose and blood pressure control, reduction in low-density lipoprotein cholesterol (LDL-C) oxidation and triacylglyceride (TAG) levels, and lowering of body mass index and waist circumference [9], [10]. Fruits are important because of their richness and the intra- and interspecific variability in phenolic compounds. However, it is worth mentioning that almost half of the fruits and vegetables produced worldwide are wasted [11]. Therefore, there is an urgent need to develop new strategies for preventing postharvest losses. One alternative for these wastes could be the production of fruit extracts enriched in phenolic compounds with

respect to the fruits capable to prevent chronic metabolic diseases and promoting their use as nutraceuticals or functional food ingredients [12].

Nevertheless, the phenolic profile and composition of vegetables and fruits are affected by various factors including plant type, environmental conditions (temperature, humidity, soil, light, etc.), transport, and storage conditions [13]. These factors affect the bioactivity of the phenolic compounds [14]. Moreover, their effects differed depending on the photoperiod. For instance, sweet cherries of the same variety but from different geographical origins and, therefore, different phenolic composition compositions, exerted different effects on plasma TAG levels in Fischer 344 (F344) rats exposed to a short photoperiod (L6, 6 h light) [15]. However, only one of the studied sweet cherries increased plasma high-density lipoprotein cholesterol (HDL-C) and non-esterified fatty acid levels in F344 rats exposed to a long photoperiod (L18, 18 h light) compared with non-treated L18-exposed animals [15].

Specific metabolic challenge tests have been developed to evaluate the effects of bioactive compounds on main metabolic alterations. In response to metabolic stressors, the organism will strive to restore homeostasis and elicit a strong response in a relatively short period of time [16]. Consequently, the quantification of the stress response reaction will be considered informative regarding the effectiveness of bioactive compound administration. This is considered a sensitive method for assesing changes in health status in response to interventions. One of the most widely used challenge tests is the oral lipid tolerance test (OLTT), used to monitor lipid metabolism [17]. In addition, lipopolysaccharide (LPS)-induced inflammatory challenge has been extensively used to study the anti-inflammatory potential of drugs and natural compounds [18]. LPS activates macrophages/monocytes and induces the production and release of proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) [19].

Therefore, the objective of this present study was to evaluate the effects of fruit extracts on postprandial lipid metabolism and inflammatory responses in F344 rats exposed to different photoperiods and subjected to different metabolic challenge tests to temporarily induce disturbances in homeostasis.

2. Materials and Methods

2.1. Seasonal-fruit extracts

Ripe summer (cherry (*Prunus avium L.*), apricot (*Prunus armeniaca*), strawberry (*Fragaria vesca L.*), plum (*Prunus domestica*)) and winter fruits (grape (*Vitis vinifera*), orange (*Citrus sinensis L.*), persimmon (*Diospyros kaki*), and pomegranate (*Punica granatum*) were acquired during the summer and winter seasons, respectively, from local producers. Pedicels were manually removed, and whole fruits, including the skin, seed, and pulp, were mechanically triturated and mixed to obtain homogenates of strawberry and grapes. For cherry, apricot, plum and persimmon seed were removed before triturated and for orange and pomegranate skin was removed before being triturated. The homogenates were stored at -20 °C until the extraction process, proximate composition, and phenolic content are listed in the Manuscript 1.

2.2. Experimental design

8-week-old F344/IcoCrl rats were purchased from Charles River Laboratories (L'Arblese, Cedex, France). This substrain of the F344 rats has shown to be responsive to photoperiod [20], [21]. After 1 week of adaptation, the rats were randomly housed in two groups (n = 72, each) and exposed to a short photoperiod or a long photoperiod. Animals were fed a standard chow diet (AO4, Panlab, Barcelona, Spain) and tap water *ad libitum* throughout all the experiment.

Experiment 1: After four weeks of photoperiod adaptation, rats were supplemented daily with 100 mg/kg of body weight (BW) of each extract or tap water (control, C group) for two weeks (n=8 per group). Their administration was

voluntary, linking them from a syringe between 8:00 a.m. and 9:00 a.m. (lights on at 7:00 a.m.). After 12 h of fasting, the animals were subjected to OLTT. For that, they were administered either the extracts (100 mg/kg BW) or tap water, and 3 h later, they were orally gavaged with lard oil (2.5 mL/kg BW). Blood was collected from the cut tail to determine TAG levels at different time points (0, 30, 60, 120, 240 min) using the Accutrend Plus instrument (Roche Diagnostics, Barcelona, Spain).

Following the principle of 3 Rs only micro sampling of blood was performed, and the animals were used again in the following experiment after 4 days of wash out where they were not supplemented with extracts.

Experiment 2: Rats were orally administered the fruit extracts or tap water (C) daily for two weeks (n=8 per group). On the last day, lipopolysaccharide (LPS, 0.5 mg/kg BW) was intraperitoneally injected into animals 3 h post-treatment administration. After 4 h, the rats were sacrificed by decapitation, and blood was collected in non-heparinized tubes and centrifuged (3,000 *×g* for 15 min at room temperature). Serum was stored at -80°C until use. A summary of the experimental design is shown in the Figure 1.

Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and Generalitat de Catalunya (permission number 11610) approved the animal procedures carried out in the study.

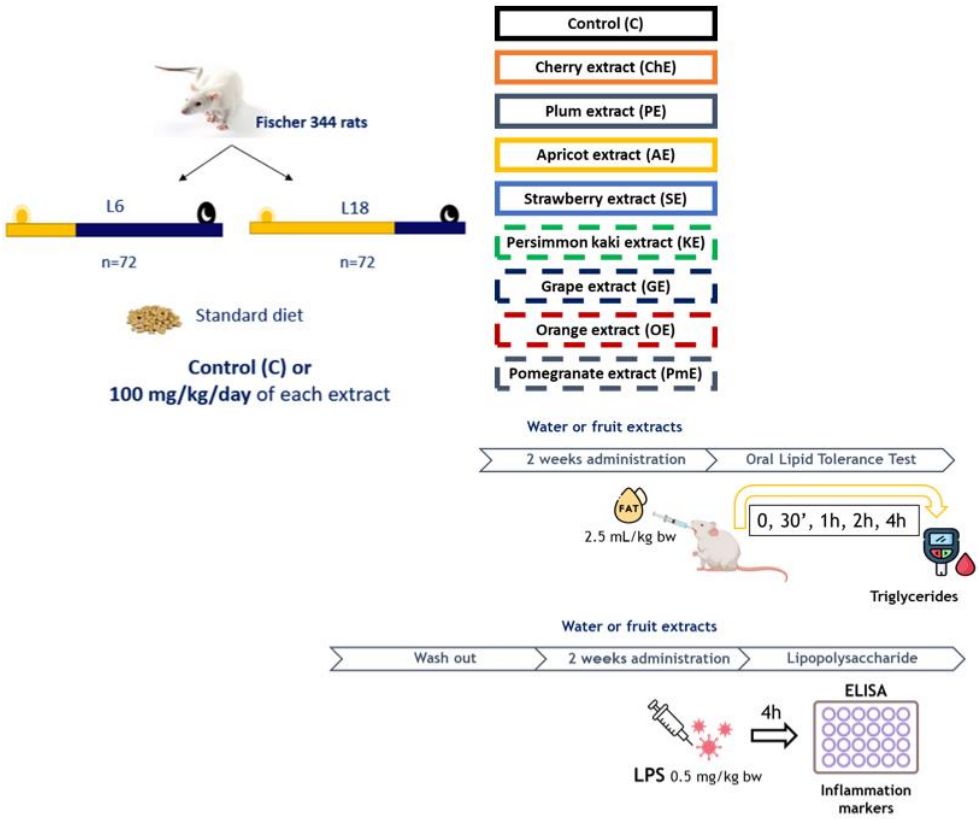

Figure 1. Experimental design carried out in Fischer 344 (F344) rats exposed to different photoperiods and subjected to different metabolic challenges.

2.3. Determination of serum inflammatory markers

Serum IL-6, IL-10, TNF-α, and C-reactive protein (CRP) levels were determined by ELISA kits Finetest following the manufacturer's instructions (Labclinics, Barcelona, Spain). All analyses were performed in duplicate.

2.4 Statistical analysis

Values are expressed as the mean ± standard deviation (SD). Outliers were identified and removed before statistical analysis. Two- and One-way analyses of variance (ANOVA) were used to determine differences among groups using the Post-hoc Tukey test. Two-way ANOVA is indicated as P: photoperiod effect, E:

extract effect, PxE: interaction between photoperiod and extract when $p \leq 0.05$. Student's t-test was used to identify significant differences ($p < 0.05$) produced by the photoperiod for each treatment. All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) software (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

Noncommunicable diseases are chronic diseases such as cardiovascular disease and diabetes, and together with obesity, they represent one of the major risk factors of death worldwide [22], [23]. Several strategies have been proposed for their prevention, including a nutritional approach towards the consumption of diets rich in bioactive compounds, such as fruits and vegetables [6]. Currently, food loss and waste are growing and serious problems [24], with fruits having the highest losses [25]. Fruits are rich in phenolic compounds, that play an important role in the prevention of metabolic disorders [13]. Thus, a strategy to reduce fruit loss and help prevent non-communicable diseases may be the use of fruit loss to produce functional ingredients. However, pre-and post-harvest conditions modify the phenolic profile and concentration of fruits [26]. Consequently, these changes may produce different health effects [15]. Moreover, their functional properties can be affected by the photoperiod of consumption [27]. The present study focused on investigating the effects of eight seasonal-fruit extracts administered to F344 rats exposed to different photoperiods for two weeks, when metabolic alterations were performed through OLTT and LPS-induced inflammatory challenges.

3.1. Fruit extracts improve the management of dyslipidemia depending on the photoperiod

OLTT is an efficient method for evaluating lipid metabolism in the body. The capacity to lower TAG levels after lipid ingestion provides information on intestinal lipid absorption, transport, and metabolism [28]. Thus, this challenge test is an

efficient method for evaluating the hypotriglyceridemic potential of various compounds. In the present study, a significant photoperiod effect and a tendency in the interaction between photoperiod and extracts were observed (two-way ANOVA, $p<0.05$ and $p=0.06$ respectively Figures 2 A and B.

The extracts obtained from two summer fruits, cherry and plum, significantly lowered the serum TAG levels in rats exposed to L6 (65,613 and 74,432.5 mg/dL x secs, respectively) compared to those shown in the L6-C group (106,613 mg/dL x secs) (Figure 2A). However, none of the fruit extracts changed the postprandial lipid response in the L18-exposed animals after OLTT compared to the L18-C group (Figure 2B).

According to the metabolic winter hypothesis, obesity and metabolic-related outcomes are more frequent during winter months in humans [29]. Moreover, seasonal fluctuations in circulating LDL-C, glucose, and blood pressure levels have been observed in diabetic patients, with higher levels in winter than in summer [30]. In Manuscript 1 it has already been shown that TAG levels were higher in L6 respect to L18 in the fasting state, however here under metabolic disruption we showed that no differences were observed in the AUC between the two control groups. However, phenolic-enriched extracts showed different effects depending on the photoperiod of consumption.

In line with the results of this study, previous clinical studies showed that fresh fruit, such as tart or sour cherry consumption, had a powerful effect on lowering TAG levels in the blood of humans after ingestion of a meal high in fats, which was independent of the exercise performed by the patients [31]. Considering the photoperiod of consumption previous studies from our research group showed that sweet cherries (100 mg/kg BW) did not have any hypotriglyceridaemic effect in F344 rats exposed to both L6 and L18 photoperiods although photoperiod played a key role in increasing their levels during the short photoperiod compared to the long photoperiod in the control groups [15]. In this present study it has been showed thatthe cherry extract resulted enriched in total (poly)phenols content

(14.50 mg GA eq/g dried weight), 1.4 times higher than those contained in the whole cherry (Manuscript 1). Thus, although animals in both studies consumed the same amount of product, the dose extract contained more phenolic compounds than the fruit dose, which may explain the differences observed among the groups Moreover, cherry extract was enriched in anthocyanins, which may be responsible for the observed effect. Previous studies have shown that anthocyanin intake improved dyslipidemia in healthy populations without taking into account the time of the year of consumption [32].

Moreover, some phenolic compounds, such as catechins, which are present in cherries [33], are known to inhibit pancreatic lipase and therefore limit the absorption of lipids from the diet [34].

Fresh plum is characterized by a high content of proanthocyanidins and chlorogenic acids [35], and previous studies have shown that extracts rich in proanthocynidins attenuated the development of cardiometabolic risk factors in rats [36]. In addition, several clinical studies showed that plum supplementation had a significant effect on reducing LDL-C; however, no effects were observed in lowering TAG levels among healthy and overweight patients supplemented with different dosage [37]. These results are not in agreement with those of our study, in which plum extract reduced TAG levels. The plum extract was enriched in phenolic compounds compared with the whole plum (1.5 times). Therefore, in addition to the *in vivo* model and duration of treatment, an increase in the amount of phenolic compounds administered to animals (plum extract vs. whole fruit) could explain the different effects observed by fruit and extract.

Therefore, these results suggest that cherry and plum extracts might be good candidates for use as supplements or functional food ingredients to lower blood postprandial TAG levels during winter season and the composition of fruit extract plays a key role in TAGs management.

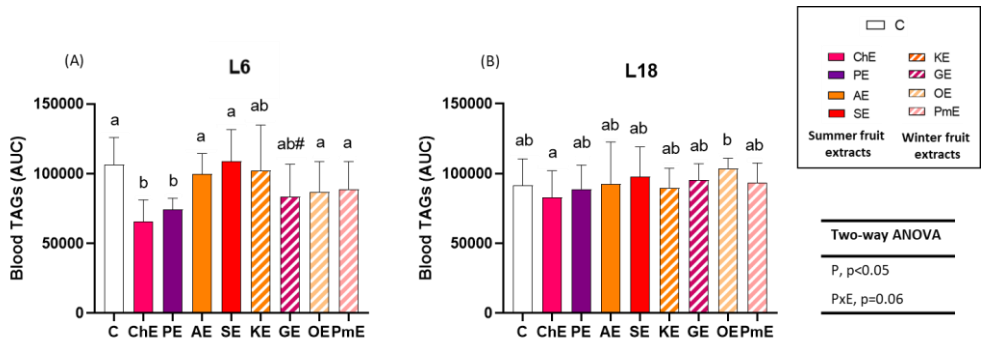

Figure 2. Oral lipid tolerance test (OLTT). Blood triacylglyceride (TAG) levels, represented as area under the curve (AUC), of 12h-fasted Fischer 344 (F344) rats exposed to short (L6, 6h light, figure A) or long (L8, 18 h light, figure B) photoperiods and administered water (control, C) or 100 mg/kg body weight (BW) of cherry (Ch), plum (P), apricot (A), strawberry (S), persimmon kaki (K), grape (G), orange (O), and pomegranate (Pm) extracts (E) for two weeks (n=8 per group, mean ± standard deviation). OLTT was performed after 3 h post-treatment administration through the oral administration of lard oil (2.5 mL/kg BW) to animals, and tail blood was analysed at different times (0-240 min). Different letters indicate significant differences between groups for a specific photoperiod (One-way ANOVA, Post-hoc Tukey test, $p < 0.05$). Two-way ANOVA analysis was used to examine differences between all groups: P (photoperiod effect); E (fruit-extract effect); PxE (interaction between photoperiod and extract) (Post-hoc Tukey test, $p < 0.05$).). # indicates tendency differences between the same control or extract administered in the L6 or L18 photoperiods (Student's t-test, $p > 0.05$

3.2. Circulating inflammatory cytokines after induced inflammation are modulated by fruit extracts depending on the photoperiod

Another outcome related to obesity and metabolic disorders is intestinal inflammation due to a defective barrier function. When intestinal barrier function is compromised, endotoxin entry is promoted, causing a chronic and systemic inflammatory state [38]. Indeed, gut permeability is strictly dependent on microbiota composition, which in turn is influenced by diet type [39]. High-fat diet intake is strongly correlated with adipose tissue expansion with an increase in several inflammatory cytokines and chemokines, such as TNF-α, IL-1, and IL-6, and

acute-phase proteins, such as CRP [40]. In the present study, an LPS-induced inflammatory challenge was performed to simulate systemic inflammation, and several inflammation markers were analysed to evaluate the possible anti-inflammatory effects of the seasonal-fruit extracts in a photoperiod dependent manner. Previous studies have pointed out that prevalence of inflammation could occur during the winter season in humans [41]. Moreover, it has been previously reported that in photoperiodic responsive species there is a proinflammatory status during short photoperiod in the *pars tuberalis* of the ewe [42].

One of the evaluated biomarkers was TNF-α, a pro-inflammatory cytokine that stimulates macrophage response in the presence of LPS [43]. A photoperiod and extract consumption effect, and interaction between both factors were observed (two-way ANOVA, P, E, PxE, $p<0.05$). Serum TNF-α levels were more than 2 times higher in the L6-C group than in the L18-C group (2,197.3 pg/mL and 853.4 pg/mL, respectively) (Figure 3A-B), indicating that the inflammatory state is favoured under the winter light/dark schedule.

Regarding the effects of the extracts on this inflammatory marker, serum TNF-α levels ranged between 427.5 and 2197.3 pg/mL in the supplemented L6-exposed animals, and between 714.0 and 1453.2 and pg/mL in the supplemented L18-exposed rats (Figure 3 A and B, respectively). All extracts obtained from winter fruits (persimmon kaki, grape, orange, and pomegranate), and one from a summer fruit, strawberry extract significantly decreased TNF-α levels when administered to L6-exposed rats in comparison with the L6-C group (Figure 3A). However, when the same extracts were administered to L18-exposed rats, most of them did not produce changes in serum TNF-α levels compared to the L18-C group, except for the persimmon kaki extract (Figure 3B). In line with persimmon kaki extract, apricot and strawberry extracts increased TNF-α levels in rats exposed to the L18 with respect to L18-C group, tending to counteract the photoperiod effect (Figure 3 A and B). Notably, a differential photoperiod-dependent effect was observed after the consumption of the strawberry extract ($p<0.05$, Student's t-test) as TNF-α levels

were reduced in the L6 photoperiod, whereas it exerted an opposite effect in the L18 photoperiod (Figures 3A and B). Previous studies conducted in preclinical models have observed that supplementation with a combination of fruits and vegetables reduced serum TNF-α concentration in 12:12 light/dark schedule [44]. Circulating TNF-α is correlated with endothelial dysfunction, and specifically, the intake of strawberry has been associated with the improvement of antioxidant markers and endothelial dysfunction associated with cardiovascular diseases by decreasing inflammation markers in humans [45]. Pomegranate, grape and strawberry extracts were the most antioxidant as they exhibited DPPH scavenging activity *in vitro* (174.90 umol/g, 107.62 umol/g and 81.78 umol/g, respectively) (Manuscript 1). Thus, these antioxidant effects could contribute to the anti-inflammatory effects of these fruit extracts during short photoperiod.

Several studies performed on fruits have previously observed a photoperiod-dependent effect on several metabolic biomarkers involved in glucose and lipid metabolism [27]. However, to the best of our knowledge, this is the first time that the anti-inflammatory effects of extracts containing phenolic compounds have been shown to depend on the photoperiod in which they are consumed.

As a non-specific response to obesity, the liver produces CRP, whose levels are modulated by different dietary patterns [46]. A high intake of cholesterol was associated with increased CRP levels [47], whereas a Mediterranean diet, rich in bioactive compounds from fruits and vegetables was associated with decreased CRP levels [48]. Moreover, high CRP levels are associated with winter season suggesting that light hours could play a role in the inflammatory state in humans [49]. Moreover, it has been showed that there is an inverse correlation between consumption of several fruits and blood CRP levels in patients suffering MetS [50]. In the present study, serum CRP levels were higher in rats exposed to a short photoperiod in the C group than in the C group exposed to long photoperiod (Student's t-test, $p<0.05$) (Figure 3C and D). A photoperiod and extract consumption effects, and interaction between both factors were observed (two-

way ANOVA, P, E, PxE, $p<0.05$). All fruit extracts, except for the cherry extract, decreased serum CRP levels when consumed in the L6 photoperiod, with the pomegranate extract being the most active in lowering this inflammation marker (0.24 ng/mL) (Figure 3C). This extract showed the highest *in vitro* antioxidant capacity (Manuscript 1), and the correlation between the intake of antioxidants and low serum CRP levels has been described in a clinical study [51].

Regarding the L18 photoperiod, all extracts produced changes in serum CRP levels in F344 rats exposed to L18 photoperiod compared with the control group, except for cherry and grape extracts. In this regard, plum, apricot, strawberry, orange, and pomegranate extracts decreased CRP levels compared to the L18-C group, whereas persimmon kaki extract increased it (Figure 3D). Among the fruits used in this present study, strawberries are one of the fruits with the highest content of (poly)phenols and antioxidant capacity [52]. Their consumption has been associated with several beneficial effects, such as antimicrobial, antioxidant, and antihypertensive effects [53], and it is inversely correlated with CRP levels in humans [54] which agrees with the results of this study. Although TNF-α levels increased after the consumption of strawberry extract in L18 photoperiod, they remained lower than those in L6-C (less than 2000 pg/mL).

In addition, the strawberry extract is the third extract with the highest total polyphenols content and antioxidant capacity (Manuscript 1). Differences between extracts could be produced by the different phenolic profiles of the eight fruit extracts (Manuscript 1). Moreover, here we showed that the ability to decrease this inflammatory marker depended on the photoperiod. Pomegranate extract also significantly lowered CRP levels in both photoperiods, although previous clinical studies in individuals supplemented with pomegranate juice did not show any improvement in CRP blood levels [55]. Overall, most fruit extracts reduced CRP levels although this effect depended on the fruit type and photoperiod of consumption.

Another circulating inflammatory cytokine, IL-6, is associated with obesity phenotype and contributes to the incidence of cardiovascular diseases [56]. The consumption of foods rich in phenolic compounds, such as the açaí fruit is associated with decreased levels [57]. On the other hand, IL-10, anti-inflammatory cytokine, is involved in the suppression of inflammation [58]. In the present study, IL-6 levels were affected by both photoperiod and extract consumption, and an interaction was observed between both factors (two-way ANOVA, P, E, PxE, $p<0.05$) (Figures 3 E and F). All winter-fruit extracts (persimmon kaki, grape, orange, and pomegranate extracts) decreased serum IL-6 levels in rats exposed to the L6 photoperiod with respect to C and all summer-fruit extracts, suggesting a relationship between the consumption of winter-fruit extracts and their anti-inflammatory properties only under the winter light/dark schedule, although the extracts do not maintain the phenolic hallmark of the original fruit. Apricot, strawberry, and persimmon kaki extracts reduced IL-6 levels in the long photoperiod (Figure 3F).

The results of the present study agree with those of previous studies carried out in fruits, in which anti-inflammatory effects of persimmon kaki were observed as it downregulated IL-6 gene expression in a model of MetS in zebrafish [59]. In addition, a clinical study showed that after the ingestion of high-fat foods, proinflammatory cytokines increased in the blood, and orange fruit supplementation partially counteracted inflammation by lowering pro-inflammatory cytokine levels such as IL-17 but not IL-6 [60]. In addition, our study revealed photoperiod-dependent effects on IL-6 level in most of fruit extracts. In this regard, the anti-inflammatory effects of apricot, strawberry and grape extracts depended on the photoperiod of consumption; however, in the case of persimmon kaki extract, its anti-inflammatory effect did not depend on photoperiod (Figures 3E and F). These results highlight the link between biological rhythms and fruit extract enriched in (poly)phenols.

Finally, a photoperiod and extract consumption effects as well as an interaction between both factors were found in the present study (two-way ANOVA, P, E, PxE, $p<0.05$) (Figures 3G and H). Anti-inflammatory cytokine IL-10 levels were almost two times higher in the L6 photoperiod in most of the groups studied than in the L18 groups (except for plum and apricot extracts, Student's t-test, $p<0.05$). Moreover, neither of the fruit-extracts increased IL-10 levels in any photoperiod. In contrast, all winter-fruit extracts and the summer-fruit extracts, cherry and apricot extracts, reduced serum IL-10 levels in L6 photoperiod, whereas plum, grape, and orange extracts reduced they in L18-photoperiod (Figures 3 G and H). These results could be attributed to the number of hours after LPS injection, indeed after 4 h of induced inflammation, the anti-inflammatory cytokine response did not reach its peak concentration [61].

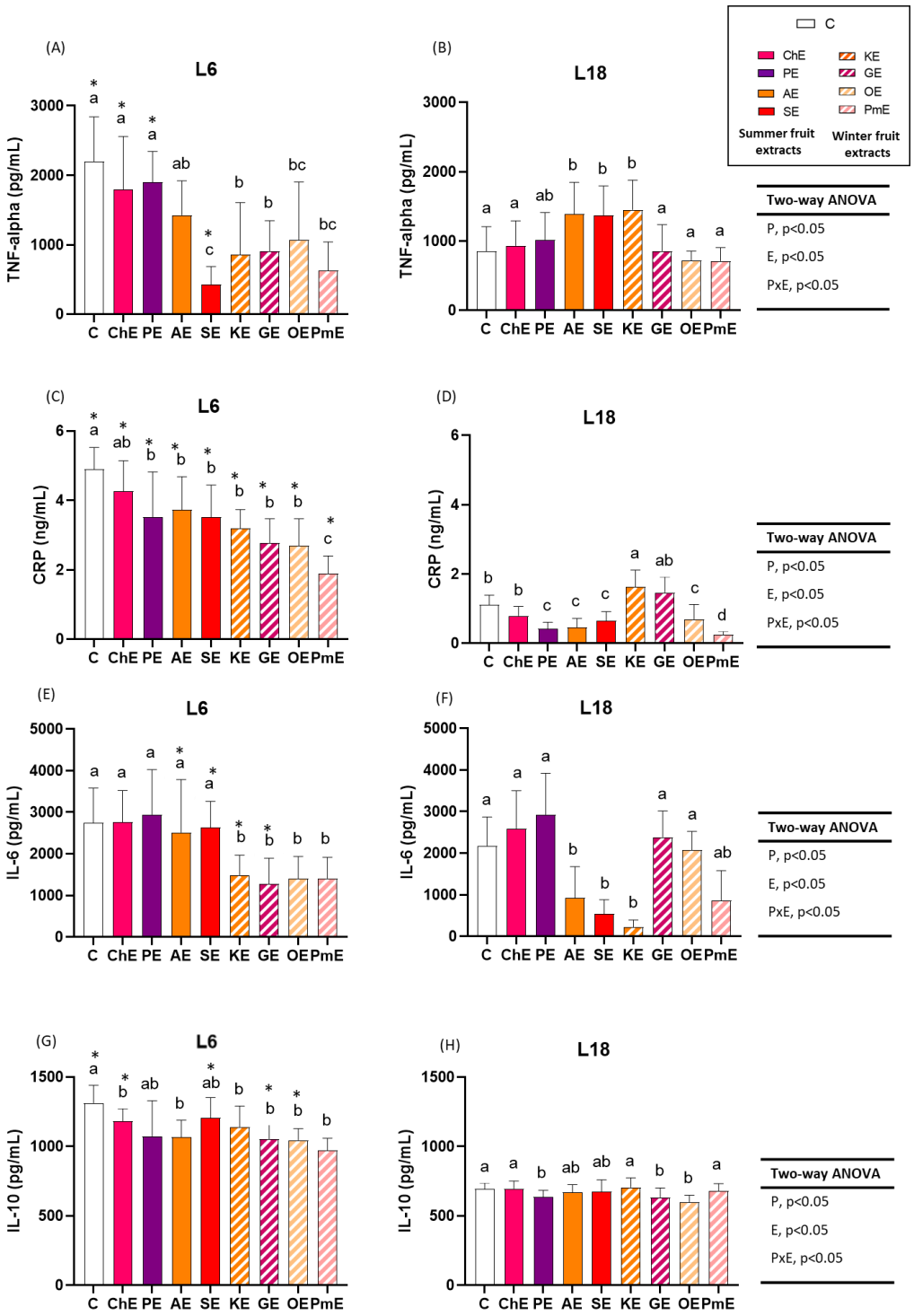

Figure 3. Lipopolisacharide (LPS) challenge**.** Serum tumour necrosis factor alpha (TNF-α, figures A and B), C-reactive protein (CRP, figures C and D), interleukin-6 (IL-

6, figures E and F), and IL-10 (figures G and H) levels of Fischer 344 (F344)rats exposed to short (L6, 6h light, figures A, C, E, and G) or long (L8, 18 h light, figures B, D, F and H) photoperiods and administered water (control, C) or 100 mg/kg body weight (BW) of cherry (Ch), plum (P), apricot (A), strawberry (S), persimmon kaki (K), grape (G), orange (O), and pomegranate (Pm) extracts (E) for two weeks (n=8 per group, mean ± standard deviation). LPS-induced inflammatory response was performed after 3 h post-treatment administration through the intraperitoneal injection of LPS (0.5 mg/kg BW) to animals. Different letters indicate significant differences between groups for a specific photoperiod (One-way ANOVA, Post-hoc Tukey test, $p < 0.05$). Two-way ANOVA analysis was used to examine differences between all groups: P (photoperiod effect); E (fruit-extract effect); PxE (interaction between photoperiod and extract) (Post-hoc Tukey test, $p < 0.05$). * indicates differences between the same control or extract administered in the L6 or L18 photoperiods (Student's t-test, $p<0.05$).

4. Conclusions

Seasonal fruit extracts might be effective in preventing or treating metabolic disorders by restoring the disturbed homeostasis. The reduction in postprandial blood TAG levels, after induced dyslipidemia, and modulation of inflammatory cytokines in a situation of induced inflammation depended not only on the fruit extracts but also on the photoperiod of consumption. The management of postprandial TAG levels is improved by the consumption of extracts, independent of the seasonality of the fruit in short photoperiod (cherry and plum extract). These results provide new insights into the role of phenolic-enriched fruit extracts with a specific interest in cherry and plum extracts in the management of dyslipidemia, a cardiovascular risk factor. However, inflammatory markers decreased after induced inflammation in short photoperiod with a stronger effect of winter-fruit than summer-fruit extracts.

These results are promising for producing seasonal fruit extracts and fighting one of the challenges in reducing fruit loss. By designing food supplements that align with different seasons, it might be possible to provide specific nutritional needs depending on the time of year. Thus, further research is needed to explore the

potential benefits of seasonal-fruits extracts in the prevention of metabolic disorders depending on the time of the year they are consumed.

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7. Conflict of Interest

The authors declare no conflict of interest.

8. Author Contributions

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

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Chapter 3

To study the impact of seasonal consumption of whole fruits with different phenolic hallmarks on cerebral and peripheral responses

Manuscript 5

Cherries with different geographical origin regulate neuroprotection in a photoperiod dependent manner in F344 rats

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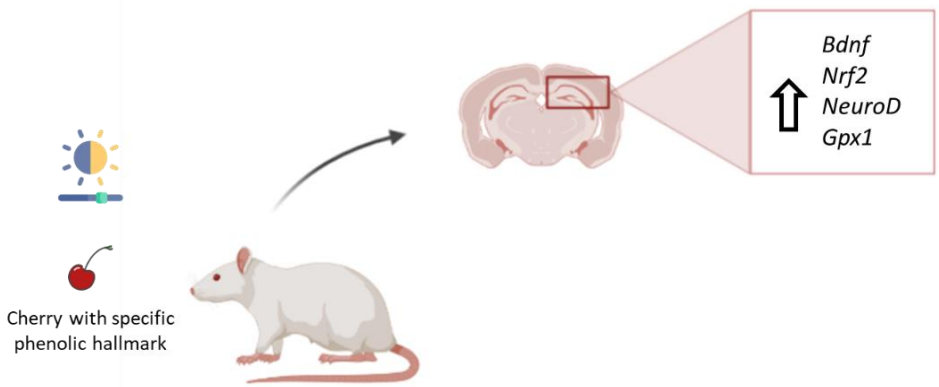
Abstract

Photoperiod is the main environmental cue that drives seasonal adaptive response in reproduction, behaviour, and metabolism in seasonal species. There is increasing evidence that polyphenols contained in fruits can modulate seasonal rhythms. Polyphenols rich diet are associated with an improvement in cognitive function and neuroprotection. However, it remains to be elucidated whether cherries rich in polyphenols affect neuroprotection in a photoperiod-dependent manner. To test this, F344 rats were exposed to short (L6, 6h of light/6h of dark), intermediate (L12, 12h of light/12h of dark) and long (L18, 18h of light/12h of dark) photoperiods and fed a standard chow diet supplemented with either control, or lyophilized cherry 1, or cherry 2 with distinct phenolic hallmark. Hippocampal markers of neuroprotection, such as *Bdnf*, *Sod1* and *Gpx1* resulted modulated by photoperiod and upregulated by the consumption of Cherry 2, richer in total flavonols but not by Cherry1 richer in total anthocyanins and flavanols, highlighting that the phenolic hallmark plays a pivotal role to exert healthy effects.

Keywords: neuroprotection, photoperiod, cherry, phenolic compounds.

Graphical abstract



1. Introduction

Many organisms adapt to seasonal fluctuations in the climate with profound changes in physiology, behaviour, and reproduction [1]. These seasonal changes are driven by changes in daylength (photoperiod) and mediated by neuroendocrine processes in the hypothalamus [2]. Briefly, photoperiod information is translated biochemically through the release of melatonin from the pineal gland during dark hours. Melatonin then acts on the pars tuberalis of the pituitary gland to regulate thyroid stimulating hormone release which in turn regulates thyroid hormone availability of deiodinase enzymes Dio2 and Dio3 in hypothalamic tanycytes lining the third ventricle of the hypothalamus. Indeed, hypothalamic tanycytes express thyroid stimulating hormone receptors [3]. Thus, they sense thyroid stimulating hormone subunit beta signals from the pituitary, which expression is upregulated by photoperiod and results in a long photoperiod-induced *Dio2* mRNA expression (12, 14 or 16 h of light) and a short photoperiod-induced *Dio3* mRNA expression (6 and 8 h of light) [4]. DIO2 coverts the inactive form of thyroid hormone, thyroxine (T4) in the active form triiodothyronine (T3), increasing hypothalamic concentration of T3 [5]. Thus, the hypothalamic-pituitary-thyroid as well as the hypothalamic-pituitary-gonadal axes are sensitive to changes in photoperiod.

In addition, Hypothalamic tanycytes express a variety of nutrients sensors [6]. Nutrients (glucose, amino acids, lipids) as well as hormones (leptin, ghrelin, and insulin), produced in response to nutrients availability, can modulate gene expression in tanycytes, which ultimately regulates energy metabolism [7]. Moreover, the hypothalamus is sensitive to phenolic compounds, which are one of the main bioactive components in fruits and vegetables [8]. In support, administration of grapes and cherries, rich in phenolic compounds, to Fischer 344 (F344) rats increase hypothalamic leptin sensitivity when they are consumed during short photoperiod [8], [9]. Moreover, phenolic compounds can exert a neuroprotective effect. A recent study showed that the consumption of tart

cherries, fruit rich in anthocyanins (a class of phenolic compounds), significantly affects the plasma metabolome associated with an improvement of cognitive function in humans consuming this fruit [10].

For example, tart cherry exerts anti-neuro-inflammatory properties and suppresses neuronal apoptosis and stimulates pro-survival signalling cascades *in vitro* [11] and in aged rats [10]. Furthermore, aged rats consuming blueberries show an improvement of working memory and an upregulation of hippocampal Brain derived neurotrophic factor (*Bdnf*) gene expression compared to control rats [12]. The BDNF protein plays a key role in neuronal plasticity and together with nuclear factor erythroid-derived 2-like (Nrf2) promotes gene expression of antioxidant enzymes in the hippocampus [13]. One of the most important activities of phenolic compounds is the antioxidant activity. Recently, our group showed that sweet cherries increased hepatic antioxidant markers in F344 rats depending on the composition profile of the cherries and the photoperiod of consumption [14]. Cherries are seasonal fruits (available from May to August), but out of season they are harvested and imported from different geographical locations to allow continuous sales [15]. Depending on the location of harvest and season they are consumed their phenolic profile is different and sweet cherries can modulate the activity of transaminase enzymes and reduce lipid peroxidation markers, depending on their phenolic profile and the photoperiod in which they are consumed [14].

However, it is yet unknown if the consumption of cherries with different geographical origins and phenolic composition could exert a neuroprotective effect in a photoperiod dependent manner. Considering these facts, the aim of this study was to elucidate if the consumption of sweet cherries can play a role in neuroprotection in a photoperiod and composition profile dependent manner.

2. Material and methods

2.1. Fruit preparation and composition

Royal Dawn sweet cherries (*Prunus avium* L.) were harvested in two different geographical locations. Cherries from the Tarragona region in Spain (Ch1) were harvested in season and kindly donated by a local farmer, cherries from Chachapoal in Chile (Ch2) were purchased in a local market out of season. Cherry pulps were frozen in liquid nitrogen, grounded and lyophilized. After lyophilisation, they were kept at -20◦C until administration to animals. Basic composition and phenolic content of the two cherries has been published in detail in Cruz-Carrion et al. 2020 [14]. Briefly, Ch1 has a higher content in total polyphenols, total anthocyanin, total flavanol than Ch2. Ch2 has more flavonol content compared to Ch1.

2.3. Experimental design

The animal experiment in this study was conducted in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and further approved by Generalitat de Catalunya (permission number 9495, FUE-2017-00499873).

Blood and brain tissue for the current publication are taken from the previously published study by Cruz-Carrion et al. 2020 [14]. 72 male Fischer 344 rats (substrain F344/IcoCrl), 7 to 8 weeks old, with body weight between 216-244 g, were purchased from Charles River Laboratories (Barcelona, Spain) and were housed in pairs at 22 °C, 50% of humidity, in three photoperiods (light density 700 lx): L6 photoperiod (short photoperiod; 6 h light/18 h dark); L12 photoperiod (12 h light/12 h dark) and L18 photoperiod (long photoperiod; 18 h light/6 h dark). Throughout the experiment, rats received a standard chow diet (AO4, Panlab, Barcelona, Spain) and tap water *ad libitum*. After 4 weeks of photoperiod acclimatisation, F344 rats in each photoperiod were randomly subdivided into

three groups. One group of F344 rats received chow diet which was supplemented with 100mg Ch1 dry weight (dw)/kg body weight (Ch1 group), group 2 received 100mg Ch2 dw/kg (Ch2 group), and the control group (C group) received 21.2mg/kg glucose and 21.2 mg/kg bw fructose to match the sugar content of the diets between the three groups (n = 8 per group). Group sizes were selected based on results from previous studies [16], [17]. During the 4 weeks of photoperiod acclimatisation, rats were trained to lick a syringe with water, which allowed us to avoid oral gavage and thus reduce stress and improve animals' welfare during the 7 weeks of fruits supplementation. Fruit supplements were dissolved in tap water and orally voluntary administered. Rats were weighed every week and food intake was measured in the last week. The weight (in gram) of pellets of standard diet was recorded when the food was provided and subtracted after 24 h to calculate how many gr of pellets rats consumed. The eating pattern index (EPI) was calculated according to the calculations used by Ruiz de Azua et al. 2022 [18]and considers the kcal consumed, as well as the fact that animals eat mainly during the hours of darkness: EPI (kcal/h): (Kcal/day)/hours of darkness/day. Health checks were carried out daily and no welfare-related issues were observed.

At the end of the experiment, rats were deprived of food after supplementation with Ch1, Ch2, or C and were killed by decapitation 1h later between ZT1 and ZT2. Paired testes weight was recorded, and brains were dissected and immediately frozen in liquid nitrogen and stored at −80◦C until use. The hippocampus and hypothalamus were dissected at -20◦C on the day of RNA keep the integrity of RNA. Figure 1 shows a graphical representation of the experimental design used in this study.

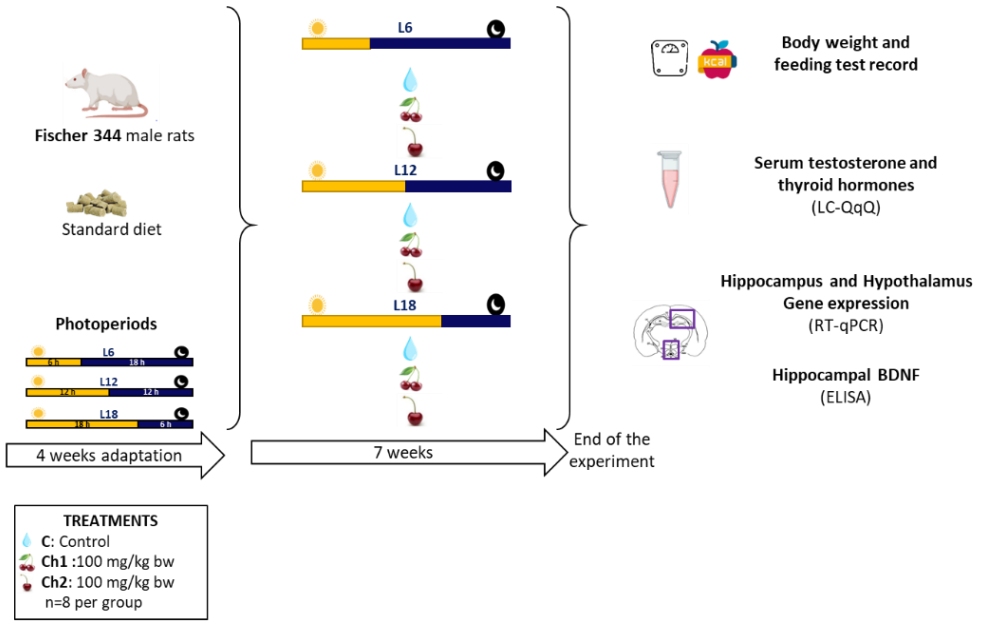


Figure 1. Experimental design carried out in the study.

2.4. High performance liquid chromatography coupled to triple quadrupole (LC-QqQ)

Serum testosterone, T3 and T4 concentrations were measured using high performance liquid chromatography coupled to triple quadrupole (LC-QqQ) following the method and the equipment described by Domenech-Coca et al., 2019 [19]. Preparation of samples was performed mixing 50 µL of serum with 250 µL of methanol containing the internal standard (2 ng/mL). Then, it was vortexed and centrifuged for 5 min at 4 °C at 20000g. The supernatant was mixed with 700 µL of 0.1 % formic acid in water and subjected to a solid phase extraction using an Oasis HLB 96-wells plate (Waters, Milford, MA, USA), previously conditioned with methanol and 0.1 % formic acid in Milli-Q water. The cartridge was washed with 0.1 % formic acid in Milli-Q water and dried under high vacuum. The compounds were eluted with 500 µL of methanol. Samples were evaporated in a SpeedVac at 45 °C and reconstituted with 50 µL of Milli-Q water:methanol (60:40, v/v) and transferred to a glass vial for analysis. After that, 5 µL of solution was injected in a spectrometry

UHPLC 1290 Infinity II Series (Agilent Technologies, Santa Clara, California, USA) coupled to a QqQ/MS 6490 Series (Agilent Technologies) using an C18 analytical column (Zorbax Eclipse, 150 x 2.1 mm, from Agilent Technologies). The chromatographic separation was performed with a gradient (40 % of B during 0.5 min, 40-80 % of B in 10.5 min, 80-100% in 0.1 min, 100% of B during 1.1 min, 100-40 % in, 40% during 3.5 min), Mobile phase A was water with 0.5 % acetic acid and 10mM ammonium acetate, and B was 100% methanol. The column temperature was set at 45ºC and flow at 3 mL/min. Standards and internal standards (3,3',5'-Triiodo-L-thyronine-13C6 solution, L-thyroxine-d4 and testosterone d3 for T3 and T4 and testosterone, respectively) were dissolved in methanol. Calibration curves were constructed in the range of 0.05-10 ng/mL for testosterone, 0.05-5 ng/mL for T3 and 1-100 ng/mL for T4.

2.5. Gene expression analysis in hypothalamus and hippocampus

Approximately 70 - 80 mg of hypothalamus and hippocampus, were dissected from each rat and transferred to cell disruption buffer, homogenized with 1.5 mm homogenization beads (Triple-Pure™, Molecular Biology Grade Zirconium Beads) using the BeadBug microtube homogenizer. After homogenization, RNA extraction was carried out according to the manufacturer's protocol using the PARIS™ kit (Invitrogen). The RNA was quantified using a Nanodrop™ spectrophotometer (Nanodrop Lite, Thermo Fisher Scientific). 500 ng of RNA were used to synthesize the cDNA using High-Capacity cDNA Reverse Transcription Kit and qPCR was performed with the Biorad real time system CFX96 to amplify gene expression levels using following parameters: 40 cycles of 2 min at 50°C, 5 min at 95°C, 10 s at 95°C, and 30 s at 60°C. mRNA levels were normalized to D-Box and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used to amplify the genes are described in the supplementary materials Table S1.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation. One-way ANOVA (Tukey post-hoc) was used to determine the differences between groups, ($p < 0.05$). Two-way repeated measures ANOVA (Tukey post-hoc) analysis Photoperiod x Fruit was used to assess the differences between groups. F, Fruit and P, photoperiod effect; PxF, effect of interaction. The interaction was considered statistically significant with a p value of ≥ 0.05. The statistical tests were performed with SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). Outliers were identified using SPSS using Tuckey's method and removed before statistical analysis.

3. Results

3.1 Effect of photoperiod on body weight, eating pattern index (EPI) and serum testosterone levels in F344 rats.

The photoperiod responsiveness of F344 rats was assessed using body weight, eating pattern index, testes weight, serum testosterone, and T3/T4 ratio (Table 1). After 11 weeks of photoperiod exposure, 2-way ANOVA revealed a significant difference in body weight between the groups ($P=0.001$) with a 6% lower body weight in L6-C compared to L12-C (Tukey post hoc, $p=0.06$). Within the photoperiod groups, no significant difference was found regarding cherry consumption, however, L6-Ch1 showed significant decrease in body weight compared to L12-Ch1. To assess eating behavior, EPI was calculated for all groups and represented as kilocalories consumed by the rats and the hours of darkness when animals are active and fed (Table 1). EPI was significantly affected by photoperiod as follows: L6-C<L12-C<L18-C (two-way ANOVA $p<0.05$). As it was observed in body weight, no differences were found in EPI when rats consumed Ch1 or Ch2.

No significant difference was found in paired testes weight (Table 1), however, two-way Anova revealed a significant interaction between photoperiod and cherry consumption in serum testosterone levels (P, PxF, two-way ANOVA $p<0.05$).

Specifically, testosterone concentration was significantly lower in L18-C compared to L6-C and L12-C (Table 1). Regarding cherry consumption, Ch1 increased serum testosterone levels in L6-Ch1 and L12-Ch1 groups compared to their controls group (L6-C and L12-C groups, respectively); however, no effects of cherry consumption was found in L18 photoperiod.

Circulating levels of thyroid hormones in serum (T4/T3 ratio) were affected by photoperiod and fruit (PxF, two-way ANOVA, $p<0.05$), with the lowest T4/T3 ratio in L6-C compared to the other photoperiod control groups (Table 1). Moreover, Ch1 significantly increased the T4:T3 ratio in F344 rats exposed to L6 photoperiod compared to L6-C animals (Table 1).

3.2. Hypothalamic gene expression mainly responds to photoperiod.

Previous studies have shown a range of photoperiodically regulated genes in the hypothalamus of F344 rats, including genes involved in thyroid hormone and retinoic acid signalling (Ross et al 2011, Helfer et al 2012, Helfer et al 2013). Here, we tested key genes involved in photoperiodic time measurements and assessed whether they are regulated by cherries consumption. Hypothalamic *Dio2* expression did not show photoperiod responsiveness in control groups after 11 weeks of photoperiod exposure; however, Ch1 consumption significantly upregulated *Dio2* expression in F344 rats exposed to L6 photoperiod compared to all other groups, unless L12-C (Figure 2A; two-way ANOVA P, PxF $p<0.05$). *Dio3* was downregulated in L18-C compared to L6-C and L12-C groups, and in L12-Ch2 compared to L12-C (Figure 2B) (P, F two-way ANOVA, $p<0.05$).

An interaction between photoperiod and fruit was found in *Retinaldehyde dehydrogenase 1 (Raldh1)* expression levels (PxF, two-way ANOVA, $p<0.05$), however we did not find differences in photoperiod (Figure 2C). After Ch1 consumption, hypothalamic *Raldh1* expression levels were downregulated in L12-Ch1 and L18-Ch1 groups compared to L6-Ch1 group.

Furthermore, hypothalamic Growth hormone-releasing hormone *(Ghrh)* and Somatostatin *(Sst)* mRNA expression levels were analysed. *Ghrh* mRNA levels were strongly affected by photoperiod with lower levels in L6-C compared to L12-C and L18-C; however, this effect disappeared after the consumption of both cherries (L6-Ch1 and L6-Ch2 groups) (Figure 2D). Interestingly, *Ghrh* mRNA expression was downregulated by the consumption of Ch2 in L18, reaching expression levels as the ones in L6-C (Figure 2D). Two-way ANOVA analysis revealed an effect of photoperiod and an interaction between photoperiod and fruit (two-way ANOVA; PxF, P, $p<0.05$). *Sst* levels were downregulated in L18-C compared to L12-C and L6-C. Moreover, Ch2 group showed a downregulation of this gene compared to all other groups (two-way ANOVA, F, $p<0.05$). Here, we showed that both genes not only responded to photoperiod but also to cherry consumption within a specific photoperiod, as Ch2 exacerbate the downregulation of *Ghrh* and *Sst* in L18.

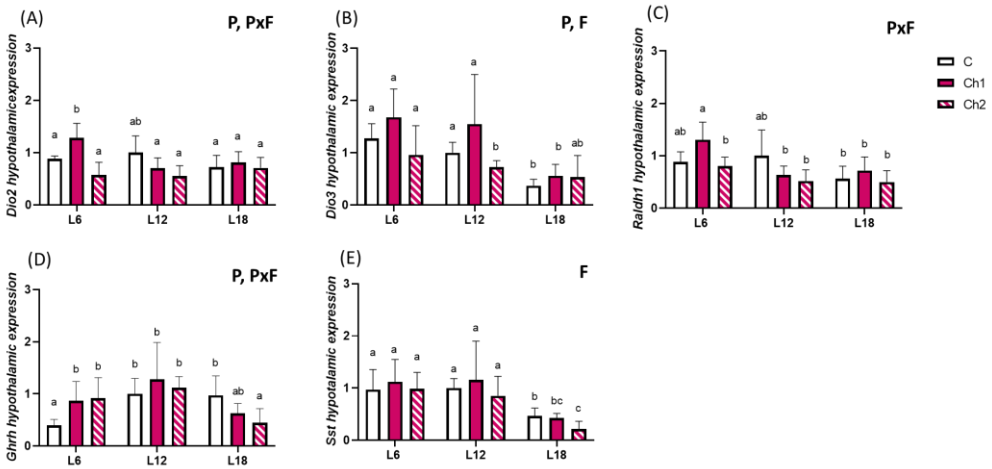


Figure 2. Hypothalamic (A) deiodinase 2 (Dio2), (B) deiodinase 3 (Dio3), (C) retinaldehyde dehydrogenase 1 (Raldh1), (D) growth hormone-releasing hormone (Ghrh), (E) somatostatin (Sst) mRNA expression of Fisher 344 rats exposed to L6 (6 h of light/ 18 h of dark), L12 (12 h of light/ 12 h of dark) or L18 (18 h of light/ 6 h of dark) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight) or cherry 2 (Ch2, 100 mg/kg body weight) during the last 7 weeks. Values are expressed as mean ± standard deviation and normalized relative to L12-C group (n = 8 per group). P and F or PxF indicate significant differences

produced by photoperiod, fruit or interaction between photoperiod and fruit, respectively (two-way ANOVA, $p<0.05$). Different letters above the bars indicate significant differences post-hoc Tukey, (one-way ANOVA).

3.3. Cherry consumption exerts a hippocampal neuroprotection effect, which was modulated by specific phenolic hallmark and photoperiod.

Next, we investigated a possible neuroprotective effect of seasonal consumption of sweet cherries with different phenolic hallmark on gene expression markers in the hippocampus. Growing evidence suggests that consumption of fruit is associated with neuroprotective effects (Habauzit & Morand, 2012; Lapuente et al., 2019) but reports on the effect of photoperiod on neuroprotection are less common. *Bdnf* mRNA levels were modulated by P, F and PxF (two-way ANOVA, $p<0.05$), with Ch2 consumption strongly upregulating *Bdnf* expression compared to all other groups (Figure 3A). *Nrf2* expression levels were affected by fruit consumption and photoperiod (PxF, $p<0.05$). L18-Ch2 group showed higher expression levels compared to L18-Ch1 group (Figure 3B). *NeuroD1* was regulated by photoperiod, with lower *NeuroD1* expression in L18-C compared to L6-C; however, Ch2 consumption significantly upregulated *NeuroD1* expression compared to all other groups (Figure 3C). In addition, *Sod1* was significantly affected by photoperiod being upregulated in L6-C compared to L12-C and L18-C. Consumption of Ch1 significantly downregulated *Sod1* levels while Ch2 consumption maintained the expression level of *Sod1* (Figure 3D). *Gpx1* showed a similar expression pattern to *Sod1*, however Ch2 consumption significantly upregulated *Sod1* expression compared to L18-C and Ch1 (Figure 3E).

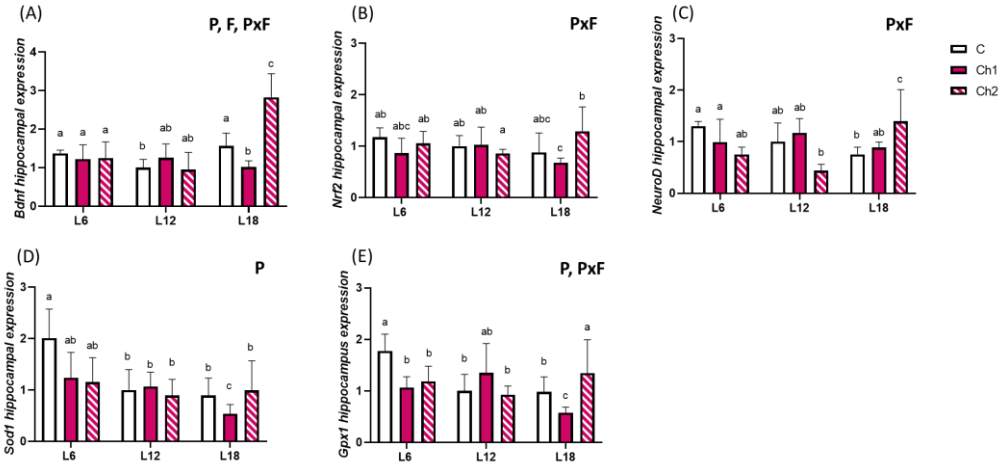


Figure 3. Hippocampal (A) brain derived neurotrophic enzyme (Bdnf), (B) nuclear factor erythroid-derived 2-like 2 (Nrf2), (C) neuronal differentiation 1 (NeuroD), (D) superoxide dismutase type 1 (Sod1), (E) glutathione peroxidase 1 (Gpx1) mRNA expression of Fisher 344 rats exposed to L6 (6 h of light/ 18 h of dark), L12 (12 h of light/ 12 h of dark) or L18 (18 h of light/ 6 h of dark) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight) or cherry 2 (Ch2, 100 mg/kg body weight) during the last 7 weeks. Values are expressed as mean ± standard deviation and normalized relative to L12-C group (n = 8 per group). P and F or PxF indicate significant differences produced by photoperiod, fruit or interaction between photoperiod and fruit, respectively (two-way ANOVA, $p<0.05$). Different letters above the bars indicate significant differences post-hoc Tukey, (one-way ANOVA).

4. Discussion

In this study, we investigated the effect of photoperiod and fruit consumption on the expression of key genes involved in photoperiod regulation in the hypothalamus and the activation of antioxidant pathways in the hippocampus in photoperiod-sensitive F344 rats. We show that markers of neuroprotection are strongly regulated by photoperiod and/or seasonal fruit consumption. The consumption of fruit is associated with several beneficial health effects, including the reduction of blood pressure or cell damage through their antioxidant effects [20], [21]. One of the main compounds responsible for the health benefits of fruit are the phenolic compounds which can have antioxidative and anti-inflammatory

effects [22]. Importantly, phenolic compounds, including resveratrol and epicatechin, can cross the blood-brain barrier [23], [24]. In fact, the phenolic compounds quercetin and naringin, have been shown to promote neuroprotection in the dentate gyrus of the hippocampus [25], the main brain region that hosts neurogenic niches [26]. In support, the consumption of blueberries, a fruit rich in phenolic compounds, exerts positive effects on the brain by enhancing memory and learning in animals and humans [12]. Firstly, to evaluate photoperiod responsiveness of the F344 rats in this study, we analysed physiological parameters (body weight, eating pattern), photoperiod responsive hormones and photoperiod regulated genes in the hypothalamus. As expected, we found that body weight and feeding behaviour respond to photoperiod. These results are consistent with previous studies in F344 rats showing that body weight and food intake are strongly regulated by photoperiod with lower body weight and food intake in short photoperiod compared to long photoperiod [4], [17], [27]. However, compared to these studies, the effect of photoperiod on body weight in our study was more subtle. Differences between studies could be attributed to a photoperiod refractoriness since this study was carried during 11 weeks of photoperiod. Refractoriness is a physiological response of seasonal species, such as hamster and sheep, to a change in photoperiod spontaneously reverting to the initial state, even if the photoperiod remains unchanged [3]. Another reason could be that different sub-strains of F344 respond differently to photoperiod, for example the F344/NHsd rats are more sensitive to photoperiod than F344/NCrHsd [28]. Interestingly, Ch1 exacerbated the photoperiod effect decreasing body weight in short photoperiod compared to L12 photoperiod.

A shared mechanism of photoperiod responsiveness among different seasonal species is the change in hypothalamic thyroid hormone availability depending on the photoperiod of exposure [29]. Here we show that hypothalamic thyroid signalling was not only affected by photoperiod but also varied depending on the cherry consumption. It has been widely described that the expression of Dio2 and

Dio3 enzymes in tanycytes regulates hypothalamic thyroid hormone availability by converting between biologically active T3 and inactive T4 [1]. In our study only hypothalamic Dio3 expression was regulated by photoperiod with a downregulation in long photoperiod as expected [30]–[32]. Species-specific differences in *Dio2* and *Dio3* expression have been discussed before [1] although previous studies in F344 rats indicate a photoperiod response with an upregulation of *Dio2* in long photoperiod [33], [34]. However, in these studies maximum photoperiod exposure was for 4 weeks, whereas in this study rats were exposed to photoperiod for 11 weeks.

Intra-hypothalamic thyroid hormone metabolism plays an important role in the seasonal response [35] but seasonal pattern of serum thyroid hormone is controversial. According to a clinical study in 700,000 people, only thyroid stimulating hormone level showed a seasonal fluctuation, while T3 and T4 levels were not affected [36]. Nevertheless, another study with over 7,000 volunteers showed monthly changes in serum T3 and T4 levels [37]. It has been suggested that diets rich in fruits (i.e., rich in polyphenols) are correlated with beneficial effect on thyroid hormones in population studies [38]. Here we show that T4/T3 ratio increased with consumption of Ch1 under short photoperiod compared to control suggesting that the effect of fruit consumption on thyroid hormone depends on the photoperiod.

Raldh1 is involved in retinoic acid synthesis and its absence is associated with a decrease in food intake and body weight in F344 rats [31], [39]. Our results contrast other studies carried out in F344 exposed to photoperiod for 3 and 14 days, where *Raldh1* showed a strong photoperiod effect and was downregulated in short photoperiod [39]. Differences between both studies could be attributed to the duration of animal photoperiod exposure considering that photoperiod refractoriness could be involved in this pathway. The growth hormone axis is seasonally regulated, and its increase is associated with an increase in daylength in seasonal species [40]. Growth hormone is involved in promoting anabolic functions

when nutrients are available. On the other hand, somatostatin (SST) inhibits its activity as a mechanism of feedback regulation [41]. Different nutrients such as amino acids, vitamins as well as minerals play a role in the regulation of this axis [41] , and it has been suggested that a Mediterranean diet rich in fruits and vegetables is correlated with a peak of growth hormone in humans [42]. In this study we demonstrated that Ghrh and Sst are regulated by photoperiod in F344 rats and the consumption of Ch2 is associated with decreased expression.

In mammals, reproduction is strongly modulated by photoperiod [43]. For example, testes weight of F344 decreases during the first weeks of short photoperiod although these changes disappear after 10 weeks of photoperiod exposure [44]. These findings agree with the results observed in the present study since no differences were observed in testes mass after 11 weeks of photoperiod. In humans, a clinical study showed that antioxidant properties of fruit are strongly associated with improved sperm quality [45], however the effect of fruit consumption on testosterone was not fully elucidated We observed that testosterone levels are modulated by Ch1 but not Ch2, in short and intermediate photoperiod which could be linked to different composition between the two cherries. Moreover, sex hormones have been correlated with neuroprotection in neuronal model cells, however in presence of oxidative stress, they increase the cell loss in both neuronal and glial cells [46].

This evidence allowed us to evaluate the photoperiod responsiveness in this study to later focus on neuroprotective role of cherries with different origin and composition.

Among the factors that regulate hippocampal homeostasis, diets rich in fruit and vegetables have been associated with an improvement in cognition and working memory. For instance, the consumption of tart cherry in aged F344 rats decreases the expression of neuroinflammatory markers such as NADPH oxidase and Cyclooxygenase 2 [47]. Other studies observed that phenolic compounds, such as quercetin and kaempferol activate key proteins involved in neuroprotection in the

hippocampus including NRF2 and BDNF. NRF2 acts in the nucleus by inducing the transcription of genes containing a specific region called ARE (antioxidant response element) [48]. This signalling promotes the expression of antioxidant enzyme such as Glutathione peroxidase 1 (GPX1) and Superoxide dismutase type 1 (SOD1), by reducing cell damage and the production of reactive oxygen species [25]. Neuroprotection in the brain is correlated with antioxidant enzymatic activity of a subset of proteins, which can be stimulated by external factors such as the intake of fruits rich in bioactive compounds [49]. Additionally, it has been observed that thyroid hormones play an important role in adult neurogenesis in the hippocampus, promoting cell survival, upregulating expression of genes involved in cell differentiation such as Neuronal differentiation 1 (*NeuroD)* [50]. Considering that thyroid hormones play a pivotal role in generating seasonal rhythms, it is plausible to hypothesize that neuronal functions in the hippocampus is affected by photoperiod. In support, we recently showed that short photoperiod impairs memory in F344 rats, [17]. In this current study, hippocampal *Bdnf*, *Nrf2*, *NeuroD*, and *Gpx1* expression levels were upregulated when rats consumed Ch2 in L18 photoperiod, promoting hippocampal neuroprotection and stimulating the expression of gene involved in antioxidant signalling, suggesting that cherry effects are regulated by photoperiod. Given Ch1 did not exert effects, composition of both cherries must be pivotal in their differential effects. Phenolic composition of the two cherries was different; Ch1 was composed of a higher content in total polyphenols contents, total anthocyanin, total flavanol than Ch2 while Ch2 showed more flavonol respect to Ch1 [14]. Previously we showed that lipid peroxidation markers due to free radicals are reduced by the consumption of both cherries during L18 in a different manner being one of the cherries more effective in lowering those markers respect to the other, highlighting that the composition of the cherry influence the different response. Moreover, the effects from one organ to another could be different since, in the case of the brain, the blood brain barrier is highly selective. It has been observed that the metabolites coming from the

digestion of polyphenols cross the blood brain barrier depending on the molecular structure and their metabolism in endothelial layer produces a variety of metabolites with additional bioactive properties [51]. While a previous study, did not show a photoperiod response in Bdnf mRNA expression in the hippocampus of hamster exposed to short or long photoperiod [52], clinical studies have shown that circulating markers of neuroprotection, such as BDNF, show a seasonal pattern [53]. To our best knowledge, this is the first study showing photoperiod-dependent regulation of neuroprotective markers in the hippocampus by a specific type of cherry.

5. Conclusions

Seasonal consumption of cherry with specific composition could play an important role in hippocampal activation of neuroprotection in a photoperiod dependent manner, by increasing expression markers of neuroprotection in long photoperiod.

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7. Conflict of Interest

The authors declare no conflict of interest.

8. Author Contributions

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

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Table 1 Final body weight (BW), testes weight, eating pattern index, serum testosterone levels, serum triiodothyronine (T3)/ thyroxine (T4) ratio of Fisher 344 rats exposed to L6 (6 h of light/ 18 h of dark), L12 (12 h of light/ 12 h of dark) or L18 (18 h of light/ 6 h of dark) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight) or cherry 2 (Ch2, 100 mg/kg body weight) during the last 7 weeks of the experiment. Values are expressed as mean ± standard deviation (n = 8 per group). P and PxF indicate significant differences in photoperiod or interaction between photoperiod and fruit, respectively (two-way ANOVA, 2wA, $p<0.05$). Different letters indicate significant differences among groups for each parameter (one-way ANOVA, post-hoc Tukey, $p<0.05$). # indicates tendency (one-way ANOVA, post-hoc Tukey, $p<0.05$-$p<0.01$).

Parameters	L6 photoperiod			L12 photoperiod			L18 photoperiod			2wA
	C	Ch1	Ch2	C	Ch1	Ch2	C	Ch1	Ch2	
Final BW (g)	$367.7\pm13.7^{ab\#}$	369.5 ± 20.0^{b}	370.8 ± 21.3^{ab}	389.8 ± 25.4^{a}	393.7 ± 14.9^{a}	381.8 ± 17.1^{ab}	383.7 ± 27.8^{a}	387.8 ± 20.7^{ab}	376.1 ± 24.0^{ab}	P
EPI*	3.00 ± 0.04^{a}	3.16 ± 0.19^{a}	3.06 ± 0.25^{a}	4.62 ± 0.27^{b}	4.68 ± 0.27^{b}	4.48 ± 0.26^{b}	9.15 ± 0.22^{c}	9.02 ± 0.22^{c}	9.19 ± 0.47^{c}	P
Testosterone (ng/mL)	2.98 ± 0.72^{a}	4.16 ± 1.02^{b}	3.45 ± 1.43^{ab}	2.95 ± 0.98^{a}	4.20 ± 1.09^{b}	3.68 ± 1.83^{ab}	1.63 ± 1.0^{c}	1.86 ± 0.98^{c}	2.43 ± 1.39^{ac}	P, PxF
Testes (g)	3.08±0.16	3.13±0.14	3.15±0.12	3.15±0.13	3.20±0.1	3.26±0.14	3.05±0.17	3.2±0.15	3.2±0.17	
T4/T3	42.46 ± 2.64^{a}	50.68 ± 4.89^{b}	$47.76\pm4.85^{ab\#}$	48.31 ± 4.41^{b}	47.45 ± 5.90^{ab}	47.89 ± 5.40^{b}	51.25 ± 6.14^{b}	51.97 ± 6.47^{b}	47.6 ± 5.22^{ab}	PxF

* Kilocalories consumed per hours of darkness in the different photoperiods

Supplementary material

Table S1. Nucleotide sequences of primers used for real time quantitative PCR.

Genes	Forward (5' to 3')	Reverse (5' to 3')
Bdnf	GTCTGTCTGTAAGGGCTAGAATG	GTCTCCTATGAAGCCACCTAATC
Dio2	GCGACCTGACCACCTTTTACTAG	GCAGCACATCGGTCCTCTTG
Dio3	AATTGCAGAGGGGCTCGAAA	TTCCTTTGGTCCTGAAGCCC
Ghrh	TTAGGGTCTGGACATCACTGG	GCCCACACTCTGTCCAAATG
Gpx1	CAGTCCACCGTGTATGCCTT	GTAAAGAGCGGGTGAGCCTT
NeuroD	AGCCCCCTAACTGATTGCAC	CCCGGGAATGGTGAAACTGA
Nrf2	CTCTCTGGAGACGGCCATGACT	CTGGGCTGGGGACAGTGGTAGT
Raldh1	ACGTGGAAGAAGGGGACAAGGCTG	GCAAAGACTTTCCCACCATTGAGTGCC
Sod1	TAACTGAAGGCGAGCATGGG	TCCCAATCACACCACAAGCC
Sst	CCCCAGACTCCGTCAGTTTC	AACGCAGGGTCTAGTTGAGC

Abbreviations: Bdnf (brain derived neurotrophic enzyme); Dio2 (deiodinase 2); Dio3 (deiodinase 3); Ghrh (growth hormone realising hormone); Gpx1 (glutathione peroxidase 1); NeuroD (neuronal differentiation 1); Nrf2 (nuclear factor erythroid-derived 2-like 2); Raldh1 (retinaldehyde dehydrogenase 1); Sod1 (superoxide dismutase type 1); Sst (somatostatin)

Manuscript 6

Photoperiod and geographical origin of sweet cherries modulate thyroid hormones and hepatic glucose metabolism in F344 rats

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Abstract

In seasonal species, metabolism and reproductive homeostasis are regulated by the photoperiod. Cherries are seasonal fruits rich in phenolic compounds, and their intake induces marked photoperiod-dependent effects. Moreover, it has been reported that cherries of different geographical origins exhibit different effects, possibly owing to their different phenolic signatures. Thyroid hormone (TH) is sensitive to changes in day length and plays a pivotal role in linking neuroendocrine responses to peripheral organs. Among other effects, TH is responsible for the regulation of hepatic glucose metabolism. Therefore, this study aimed to investigate whether cherries with different phenolic hallmarks exhibit a seasonal response to TH and potential metabolic consequences on hepatic glucose metabolism. To accomplish this, Fischer 344 rats fed a standard diet were chronically exposed to short (L6, 6h light), intermediate (L12, 12h light), and long (L18, 18h light) photoperiods and supplemented with either control (C) or lyophilized cherries from different geographical origins (Ch1 and Ch2). Both cherries showed differential and photoperiod-dependent effects on the serum concentrations of TH and metabolites related to glucose metabolism, whereas the hepatic expression of TH target genes related to gluconeogenesis was mainly affected by photoperiod. Specifically, Ch1 affected mainly in L6 rats; however, Ch2 caused effects in L18 and, to a lesser extent, in L6, tending to cancel out the differences between photoperiods.

Keywords: fruit seasonality, phenolic hallmark, photoperiod, (poly)phenols, seasonal rhythms.

1. Introduction

Most vertebrates have adapted their physiology to external conditions as a survival response to seasonal changes [1]. Despite their short lifespan, seasonal variations in reproduction, body weight (BW), and food intake have been shown to occur even in small animals [2]. In addition, different tissues have peripheral patterns that are sensitive to the photoperiod. Specifically, chronic exposure to a short photoperiod (6h light/18h dark) resulted in lower insulin levels in rats fed a standard diet than in those exposed to a long photoperiod exposure (18h light/6h dark). This effect was accompanied by hyperglycemia, decreased phosphorylation of protein AKT2 in the soleus and gastrocnemius muscles, as well as downregulation of genes involved in glucose metabolism and insulin signalling [3].

Day length is one of the main seasonal cues [4]. The change in photoperiod affects nocturnal melatonin production in the pineal gland, which in turn stimulates the production of thyroid-stimulating hormone (TSH) in the hypophysis (*pars tuberalis)* and controls the availability of local thyroid hormone (TH) in hypothalamic tanycytes by acting on deiodinase (DIO) enzymes [5]. This photic information is translated as a loss of BW and a decrease in food intake during a short photoperiod in Fisher 344 (F344) rats [6]. The active form of TH, triiodothyronine (T3), exerts a variety of physiological roles associated with metabolism, thermogenesis, heart rate [7], and glucose metabolism in the liver [8].

Eating cherries, seasonal fruits consumed between spring and summer, have been linked to health-promoting effects, including antihypertensive properties [9] and protection against high-fat-induced steatosis in the liver [10]. Moreover, an improvement in the activity of antioxidant blood and hepatic enzymes [11], as well as a decrease in different markers of inflammation such as C-reactive protein [12] have been demonstrated after cherry consumption in animal or clinical studies, respectively, demonstrating their anti-inflammatory and antioxidant effects. Nevertheless, it has been demonstrated that their intake induces marked

photoperiod-dependent effects [3] Regarding to the molecules responsible of their beneficial effects, cherries are a source of vitamins, minerals, tryptophan and phenolic compounds [13], and it has been widely reported that phytochemicals consumption, particularly phenolic compounds, contained in plants and fruits, exert healthy benefits on metabolism [14]. The phenolic profile of cherries has been extensively studied, containing high levels of hydroxycinnamic acids, anthocyanins, flavan-3-ols, and flavonols [15]. However, the phenolic content of plants and fruits varies according to environmental conditions (temperature, humidity, light, soil, etc.), maturity, processing, and storage [16] According to this, cherries from different geographical origins showed differences in the abundance of total phenolic compounds, anthocyanins and flavanols [17]. In addition, the administration of cherries with different phenolic hallmarks to F344 rats under different photoperiods caused differential effects. For instance, both the origin of the fruit and photoperiod significantly affected the antioxidant status and oxidative stress of these animals [17]. In addition, the intake of cherries from different origins had also differential effect on the rat metabolic response, including hepatic lipid metabolism, which is in turn regulated by TH [18].

Considering all the evidence, the purpose of the present study was to study the effect of the consumption of cherries with different phenolic signatures on both TH levels and hepatic glucose metabolism in rats exposed to different photoperiods.

2. Material and methods

2.1. Chemical and Reagents

Liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ) analysis standards; L-thyroxine-d4, L-thyroxine (T4), 3,3’,5-triiodo-L-thyronine (T3), and 3,3’,5'-triiodo-L-thyronine-13C6 solution were provided by Sigma-Aldrich (St. Louis, MO, USA). Water (Sharlab, Barcelona, Spain), acetic acid (Sigma-Aldrich), methanol (MERK, Madrid, Spain), and ammonium acetate (Sigma-Aldrich) were HPLC grade. All other reagents were of analytical grade.

2.2. Fruits preparation and composition

Brooks sweet cherries (*Prunus avium* L.) from different geographical locations, Spain (Ch1) and Chile (Ch2), were used in this experiment to obtain cherries with different phenolic hallmarks. The frozen cherries were ground and lyophilized. After lyophilisation, the samples were frozen at -20◦C until use. Basic composition and phenolic content were previously quantified by Cruz-Carrión et al., 2021 [17].

2.3. Experimental design

Male F344 rats (7-8 weeks old) were provided by Charles River Laboratories (Barcelona, Spain). Animals were housed at 22 °C, two rats per cage, and divided into three photoperiods: L18 (long; 18 h light/6 h dark), L12 (intermediate; 12 h light/12 h dark), and L6 (short; 6 h light/18 h dark), which simulate the seasons. The rats were fed a standard chow diet (AO4, Panlab, Barcelona, Spain) and tap water ad libitum. After a 4-weeks photoperiod adaptation where they were trained to lick a syringe with water only, rats were subdivided into three groups (n= 8 per group) and supplemented daily for 7 weeks with 100 mg Ch1 dry weight (dw)/kg BW (Ch1 group) and 100 mg Ch2 dw/kg BW (Ch2 group). Moreover, control group (C group) was administered 42.4 mg/kg BW of a mixture of glucose and fructose (1:1, w/w; vehicle), to simulate the sugar content contained in cherries. Because the vehicle provides 0.11% of the daily energy, its effect is not considered relevant because of its low energy value. The dry cherries were dissolved in tap water. All treatments were administered by voluntarily licking a syringe. During the experiment, rats were weighed weekly until the end of the experiment. At the end of the experiment, rats were fasted for 1h after supplementation with Ch1, Ch2, or C and were killed 1 h post-administration by decapitation. Serum was obtained by centrifugation (2,000×g at 4°C for 15 min) of non-heparinized blood stored for 1 h at room temperature. The liver was immediately frozen in liquid nitrogen and stored at −80 °C. Figure 1 shows a representative view of the experimental design carried out in this study.

The animal protocols were in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and the Generalitat de Catalunya (permission number 9495, FUE-2017-00499873).

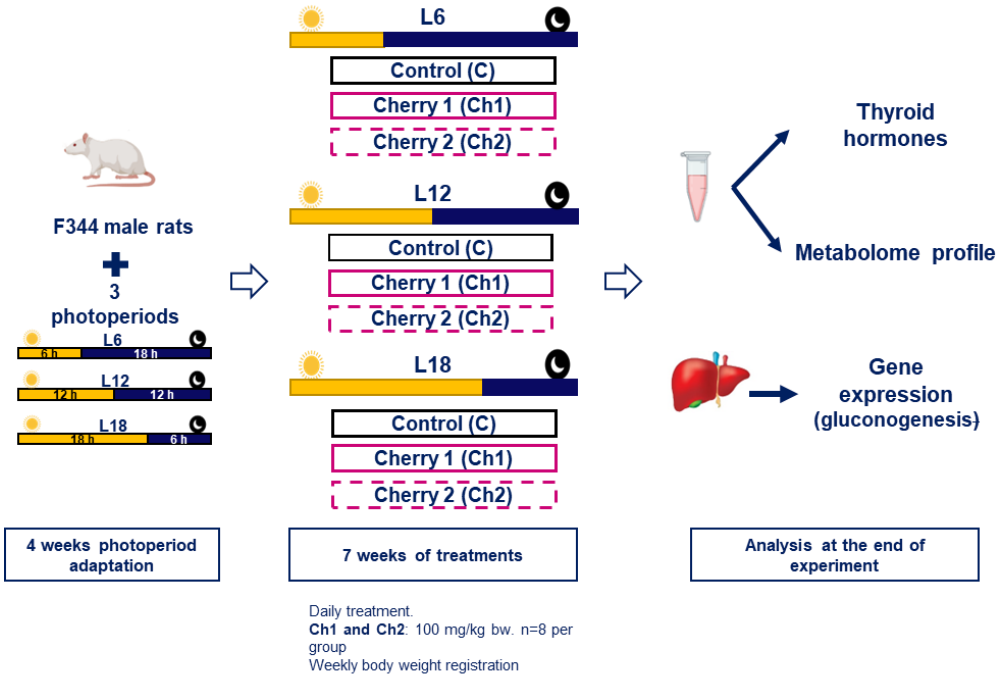

Figure 1. Experimental design.

2.4. Serum thyroid hormone analysis by LC-QqQ

Serum T3 and T4 concentrations were measured using high-performance liquid chromatography coupled to triple quadrupole (LC-QqQ) following the procedure and method described by Soliz-Rueda et al. 2022 [19]. Briefly, methanol (250 µL) containing 2 ng/mL of the internal standards (3,3',5'-Triiodo-L-thyronine-13C6 solution and L-thyroxine-d4 for T3 and T4, respectively) was added to serum (50 µL). After centrifugation (27,670 x g at 4 °C for 5 min), the supernatant was mixed with 0.1 % formic acid (700 µL) and added to an Oasis HLB 96-wells plate (Waters Milford, MA, USA), which was previously conditioned. The serum compounds were

eluted with methanol (500 µL). Methanol was removed by evaporation in a SpeedVac at 45 °C and the residue was reconstituted with 50 µL of Milli-Q water:methanol (60:40, v/v)After that, 5 µL of solution was injected in a spectrometry Ultra-High-Performance Liquid Chromatography (1290 Infinity II Series; Agilent Technologies) coupled to a QqQ/MS (6490 Series; Agilent Technologies) using an C18 analytical column (Zorbax Eclipse, 150 x 2.1 mm, Agilent Technologies). The compounds were separated using a gradient (40 % of B during 0.5 min, 40-80 % of B in 10.5 min, 80-100 % in 0.1 min, 100 % of B during 1.1 min, 100-40 % in 0.1, 40 % during 3.5 min), water with 0.5 % acetic acid and 10mM ammonium acetate as mobile phase A and 100 % methanol as phase B. The column temperature and flow rate were set to 45 ºC and 3 mL/min, respectively. The hormone standards and internal standards were dissolved in methanol. Calibration curves were generated for T3 and T4 in the range of 0.05-5 ng/mL and 1-100 ng/mL, respectively.

2.5. Gene expression analysis

50 mg of liver tissue was homogenized with TRIzol reagent (Thermo Fisher Scientific, Illkirch Graffenstaden, France) following the manufacturer's instructions for RNA extraction. cDNA was synthesized by reverse transcription using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Real-time polymerase chain reaction (RT-qPCR) and iTaq Universal SYBR Green Supermix (Biorad, Madrid, Spain) were used to amply specific cDNA fragments. Specifically, phosphoenolpyruvate carboxykinase 1 (Pck1), glucose-6-phosphatase catalytic subunit (G6pc), iodothyronine deiodinase 1 (Dio1), glucokinase (Gk), glucose transporter 2 (Glut2), and nicotinamide phosphoribosyltransferase (Nampt) genes. Relative expression levels were calculated as a percentage of the L12 Control group using peptidylprolyl isomerase A (Ppia) as a reference gene. The nucleotide sequences of primers used were provided by Bioprimers (Ulm, Germany) and are listed in Table S1.

2.6. Serum extraction and 1H-NMR spectrometry

Serum metabolites were analysed by Proton Nuclear Magnetic Resonance (1H-NMR) as previously described by Palacios-Jordan et al., 2020 and using the same equipment [20]. Hydrophilic metabolites were obtained using a twice-steps process, in which firstly, serum was mix with methanol and Milli-Q water to obtain a monophasic solution, and secondly, this solution was mix with chloroform and Milli-Q water. After centrifugation, the upper aqueous phase was freeze-dried and stored at −80 °C until 1H-NMR analysis. The 1H-NMR spectra were compared with pure compound references obtained from the metabolic profiling AMIX spectra database (Bruker), HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, AN, Canada), and using databases for metabolite identification. Specific 1H-NMR regions identified in the spectra were integrated using AMIX 3.9 software package.

2.7. Statistical analysis

Data were presented as the mean ± standard deviation. Differences between groups were identified using one-way ANOVA (Tukey's post hoc test; $p < 0.05$) and two-way ANOVA (Tukey's post-hoc test): photoperiod (*P*), fruit (*F*), or interaction of both factors (*PxF*). The interaction was considered statistically significant at $p < 0.05$. Statistical analysis was performed using the SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). The outliers were identified using SPSS software.

3. Results

3.1. Effects of seasonal consumption of sweet cherries from different geographical origins on body weight

Figure 2 shows the evolution of BW of animals subjected to the different photoperiods and supplemented C, Ch1 and Ch2 during the study. As expected, the BW increased in all groups during the 11 weeks of study. At the end of the

experiment, the L6-C group showed a tendency for decreased BW compared to the L12-C group after 11 weeks of photoperiod exposure (Figure 2A). Ch1 significantly lowered BW at L6 compared to that at L12 (Figure 2B). No differences were observed between photoperiods neither when rats did not consume Ch2 (Figure 2C) or when the three groups were compared for each photoperiod.

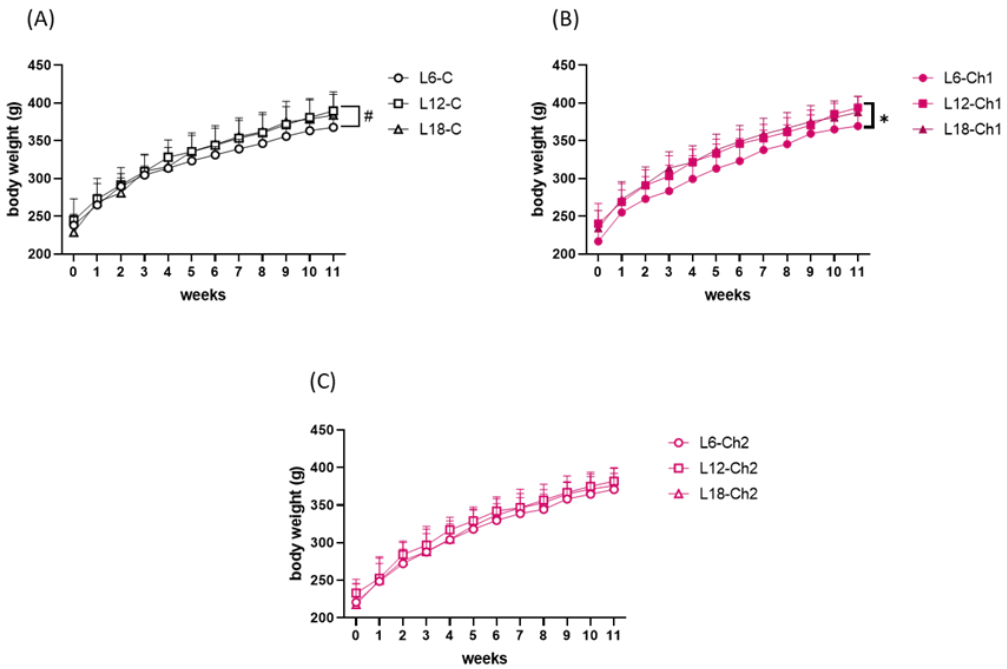

Figure 2. Body weights of Fisher 344 rats exposed to L6 (6 h light/ 18 h dark), L12 (12 h light/ 12 h dark), or L18 (18 h light/ 6 h dark) photoperiods for 11 weeks and administered vehicle (C, figure A), cherry 1 (Ch1, 100 mg/kg body weight, figure B) or cherry 2 (Ch2, 100 mg/kg body weight, figure C) for the last 7 weeks. * and # indicate significant differences ($p<0.05$) and tendency ($0.05<p<0.1$) between the L6 group and the L12 and L18 groups at the 11th week (one-way ANOVA, post-hoc Tukey).

3.2 Impact of seasonal consumption of sweet cherries from different geographical origins on thyroids hormones

Interactions between photoperiod and cherry effect were observed in serum T3 and T4 levels (two-way ANOVA, *PxF,* p=0.002 and p=0.03, respectively). T3 levels

significantly increased in L6-C group (119.0 ng/dL) respect to L12-C (100.2 ng/dL) and L18-C (87.7 ng/dL) groups (Figure 3A). Moreover, cherry effect consumption was observed in both L6-Ch1 and L6-Ch2 by decreasing in a different manner T3 levels. No differences were observed when the cherries were consumed in L12; however, Ch2 consumption increased T3 levels in L18 compared to the L18-C and L18-Ch1 groups, nullifying the photoperiod effect (Figure 3A). Regarding T4 levels, a very similar pattern was observed for the active thyroid hormone (T3). A photoperiod effect was observed when L6-C (5.1 µg/dL) and L18-C (4.0 µg/dL) were compared. In addition, Ch1 reduced T4 levels in the L6-Ch1 group compared to that in the L6-C group (Figure 3B). As shown for T3, the inactive form in L18 also showed significantly increased levels in the L18-Ch2 group compared to those in the L18-C group. To study the hepatic conversion of T4 to T3, hepatic Dio1 mRNA expression was determined. Photoperiod effect was also observed in hepatic Dio1 mRNA expression (two-way ANOVA: P, $p<0.05$) as L6-C animals showed a significantly upregulated hepatic Dio1 expression in comparison with L12-C (Figure 3C), and cherry effect was observed when Ch1 was consumed in L6, counteracting the effect of the photoperiod.

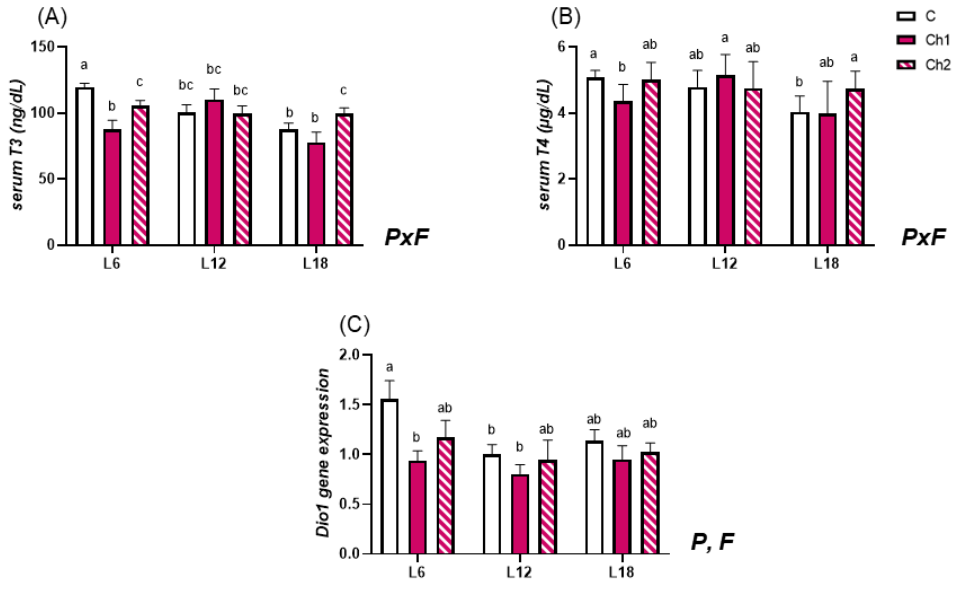

Figure 3. Effect of photoperiod and cherry consumption on thyroid hormone levels. Serum thyroid hormone levels triiodothyronine (T3, A) and thyroxine (T4, B) and hepatic expression levels of *Dio1* of Fisher 344 rats exposed to L6 (6 h of light/ 18 h of dark), L12 (12 h of light/ 12 h of dark) or L18 (18 h of light/ 6 h of dark) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight) or cherry 2 (Ch2, 100 mg/kg body weight) during the last 7 weeks. Values are expressed as the mean ± standard deviation (n = 8 per group). *P, F,* or *PxF* indicate significant differences produced by photoperiod, fruit, or interaction between photoperiod and fruit, respectively (two-way ANOVA, $p<0.05$). Different letters indicate significant differences (one-way ANOVA, post-hoc Tukey test, $p<0.05$).

3.3. Impact of seasonal consumption of sweet cherries from different geographical origins on erum metabolic profile

Serum metabolomic profiles were determined using high-resolution 1H-NMR spectroscopy (Table S2), and metabolites related to gluconeogenesis were selected and plotted in heatmaps (Figure 4). In the L6 photoperiod, Ch2 consumption increased the level of serum metabolites compared with the L6-C and L6-Ch1 groups. In L12, a remarkable shift was not observed; however, some metabolite levels, such as valine and asparagine were differentially altered by cherry

consumption. In L18, both Ch1 and Ch2 showed notably different patterns compared to those in L18-C. Statistical analyses (one- and two-way ANOVA) are presented in Table S2.

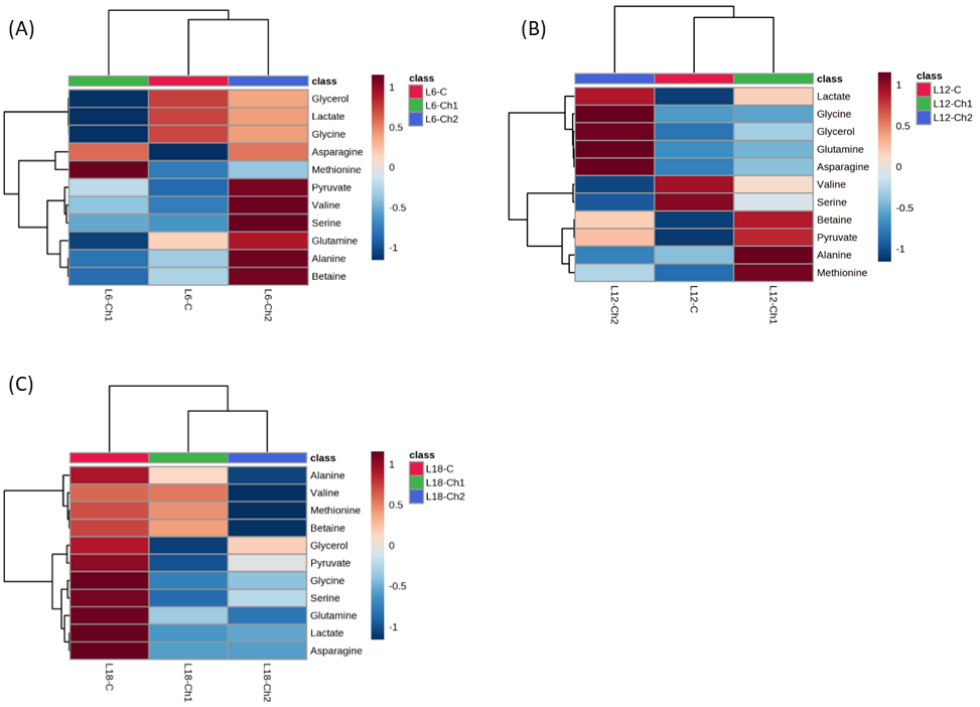

Figure 4. Heatmap plot of hierarchical clustering analysis of the serum gluconeogenic-related metabolites of Fisher 344 rats exposed to L6 (6 h light/ 18 h dark, figure A), L12 (12 h light/ 12 h dark, figure B), or L18 (18 h light/ 6 h dark, figure C) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight), or cherry 2 (Ch2, 100 mg/kg body weight) for the last 7 weeks (n=8 per group).

3.4. Effects of seasonal consumption of sweet cherries from different geographical origins on hepatic genes

A subset of genes related to gluconeogenesis (*Glut2, Gk, Pck1*, *G6pc, and Nampt*) was analysed in the liver (Figure 5). Hepatic Glut2 expression did not exhibit a photoperiod expression pattern (Figure 5A). Regarding cherry consumption, changes in Glut2 expression were observed only in L6, where the L6-Ch2 group showed significantly higher expression than the L6-Ch1 and L6-C groups. In addition, gene expression analysis revealed that Gk is part of a subset of genes affected by photoperiod exposure (two-way ANOVA, $p<0.01$). Specifically, it was upregulated in the L18-C compared to both the L12-C and L6-C groups (Figure 5B). The fruit consumption effect was observed on this hepatic gene only in L6, where Ch1 significantly downregulated its expression compared with the L6-C group. As shown in Figures 5 C and D, G6Pc (two-way ANOVA: P, $p<0.001$; F $p<0.05$) and Pck1 (two-way ANOVA: P, $p=0.01$) genes encoding key gluconeogenic enzymes were significantly downregulated in the L18-C compared with the L6-C and L12-C groups. Regarding the cherry consumption effect, cherries upregulated the expression of both genes, counteracting the photoperiod effect on G6Pc (Figures 5C and D). Specifically, Ch2 upregulated Pck1 in L18-Ch2 animals in comparison with the L18-C group and upregulated G6Pc expression in Ch2-L6 and Ch2-L18, whereas Ch1 only upregulated G6Pc expression in the L6 photoperiod. Differences between the types of cherries were only observed at L18 for Pck1 (Figure 5D).

Finally, Nampt mRNA expression (Figure 5E) was upregulated in the L18-C group compared to the L6-C and L12-C groups, highlighting the photoperiod effect (two-way ANOVA: P, $p=0.007$). The cherries effect was observed in the L6 and L18 groups, as Nampt expression was downregulated in the L6-Ch1 group and in L18-Ch2 compared with L6-C and L18-C, respectively. L18-Ch2 animals showed levels of Nampt expression similar to those observed in the L12-C group. The differential

effect of cherries on the expression of this gene was observed in L18 (Ch1 group vs. Ch2 group).

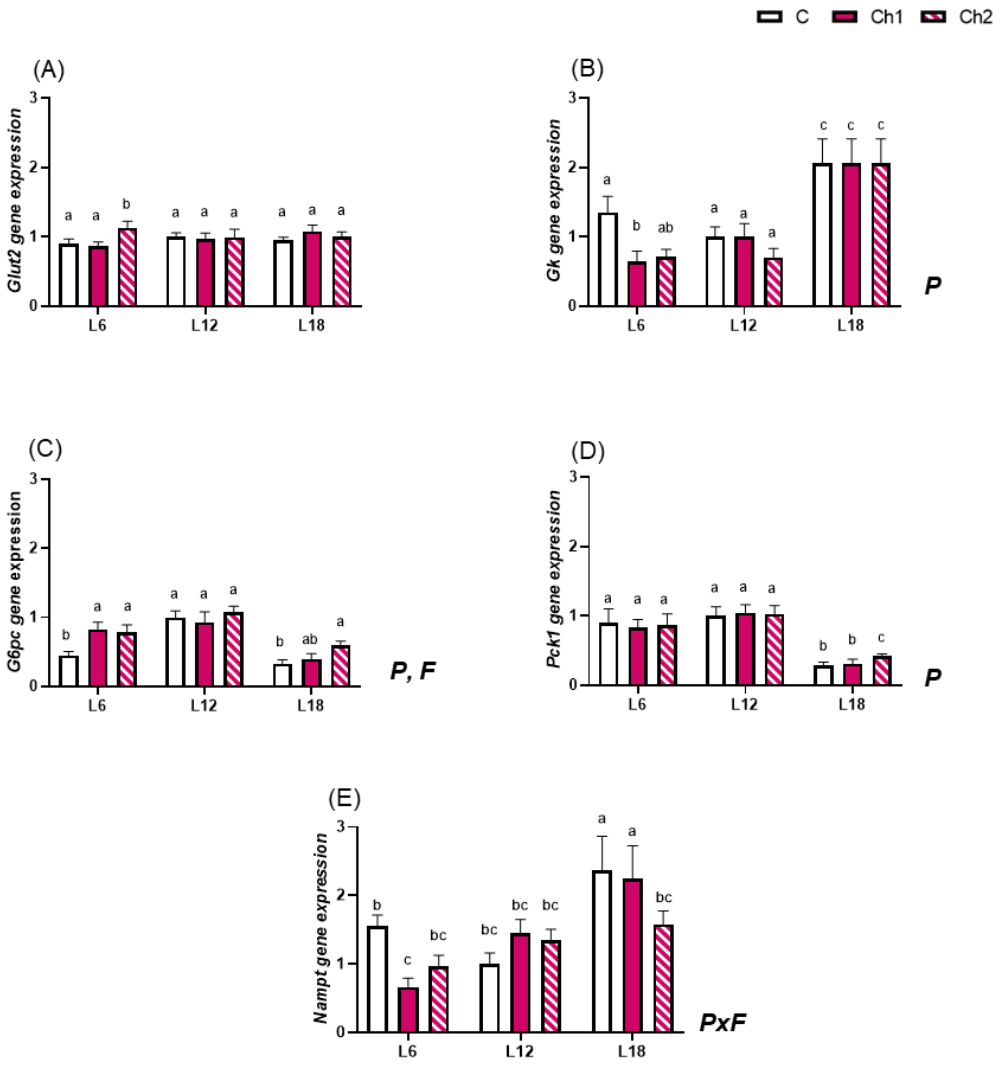

Figure 5. Hepatic expression levels of *Glut2* (A), Gk (B), G6pc (C), Pck1 (D), and Nampt (E) in Fisher 344 rats exposed to L6 (6 h light/ 18 h dark), L12 (12 h light/ 12 h dark) or L18 (18 h light/ 6 h dark) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight), or cherry 2 (Ch2, 100 mg/kg body weight) for the last 7 weeks. *P, F* or *PxF* indicates significant differences produced by photoperiod, fruit or interaction between photoperiod and fruit, respectively (two-way ANOVA, $p<0.05$). Different letters indicate significant differences (one-way ANOVA, post-hoc Tukey, $p<0.05$). Abbreviations: Gk (glucokinase); Glut2 (glucose transporter 2); G6pc (glucose-6-phosphatase catalytic

subunit); Nampt (nicotinamide phosphoribosyltransferase); Pck1 (phosphoenolpyruvate carboxykinase 1).

4. Discussion

Seasonality is plastic and reversible, allowing animals to adapt their physiology to the external environment, such as changes in light duration, temperature, and nutrient availability [21]. Previous studies in hamsters and F344 rats suggested that seasonal changes in the hypothalamus on local TH availability, among other factors, control energy balance [22], [23]. Nevertheless, the effects of the photoperiod on circulating TH levels and possible metabolic outcomes have not yet been described. In addition, recent research suggests that thyroid function can be regulated not only by iodine, but also by several food compounds including melatonin, vitamins and phenolic compounds [24], [25]. Moreover, phenolic compounds can modulate both circadian and circannual rhythms [26], a phenomenon explained by the xenohormesis theory [27]. According to xenohormesis, plants produce phenolic compounds under stress conditions depending on the external environment, and consumers can sense these chemical cues, which in turn allows them to adapt to the environmental conditions [28]. Crosstalk between nutrition and metabolism regulation in a photoperiod-dependent manner remains an outstanding question. In recent years, our group observed that the administration of seasonal fruits rich in phenolic compounds, such as cherries, to healthy animals adapted to different photoperiods produced different metabolic responses depending on the photoperiod of exposure [18], [3], and sweet cherries from different geographical origins exerted effects on liver lipid metabolism, which depended on the photoperiod and geographical origin [18]. Furthermore, the phenolic signatures of these cherries from different geographical origins may explain the differences in the effects exerted [17]. Considering that TH have been described as a seasonal rhythmic generator [29] and play a key role in linking the neuroendocrine response to peripheral organs by regulating lipid and glucose metabolism [8], and sweet cherries have shown photoperiod-dependent effects on hepatic lipid metabolism

[18], we aimed to investigate whether cherries with different phenolic hallmarks show a seasonal response to TH and potential metabolic consequences on hepatic glucose metabolism.

As expected, animals increased BW over the 11 weeks as a physiological response of normal growing. Moreover, the control group exposed to the L6 photoperiod (L6-C group) showed a tendency to reduce BW compared to the L12-C group. Loss of BW and a decrease in food intake during a short photoperiod have been previously observed in F344 rats [6]. Moreover, Mariné et al. 2019 also observed an increase in fat mass and a decrease in lower lean/fat mass ratio in L18 photoperiod-exposed rats than in L6 photoperiod-exposed rats [3]. The L12 photoperiod was previously shown to induce a long-day physiology [6]. Regarding the effect of cherry on animals, similarly to our study, L18 and L6 photoperiod-exposed F344 rats consuming cherry (100 mg/kg BW) for 10 weeks, not produced effects on BW in comparison with their respective control groups [3]. In contrast, Ch1 consumption significantly reduced BW in the L6-Ch1 group compared with L12-Ch1 group in our study differently of Ch2 administration. Interestingly, these results suggest that Ch1 consumption could improve photoperiod signals for BW change whereas Ch2 would counteract the effect of photoperiod. Phenolic compounds contained in the cherries could be responsible for the observed effect on BW and the reason why both cherries showed a differential effect. Although cherry consumption had not been previously associated to this effect [3], there are evidence that different phenolic-rich extract from walnut [30] and *Alpinia galangal* [31], with *in vitro* inhibitory activity for pancreatic lipase, reduced BW (up to 40 %) of obese animals in comparison with control animals. Anti-obesity effects of phenolic compounds have been also observed in humans, reducing up to 3.48 kg the BW of obese volunteers consuming an isocaloric diet and supplemented with a phenolic-rich extract of *Lippia citriodora* and *Hibiscus sabdariffa* for 60 days [32].

Given that TH (T3 and T4) availability has seasonally dependent production at the hypothalamic level [33]. In the present study, we investigated whether seasonal

consumption of cherry could modulate the hypothalamus-pituitary-thyroid (HPT) axis, acting through TH output. Our results showed a photoperiod effect on circulating levels of both hormones (L6>L12=L18 for T3 and L6=L12>L18 for T4), indicating that the change in day length significantly altered TH levels in the periphery. In addition, administration of cherries to animals exerted different effects on serum T3 levels, as both cherries reverted the L6 effect observed in the L6-C group in a different manner, and L18-Ch2 group showed increased T3 levels with respect to the L18-C and L18-Ch1 groups; however, the tendency in all the cases was to set the TH levels equal to L12-C, tending to counteract the photoperiod effect. Different studies have shown that phenolic compounds can interfere with several enzymatic activities related to thyroid function, including thyroperoxidase (TPO) inhibition [34]. This enzyme facilitates the addition of iodine to thyroglobulin, which is crucial for the TH generation [35]. However, more recently, it has been observed that certain phenolic compounds such as ferulic, *trans*-cinnamic, and syringic acids exert a contrary effect on TPO, producing its activation in a dose-dependent manner [36]. Thus, the differential effects of the consumption of Ch1 and Ch2 could be attributed to the sum of the inhibitory and activating signals on TH synthesis produced by their different profiles and concentrations of different phenolic compounds. To the best of our knowledge, this is the first study to show how cherries with different phenolic hallmarks modulate TH levels in the blood in a photoperiod-dependent manner.

One of the target organs of TH is the liver, which is finely regulated by multiple nutrients, hormones, and neuronal signals, which in turn regulate glucose and lipid metabolism at the hepatic level [37], [38]. Thus, the potential role of TH as a key mediator of the metabolic hepatic effect and the effect of cherry consumption on it was investigated. First, hepatic Dio1 expression was quantified, as a marker of T3 status at the hepatic level [39]. DIO1 is responsible for the activation of T4 in T3 in the liver [40]. Our results showed that it is upregulated during a short photoperiod

in the C group, indicating that T4 conversion in the active form is regulated in a photoperiod-dependent manner.

In addition, genes involved in glucose metabolism were studied, starting with *Glut2*. GLUT2 mediates the uptake and release of glucose in hepatocytes [41]. No effects of photoperiod were observed in this gene; however, gene expression was resulted significantly different between Ch1 and Ch2 under a short photoperiod. According to these results, it seems that Glut2 was not modulated by TH, given that a photoperiod effect was observed in both T3/T4 blood levels and hepatic Dio1 gene expression. Our results do not seem to agree with a previous in vivo study that observed an increase in GLUT2 and *Glut2* expression levels in the liver of hyperthyroid rats compared to euthyroid and hypothyroid rats [42]. Considering that the deletion of Glut2 at the hepatic level does not compromise hepatic glucose production in the fasting state [43], we examined the possible activation of key gluconeogenic enzymes by TH. In this regard, it has been reported that TH regulate gluconeogenesis in the liver, directly upregulating TR target genes such as the gluconeogenic transcription factor *Forkhead Box O1* (Foxo1) and key gluconeogenic enzymes (Pck1 and G6pc) in the liver [8]. During a long photoperiod, downregulation of Pck1 and G6pc expression was observed, which was slightly reversed by Ch2 consumption in both the cases. This could be attributed to the lower concentration of T3 in the blood found in the L18-C group than in the L6-C group. A photoperiod effect was also observed in liver Pck1 (not G6pc) expression in F344 rats exposed to L6, L12, and L18 photoperiods for 14 weeks (longer than in our study); however, they observed an upregulation of this gene in the L12 group with respect to the L6 and L18 groups [44]. Previous studies have already shown that cherry consumption acts on hepatic metabolism regulation, specifically regulating the gene expression of lipogenic enzymes such as sterol regulatory element binding protein 1 and fatty acid synthase in a photoperiod-dependent manner [18].

In the present study, Nampt gene expression was also investigated as NAMPT is the rate-limiting enzyme of NAD+ production. NAD is essential for the oxidation and reduction reactions and signalling pathways [45]. We found that this gene was upregulated in all groups with long photoperiod compared with short and intermediate photoperiods, which is not in concordance with the Pck1 and G6pc expression results. These results are not in agreement with those previously observed by Mariné-Casadó and colleagues, 2019 [3], where no statistical effects were observed in this gene by photoperiod, although expression levels in the long photoperiod group were higher than in the short and intermediate groups. In addition, in our study a significant downregulation was observed in L18 animals consuming Ch2, which counteracted the L18 photoperiod effect observed in the L18-C group, while Ch1 administration did not produce any effects on this gene. Neither liver nor muscle Nampt expression was altered in L6 and L18 photoperiod-exposed Fisher 344 rats supplemented with sweet cherry for the last 10 weeks of the study [3]. However, changes in hepatic and hypothalamic *Nampt* expression have been observed following the consumption of grape seed proanthocyanidin extract in Wistar rats [46]. Moreover, a dose-dependent increase in hepatic NAD+ was observed in Wistar rats consuming grape seed proanthocyanidin extract for 21 days at different doses (up to 50 mg/kg BW) [47]. As mentioned above, our previous studies showed that cherries with different phenolic compositions could exert different effects on hepatic oxidative stress [17] and lipogenic enzyme gene expression [18]. Thus, the different phenolic profiles of both tested cherries could explain the differences in liver *Nampt* expression between the two. Gk levels were upregulated during L18, but no differences were observed when the rats consumed Ch1 or Ch2. These results suggest that hepatic gluconeogenesis is downregulated under long photoperiod exposure and can be reversed by the consumption of cherries with a specific phenolic hallmark.

Finally, gluconeogenesis-related metabolites, such as alanine, valine, and glycerol, were analysed in the serum to further investigate the crosstalk between TH and

glucose metabolism. 1H-NMR-based metabolomic profiles indicated that the Cori cycle and triglyceride hydrolysis might be upregulated in the C group with respect to the Ch1 group during a short photoperiod, explaining the higher levels of lactate and glycerol. However, further studies investigating muscles metabolism are required to elucidate the exact meaning and impact of these results. Moreover, as suggested by hepatic gene expression, the downregulation of gluconeogenesis under a long photoperiod is supported by a decrease in circulating glucogenic amino acids, such as valine and alanine, with respect to a short photoperiod. Here, we confirmed that the metabolome profile is modulated in a photoperiod-dependent manner. Indeed, as has been previously observed, the adaptation to seasonal variation in humans, who live far from the equator showed that there is an increase in blood levels of cholesterol, triacylglyceride, glucose, leptin, and insulin during winter [48], [49].

5. Conclusions

Based on our research, we observed that sweet cherries can modulate TH levels depending on both photoperiod and geographical origin. Moreover, TH metabolic outcomes could be related to hepatic gluconeogenesis in a photoperiod- and cherry-type-dependent manner. Specifically, Ch1 showed effects mostly during a short photoperiod, while Ch2 was mainly during a long photoperiod, tending to minimize the differences between photoperiods. Further research is needed to elucidate the crosstalk between the consumption of fruits rich in phenolic compounds and the photoperiodic peripheral modulation of metabolic pathways.

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7. Conflict of Interest

The authors declare no conflict of interest.

8. Author Contributions

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

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SUPPLEMENTARY MATERIAL

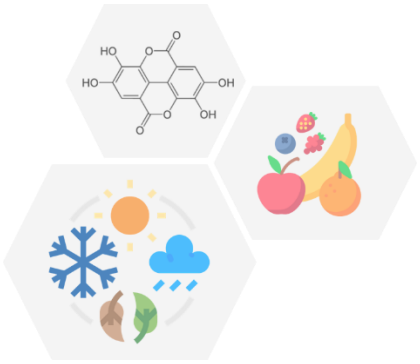
Table S1. Nucleotide sequences of primers used for real time quantitative PCR.

Genes	Forward (5' to 3')	Reverse (5' to 3')
Dio1	CCACCTTCTTCAGCATCC	AGTCATCTACGAGTCTCTTG
Gk	CTGTGAAAGCGTGTCCACTC	GCCCTCCTCTGATTCGATGA
Glut2	AGTCACACCAGCACATACGA	TGGCTTTGATCCTTCCGAGT
G6pc	ATTCCGGTGCTTGAATGTCG	TGGAGGCTGGCATTGTAGAT
Nampt	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG
Pck1	GCAAACCAGCAAGCACAATG	CTCGAAGTGGAACCAAACCC
Ppia	CCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCAAAACGCT

Abbreviations: *Dio1 (*iodothyronine deiodinase 1), *Gk* (glucokinase), *Glut2* (glucose transporter 2), *G6pc* (glucose-6-phosphatase catalytic subunit), *Nampt* (nicotinamide phosphoribosyltransferase), *Pck1* (phosphoenolpyruvate carboxykinase 1), *Ppia* (peptidylprolyl isomerase

Table S2. Serum parameters determined by nuclear magnetic resonance in Fisher 344 rats exposed to L6 (6 h of light/ 18 h of dark), L12 (12 h of light/ 12 h of dark) or L18 (18 h of light/ 6 h of dark) photoperiods for 11 weeks and administered vehicle (C), cherry 1 (Ch1, 100 mg/kg body weight) or cherry 2 (Ch2, 100 mg/kg body weight) during the last 7 weeks. Values are expressed as mean ± standard deviation (n = 8). The statistical analysis is represented as P, photoperiod effect; F, fruit effect; P x T, photoperiod effect and treatment (two-way ANOVA; 2wA, p<0.05) between groups. Different letters above the bars indicate significant differences post-hoc Tukey (one-way ANOVA, p<0.05). # represents a tendency (p>0.05<0.1).

	L6			L12			L18			2wA
mM (x10¹)	C	Ch1	Ch2	C	Ch1	Ch2	C	Ch1	Ch2	
Alanine	0.43±0.01 abc	0.41±0.02 a	0.46±0.01 b	0.42±0.01 b	0.43±0.01 ab*	0.42±0.03 ab	0.41±0.02 b	0.40±0.01 b	0.43±0.00 b	
Valine	0.15±0.01 a	0.15±0.01 a	0.16±0 a	0.15±0.01 #b	0.16±0.01 a	0.14±0.01 b	0.14±0.01 b	0.13±0 b	0.13±0.01 b	P, PxF#
Lactate	5.10±0.75 b	3.83±0.30 a	4.45±0.21 ab	3.01±0.22 a	4.18±0.38 ab	4.15±0.47 ab	5.29±0.35 b	4.81±0.22 #b	5.55±0.70 ab	P, PxF
Methionine	0.04±0.00	0.05±0.00	0.05±0.00	0.04±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.04±0.00	0.04±0.00	
Glutamine	0.44±0.01 ab	0.42±0.01 ab	0.45±0.01 a	0.44±0.01 #ab	0.44±0.01 #ab	0.40±0.02 b	0.49±0.02 a	0.45±0.01 ab	0.43±0.01 #a	
Asparagine	0.04±0.01 ab	0.05±0.01 a	0.05±0 a	0.04±0.01 ab	0.05±0.01 ab	0.20±0.01 c	0.04±0.00 ab	0.04±0.00 ab	0.05±0.00 a	
Glycine	0.19±0.01 ab	0.18±0.01 a	0.20±0 b	0.19±0.01 ab	0.19±0.01 ab	0.20±0.01 ab	0.18±0.00 a	0.18±0.01 a	0.18±0.01 ab	
Serine	0.18±0.00 a	0.18±0.00 a	0.20±0.00 b	0.19±0.00 a	0.18±0.00 a	0.19±0.01 ab	0.19±0.01 ab	0.17±0.00 a	0.18±0.01 a	F
Pyruvate	0.046±0.00 c	0.041±0.00 a	0.050±0.00 b	0.044±0.00 ab	0.049±0.00 b	0.047±0 ab	0.053±0.00 c	0.051±0.00 bc	0.052±0.00 bc	P
Betaine	0.11±0.01 ab	0.11±0.00 a	0.12±0.01 a	0.10±0.00 a	0.10±0.00 a	0.10±0.01 a	0.12±0.01 b	0.13±0.01 b	0.11±0.01 ·ab#	P
Glycerol	0.18±0.01 ab	0.14±0.01 a	0.16±0.01 ab	0.17±0.01 ab	0.17±0.01 ab	0.16±0.01 ab	0.16±0.01 ab	0.14±0.01 b	0.15±0.02 ab	PxF



GENERAL DISCUSSION

General discussion

Seasonal animals have evolved diverse variations in physiology, reproduction, and behaviour to accommodate annual changes in environmental and climatic conditions [1], [2]. These changes are initiated by variations in photoperiod (day length). Consequently, most animals have developed deep seasonal variations in their behaviour and physiology, which allow them to anticipate these patterns in food supply and improve their survival [3]. The mechanisms underlying the regulation of seasonal rhythms rely on plastic and reversible molecular changes in the pituitary-hypothalamic axis, which in turn regulate metabolism, energy expenditure, and reproduction [4]–[6].

According to xenohormesis theory, animals can sense chemical cues in plants to adapt their metabolism to incoming environmental conditions [7]. One of these chemical cues are phenolic compounds, non-nutrients of fruits and vegetables which exert many beneficial effects. Their consumption is known to play a role in the regulation of adaptation to the photoperiod, acting differently depending on the photoperiod of consumption. According to this, findings from our group show that consumption of phenolic-rich fruits such as cherries and oranges exhibits a photoperiod-dependent effect on metabolism [8]. However, because phenolic compounds are plant stress metabolites, their fruit profile changes depending on different factors, including fruit type and variety and pre- and post-harvest conditions [9], [10]. In fact, sweet cherries of the same variety but from different geographical origins and, therefore, different phenolic composition, exerted different effects depending on photoperiod consumption [11], [12] (**Manuscript 1**). Therefore, specific phenolic fruit hallmarks could cause different metabolic responses depending on the photoperiod of consumption. Thus, the main objective of this PhD thesis was to determine the differential effects on the organism of phenolic-enriched extracts and sweet cherries with different phenolic compositions in Fischer 344/IcoCrl (F344) rats exposed to different photoperiods.

Previous studies of the group have shown that seasonal consumption of fruits has a photoperiod-dependent effect in cafeteria F344 [8]. Nevertheless, the benefits of bioactive compounds are more difficult to observe in healthy individuals than in those with diseases. Consequently, the use of higher levels of bioactive compounds in phenolic-enriched extracts than in whole fruit could provide more information about the effectiveness of specific phenolic hallmarks. In addition, the use of extracts is considered an alternative strategy for the waste of postharvest fruits, which is currently a serious and growing problem [13], [14]. Therefore, in the first chapter of this PhD thesis, phenolic-enriched fruit extracts were obtained and used to test their effects on metabolism in healthy rats. The animals were exposed to short and long photoperiods to simulate winter and summer seasons, respectively. The fruits chosen to obtain the extracts were cherry, plum, apricot, and strawberry which are typical of the spring-summer season, and persimmon kaki, grape, orange, and pomegranate which are typical of autumn-winter. The results demonstrated that the consumption of fruit extracts modulated fasting blood lipid, glucose, and insulin levels, depending on the photoperiod of consumption, seasonality of the fruits used to obtain the extracts, and extract phenolic composition. Specifically, extracts obtained from winter fruits showed greater hypocholesterolemic and hypoglycemic activity when administered under short photoperiod (**Manuscript 2**). This is consistent with previous studies, in which fresh fruits, such as cherry, modulated biochemical parameters depending on photoperiod [15]. Moreover, in accordance to other studies, it was observed that the serum metabolome profile was widely affected by animal photoperiod exposure, indicating that the season of consumption played a key role in the effectiveness of these extracts [16] (**Manuscript 3**). According to this, previous studies from our group showed that the bioavailability of phenolic compounds from whole grapes and a grape seed extract, is strongly influenced by the photoperiod of consumption [17], [18]. In addition, the differential effects observed for the studied extracts can be attributed to their distinctive phenolic hallmarks. In fact, it is known that the phenolic profile and

composition of fruits are affected by various factors including plant type, environmental conditions (temperature, humidity, soil, light, etc.), transport, and storage conditions [9]. In addition, phytochemical composition is also dependent on fruit seasonality [10].

The consumption of fruits rich in phenolic compounds is also associated with improvements in lipid management and the reduction of pro-inflammatory markers in obesity and related pathologies [19], [20]. Therefore, the second chapter of this PhD thesis aimed to investigate the photoperiod-dependent effects of fruit extracts on the management of metabolic alterations related to obesity in animals subjected to metabolic challenge tests (**Manuscript 4**). The results showed that some fruit extracts might be effective in preventing or treating metabolic disorders by restoring disturbed homeostasis. Regarding the management of blood TAG levels, only two summer-fruit extracts (cherry and plum) showed TAG-lowering properties, these effects were observed only in animals exposed to short photoperiod. Levels of blood inflammatory markers after LPS-induced inflammation were found to be higher during short photoperiod. In line with our findings, previous results showed that inflammatory cytokine mRNAs were higher during winter than during summer in humans [21]. Moreover, fruit extracts decreased blood inflammatory markers, mainly those obtained from winter fruits. Anti-inflammatory effects were more evident in animals exposed to short photoperiod. The higher inflammatory state related to the short photoperiod could explain why fruit extracts are more effective when they are consumed in this photoperiod. Therefore, the beneficial effects of fruit extracts depend not only on their phenolic composition, but also on the photoperiod when they were consumed. To the best of our knowledge, this is the first study to demonstrate the photoperiod-dependent effects of fruit extracts. In addition to the fact that some of these fruit extracts are promising candidates for the prevention of metabolic disorders, these findings open the door to considering the season of consumption for functional ingredient design. This novel approach can open new perspectives in the fields of nutrition and health.

As aforementioned, fruit extracts are a valuable source of phenolic compounds. However, the beneficial effects of the seasonal consumption of fresh fruits should also be considered to establish dietary recommendations. The availability and variety of fresh fruits can vary by season, and the composition of the same fruit can vary based on factors such as ripeness, growing conditions, and storage methods [22]. These different compositions are related to changes in their beneficial effects. In fact, sweet cherries harvested from two geographical origins, which showed different phenolic compositions, did not exert the same effects on oxidative stress and hepatic fatty acid metabolism. These differential beneficial effects depended on the photoperiod of consumption and fruit composition [11], [15]. Cherries are one of the most consumed seasonal fruits [23], and their intakes has been related to neuroprotective properties [24]. Therefore, to further investigate the crosstalk between central and peripheral adaptations to photoperiod and the consumption of the sweet cherry chapter 3 of this PhD thesis aimed to investigate whether the neuroprotective effects attributed to this fruit could depend on its composition and the photoperiod in which cherries are consumed. For this, two different sweet cherries with different compositions were administered to F344 rats exposed to short or long photoperiod. The results suggested that consumption of sweet cherry shows neuroprotective properties in the hippocampus in a photoperiod-dependent manner, by upregulating the gene expression of *brain-derived neurotrophic factor* (*Bdnf*), *superoxide dismutase type 1* (*Sod1*) and *glutathione peroxidase 1* (*Gpx1*). However, these beneficial effects were only observed for one of the two cherries studied, and when it was consumed in season (long photoperiod). These findings highlight the importance of cherry composition and season of consumption for their beneficial effects (**Manuscript 5**). Other types of fruits rich in (poly)phenols, such as blueberries, have been previously demonstrated to improve cognitive function in the hippocampus by upregulating *Bdnf* expression [25]. However, this is the first time that neuroprotective markers have been shown to respond to specific types of cherry consumption and photoperiod exposure in healthy animals. These results

are relevant because they suggest that the intake of cherries with a specific composition could be useful for the prevention of neurodegenerative diseases.

To further elucidate the crosstalk between the neuroendocrine response and peripheral outcomes, thyroid hormones (TH) action on hepatic metabolism was investigated. TH is sensitive to changes in photoperiod and plays a pivotal role by linking the neuroendocrine response to peripheral organs [26], [27], including the regulation of hepatic glucose metabolism [28]. The results of this study, demonstrated for the first time that cherries with different phenolic hallmarks had different effects depending on the photoperiod of consumption, blood TH levels, and metabolites related to glucose metabolism. Moreover, the hepatic expression of TH target genes related to gluconeogenesis, such as *Phosphoenolpyruvate Carboxykinase 1* (Pck1) and *glucose-6-phosphatase catalytic* (*G6pc*), was mainly affected by the photoperiod of exposure (**Manuscript 6**).

Overall, these results suggest that there is a complex interaction between consumption of fruit phenolic compounds and seasonal rhythms, either the phenolic compounds are consumed in the form of extracts (**Manuscripts 2, 3 and 4**) or as a whole fruit (**Manuscripts 5 and 6**) and in health status (**Manuscripts 2, 3, 5 and 6**) or in metabolic stress conditions (**Manuscript 4**), and the time of the year when they are consumed plays a fundamental role. As future perspectives, more research is needed including long-term animal studies. In addition, clinical trials are necessary to elucidate how the time of the year could affect the benefits of fruit phenolic compounds in humans.

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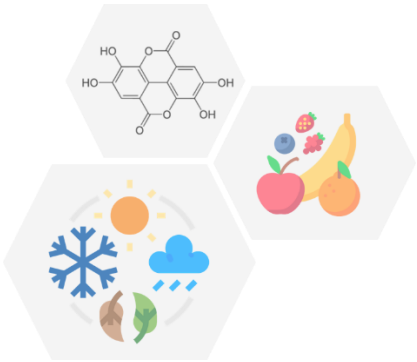

CONCLUSIONS

Conclusions

Based on the results obtained in this PhD thesis, it can be concluded that:

- The selected fruits yielded new extracts capable of modulating blood biochemical markers and metabolomic profiles in healthy F344 rats, depending on the extract composition and the photoperiod in which they were administered.
- The effects of the new fruit extracts on biochemical markers were mainly detected when the animals were exposed to a short photoperiod. Moreover, the seasonality of the fruits used to obtain the extracts appears to play a role in the activity of the extracts.
- The new extracts restored the disrupted homeostasis in F344 rats subjected to different metabolic challenges, depending on the composition of the administered fruit extracts and the photoperiod in which they were consumed.
- A higher inflammatory state was observed in animals exposed to short photoperiod than long photoperiod, which was counteracted by the consumption of winter-fruit extracts.
- Sweet cherry consumption upregulated the gene expression of several hippocampal neuroprotective markers when administered under long photoperiod, and these effects depended on the cherry composition.
- Sweet cherries modulated circulating thyroid hormone levels depending on both fruit composition and the photoperiod of their consumption.
- The metabolic outcomes of thyroid hormones may be related to hepatic gluconeogenesis in a photoperiod- and cherry composition-dependent manner.

List of publications

Published

- **Francesca Manocchio**, Jorge R. Soliz-Rueda, Aleix Ribas-Latre, Francisca Isabel Bravo, Anna Arola-Arnal, Manuel Suarez, Begoña Muguerza. 2022. Grape seed proanthocyanidins modulate the hepatic molecular clock via microRNAs. *Molecular Nutrition and Food Research* 66 (23): e2200443. 10.1002/mnfr.202200443.
- Ma. Josefina Ruiz de Azua**, Francesca Manocchio**, Alvaro Cruz-Carrion, Anna Arola-Arnal, Cristina Torres-Fuentes, Claudio Adrían Bernal, Juliana Saín, Manuel Suarez. 2023. Fatty acid metabolism in liver and muscle is strongly modulated by photoperiod in Fischer 344 rats. *Journal of Photochemistry and Photobiology B: Biology* 238: 112621. 10.1016/j.jphotobiol.2022.112621.
- Èlia Navarro-Masip, Marina Colom-Pellicer, **Francesca Manocchio**, Anna Arola-Arnal, Francisca Isabel Bravo, Begoña Muguerza and Gerard Aragonès. 2023. Grape-seed proanthocyanidins modulate adipose tissue adaptations to obesity in a photoperiod-dependent manner in Fischer 344 rats. *Nutrients* 15 (4): 1037**.** 10.3390/nu15041037.

Submitted

- **Francesca Manocchio**, Maria Josefina Ruiz de Azua, Èlia Navarro-Masip, Diego Morales, Gerard Aragonès, Begoña Muguerza, Francisca Isabel Bravo. Photoperiod and geographical origin of sweet cherries modulate thyroid hormones and hepatic glucose metabolism in F344 rats. *Food Science and Human Wellness.*
- Èlia Navarro-Masip, **Francesca Manocchio**, Marina Colom-Pellicer, Xavier Escoté, Lisard Iglesias-Carres, Enrique Calvo, Francisca Isabel Bravo, Begoña Muguerza, Yves Desjardins and Gerard Aragones. *Vitis vinifera L.* (Poly)phenols

Regulate Energy Metabolism and Adipose Tissue Type of Healthy Rats. *Molecular Nutrition and Food Research.*

- Èlia Navarro-Masip, **Francesca Manocchio**, Romina M. Rodríguez, Francisca Isabel Bravo, Cristina Torres-Fuentes, Begoña Muguerza, Gerard Aragonès Photoperiod-dependent effects of grape-seed proanthocyanidins on adipose tissue metabolic markers in healthy rats**.** *Molecular Nutrition and Food Research.*

In preparation

- **Francesca Manocchio***,* Diego Morales, Francisca Isabel Bravo and Begoña Muguerza. Seasonal fruits as signals of circannual rhythms.
- **Francesca Manocchio***,* Diego Morales, Elia Navarro-Masip, Gerard Aragonès, Cristina Torres-Fuentes, Francisca Isabel Bravo and Begoña Muguerza. Photoperiod-dependent effects on serum biochemical markers of phenolic-enriched fruit extracts.
- **Francesca Manocchio***,* Elia Navarro-Masip, Diego Morales, Gerard Aragonès, Manuel Suarez, Begoña Muguerza and Francisca Isabel Bravo. Seasonal fruit extracts regulate metabolome serum profile depending on photoperiod.
- **Francesca Manocchio***,* Diego Morales, Elia Navarro-Masip, Gerard Aragonès, Cristina Torres-Fuentes, Francisca Isabel Bravo and Begoña Muguerza. New phenolic-enriched fruit extracts with photoperiod-dependent effects for the prevention of metabolic disorders.
- **Francesca Manocchio**, Francisca Isabel Bravo, Gisela Helfer, Begoña Muguerza. Cherries with different geographical origin regulate neuroprotection in a photoperiod dependent manner in F344 rats.

Oral communications

- **Francesca Manocchio**, Gisela Helfer, Francisca Isabel Bravo, Begoña Muguerza. *Seasonal Consumption of polyphenols in F344 rats.* Invited speaker 5th Annual Seasonality Symposium. University of Glasgow, online, December 2022.
- **Francesca Manocchio***,* Diego Morales, Elia Navarro-Masip, Gerard Aragonès, Begoña Muguerza and Francisca Isabel Bravo. *Effect of Seasonal Phenolic-rich Fruit Extracts on the Regulation of Metabolic Homeostasis*. Nugo week 2022, Tarragona (Spain).
- **Francesca Manocchio**, J. R. Soliz-Rueda, A. Arola-Arnal, F.I. Bravo, M. Suárez, B. Muguerza. *Grape seed proanthocyanidins modulate the hepatic circadian clock via microRNAs*. *W*orkshop "Stilbenes, bioactive molecules with interest for health New opportunities for nutraceutical and food industry". Euskampus, University of the Basque Country, Université de Bordeaux, online, July 8th 2021.

Poster communications

- **Francesca Manocchio***,* Diego Morales, Elia Navarro-Masip, Gerard Aragonès, Begoña Muguerza and Francisca Isabel Bravo. Metabolic effects of seasonal fruit extracts in F344 rats exposed to different photoperiods and subjected to metabolic disruption. II Jornadas de Nutraceutica, 2023, Tarragona (Spain).
- **Francesca Manocchio***,* Diego Morales, Elia Navarro-Masip, Gerard Aragonès, Begoña Muguerza and Francisca Isabel Bravo. *Effect of Seasonal Phenolic-rich Fruit Extracts on the Regulation of Metabolic Homeostasis.* Nugo week 2022, Tarragona (Spain).
- Èlia Navarro-Masip, **Francesca Manocchio**, Marina Colom-Pellicer, Xavier Escoté, Begoña Mugueza, F Isabel Bravo, Enrique Calvo, Gerard Aragonès. *Cultivation Conditions of Red Grape Modulate the Seasonal Regulation of Energy Metabolism and the Phenotype of White and Brown Adipose Tissues in Fisher 344 Rats*. Nugo week 2022, Tarragona (Spain).

- **Francesca Manocchio**, Jorge Soliz-Rueda, Anna Arola-Arnal, Francisca-Isabel Bravo, Manuel Suárez and Begoña Muguerza. Grape seed proanthocyanidins modulate the hepatic molecular clock via microRNAs. International Congress of Polyphenols and Health 2022, London. **Best poster prize**.
- **Francesca Manocchio**, Elia Navarro-Masip, Gerard Aragonès, Francesca Isabel Bravo, Begoña Muguerza. *Impact of seasonal consumption of cherries with different phenolic profiles on thyroid hormones and hepatic metabolism in Fischer 344 rats.* I Jornadas de Nutraceutica, Tarragona 3-4 March 2022.

Seasonal animals have evolved diverse variations in physiology to adapt to changes in the environment annually. The consumption of phenolic compounds can modulate metabolic photoperiodic adaptation. The results of this PhD thesis suggest that there is a complex interaction between consumption of fruit phenolic compounds and seasonal rhythms, either the phenolic compounds are consumed in the form of extracts or as a whole fruit and in health status or in metabolic stress conditions, and the time of the year when they are consumed plays a fundamental role.

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