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Albert Gibert Ramos

Response of the Adipose Tissue to Nutritional and Photoperiodic Challenges

Doctoral Thesis

Directed by Dr. Anna Crescenti Savall

And

Prof. Maria Josepa Salvadó

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

NUTRIGENOMICS RESEARCH GROUP

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Departament de Bioquímica i Biotecnologia
Campus Sescelades (Edif. N4)
c/ Marcel·lí Domingo, 1
43007 Tarragona
Tel. +34 977 559 521
Fax + 34 977 558 232
A/e sdbio@urv.cat

FAIG CONSTAR que aquest treball, titulat "Response of the Adipose Tissue to Nutritional and Photoperiodic Challenges", que presenta Albert Gibert Ramos per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per a poder optar a la Menció Internacional de Doctorat.

HAGO CONSTAR que el presente trabajo, titulado "Response of the Adipose Tissue to Nutritional and Photoperiodic Challenges", que presenta Albert Gibert Ramos para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de esta universidad y que cumple los requisitos para poder optar a la Mención Internacional de Doctorado.

I STATE that the present study, entitled "Response of the Adipose Tissue to Nutritional and Photoperiodic Challenges", presented by Albert Gibert Ramos for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biochemistry of this university and that this thesis is eligible to apply for the International Doctorate Mention.

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Les directores de la tesi doctoral
Las directoras de la tesis doctoral
Doctoral Thesis Supervisors

Maria Josepa Salvadó Rovira

Anna Crescenti Savall

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TABLE OF CONTENT

SUMMARY

The adipose tissue is an organ specialized in metabolism and energy storage, however, in obesity, its dysfunction is associated with several important metabolic disturbances. The increasing prevalence of obesity remarks the importance of its prevention and treatment. The photoperiod influences many metabolic processes synchronizing the molecular clock, which, if disturbed, increases the risk of obesity. Also, according to the xenohormesis theory, fruits and vegetables signal heterotrophs about mild stresses like changes in seasons, suggesting that fruit origin should be considered in obesity preventing diets. The aim of this thesis was to evaluate whether photoperiod and consumption of fruit out of season, together with diet, could be risk factors for obesity development, focusing on the adipose tissue. Additionally, we studied whether the combination of different bioactive ingredients could improve the dysfunctional adipose tissue of obese individuals. We report that the adipose tissues of Fischer 344 rats adapt to the photoperiod, with the long day promoting fat accumulation and lipid metabolism in relation to a short day. An obesogenic diet masks some of the adaptations observed, it stimulates fat accumulation, however, the mechanism is different depending on the photoperiod. Concerning consumption of fruit out of season, orange from the southern hemisphere, with a molecular signature of a long day, stimulates fat accumulation when consumed during a short day. Furthermore, cherry consumed out of season desynchronizes the molecular clock genes and, when accompanied by an obesogenic diet, stimulates changes linked to obesity. Finally, the mix of bioactive compounds shows to be beneficial for obesity treatment, as it reduces fat accretion and stimulates an overall healthier gene expression profile in the adipose tissue. In conclusion, this work shows the importance of photoperiod and consumption of fruits out of season as risk factors for obesity, and the usefulness of a particular mix of bioactive ingredients for the treatment of obese individuals.

ABBREVIATIONS

ACACα	Acetyl-CoA carboxylase-α
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BMAL	Brain and muscle ARNT-like 1
BMI	Body mass index
CAF	Cafeteria diet
CD36	Cluster of differentiation 36
C/EBP	CCAAT/enhancer-binding protein
CPT1b	Carnitine palmitoyltransferase 1B
CRY	Cryptochrome circadian clock 1
EWAT	Epididymal white adipose tissue
FASN	Fatty acid synthase
FATP1	Fatty acid transport protein 1
GPAT	Glycerol-3-phosphate acyltransferase
HAD	Hydroxyacyl-CoA dehydrogenase
HFD	High-fat diet
HSL	Hormone sensitive lipase
IWAT	Inguinal white adipose tissue
LPL	Lipoprotein lipase
MGLL	Monoglyceride lipase
ON	Orange from the northern hemisphere
OS	Orange from the southern hemisphere
PER	Period Circadian clock
PPAR	Peroxisome proliferator-activated receptor
PRDM16	PR domain containing 16
PREF-1	Preadipocyte factor 1
RWAT	Retroperitoneal white adipose tissue
SCN	Suprachiasmatic nucleus
SVF	Stromal vascular fraction
TAG	Triglycerides
UCP1	Uncoupling protein 1
WAT	White adipose tissue

I. INTRODUCTION

1.THE ADIPOSE TISSUE

The adipose tissue is an organ with a high metabolic and endocrine activity. It contributes in the regulation of the organism homeostasis and it is primarily formed by adipocytes (1). This tissue increases in size during the development of obesity, being associated with many pathologic conditions, including hyperglycaemia, hypertension or insulin resistance. However, its deficiency is also associated with harmful metabolic states (2).

The adipose tissue can be classified in two main categories depending on its function: white and brown adipose tissues. The white adipose tissue (WAT) specialises in energy storage and mobilization, while the brown adipose tissue (BAT) manages lipid oxidation and releases energy in the form of heat. Additionally, some adipocytes in the WAT can obtain characteristics typical of brown adipocytes, a process known as browning, brown in white, or beige adipocytes (3).

1.1 White Adipose Tissue

1.1.1 Composition, localisation and function.

The **WAT is principally composed** of white adipocytes. However, it also includes macrophages, preadipocytes, fibroblasts, mast cells, mesenchymal cells, and nervous and vascular elements. White adipocytes are spherical cells with a lipid droplet that occupies an 80 or 90% of the cytoplasm, and have different sizes depending on the region of the fat depot (Figure 1) (1,4).

WAT localisation can usually be divided in visceral or subcutaneous, whether it is found around internal organs or under the skin, respectively (1,4). The principal visceral depots in humans are the mesenteric and the retroperitoneal, and in rats, the epididymal is also prominent. More specifically, *Cinti* (1) names the following visceral depots in small mammals: mesenteric, perirenal, retroperitoneal, parametrial, periovaric, epididymal and perivisceral. Furthermore, the author distributes the subcutaneous depots in two main regions: anterior and posterior (Figure 2).

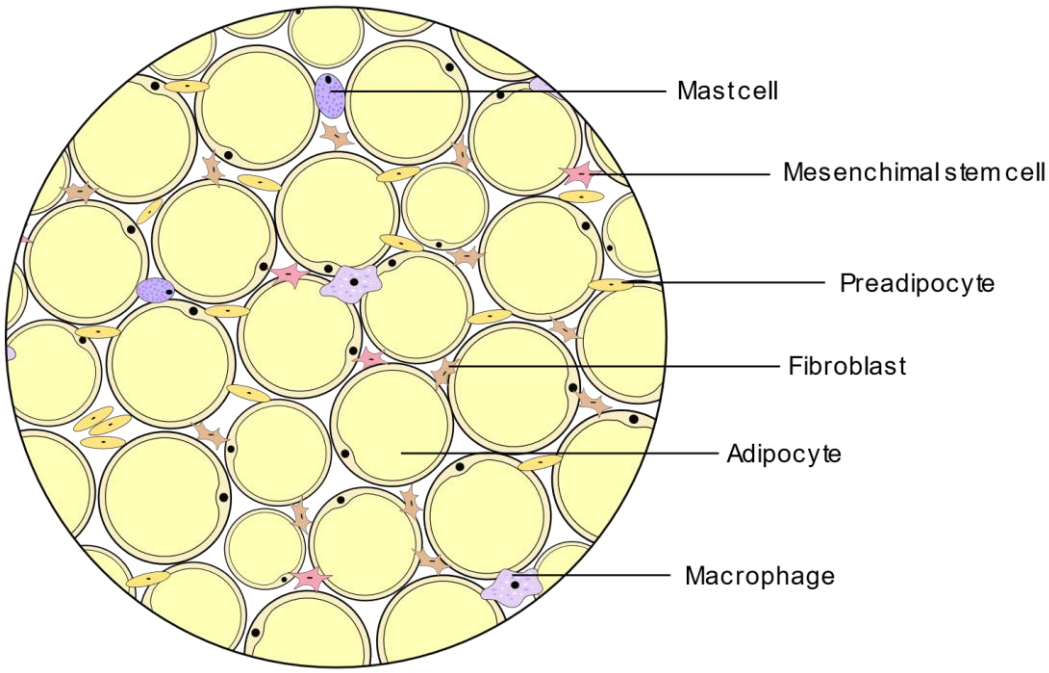

Figure 1. The heterogeneity of the adipose tissue and its major cell types.

Even though the principal **function of WAT** is **energy storage** through the lipogenesis pathway **and the release of triglycerides** through the lipolysis pathway, the WAT is known to express and secrete many cytokines, known as adipokines, with relevant roles coordinating metabolic processes in the organism. In addition to adipocytes, other cell types in the WAT are implicated in the secretion of adipokines and other factors or enzymes, such as macrophages, lymphocytes, and fibroblasts. The primary adipokine released by the adipose tissue is leptin, secreted in response to energy sufficiency and insulin, among other factors (2). On the other hand, adiponectin's main function is insulin sensitization, and it is necessary for correct energy homeostasis (5). Other adipokines such as vascular endothelial growth factor (VEGF), implicated in angiogenesis of the adipose tissue, or apelin, which inhibits insulin secretion, remarking the importance of the adipose tissue as an endocrine organ (6).

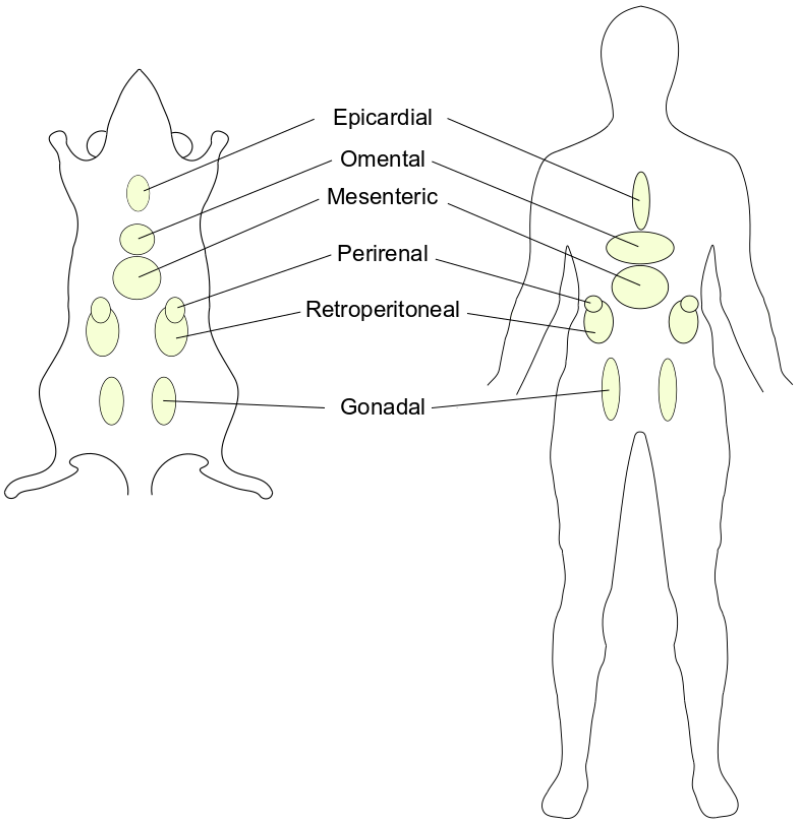

Figure 2. Drawing of the principal visceral WAT depots in rodents and human. Localisation of the main visceral WAT depots: epicardial, omental, mesenteric, perirenal, retroperitoneal and gonadal.

1.1.2 Adipogenesis

Adipogenesis consists in the differentiation process of preadipocytes into fully mature adipocytes (7). This mechanism begins during the embryo development and persists throughout life. In humans, adipose tissue growth starts during the second trimester of gestation while in rats it is initiated during the perinatal period (8). The adipogenesis process starts with the differentiation of mesenchymal stem cells (MSC), which will develop into adipocytes in two main phases, known as determination and terminal differentiation (Figure 3). The first phase consists in the conversion from a pluripotent cell into preadipocyte, which will lose its capacity to differentiate into other mesenchymal lineages, but is still able to

perform mitosis and grow. This stage is primarily ruled by bone morphogenetic proteins (BMPs), which activate transcription factors that commit the cell into the adipocyte lineage. These preadipocytes will divide and form the main pool where mature adipocytes will generate. At this point, the activation of peroxisome proliferator-activated receptor gamma (**PPARγ**) and CCAAT/enhancer binding proteins (**C/EBPs**) will start the terminal differentiation, which will activate the transcription of genes implicated in the adipocyte metabolism, lipid transport and synthesis, among others, developing the mature adipocyte (9–11).

Adipogenesis is stimulated by many factors that are generated when overfeeding, and at the same time, several other elements act as repressors, including dietary factors. Thus, adipogenesis is a tightly regulated process and preadipocyte fate is controlled by opposing signals that decide whether to remain quiescent, divide or start differentiation (11).

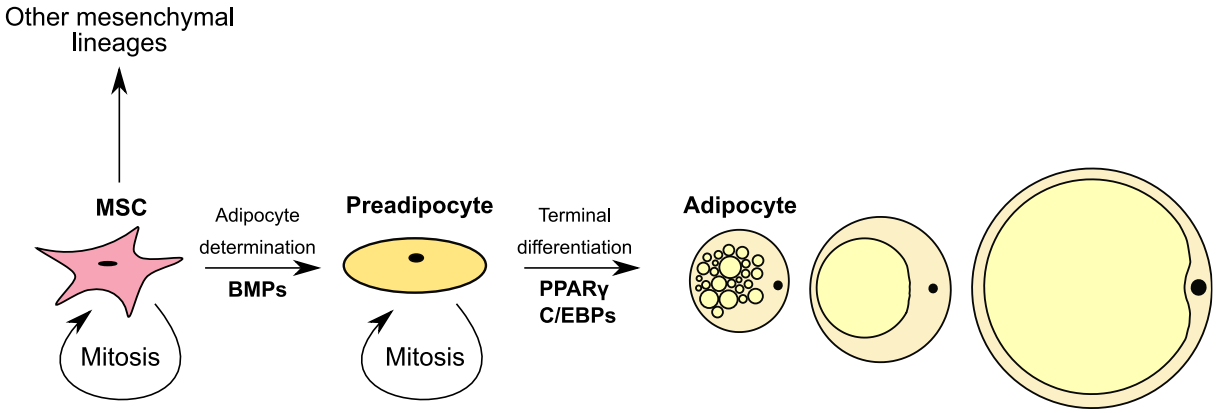

Figure 3. Adipogenesis process. The mesenchymal stem cells (MSC) can start differentiation into preadipocytes through the process known as adipocyte determination when stimulated by bone morphogenetic proteins (BMP). Preadipocytes can keep dividing by mitosis, or terminally differentiate into adipocytes (terminal differentiation phase), through the expression of PPARγ and C/EBPs, that will stimulate the transcription of genes implicated in adipocyte metabolism. PPARγ: peroxisome proliferator-activated receptor gamma; C/EBPs: CCAAT/enhancer binding proteins.

1.1.3 Metabolism

1.1.3.1 Lipogenesis

The main goal of lipogenesis is the synthesis of triacylglycerol (TAG), which will be incorporated in the lipid droplet of the adipocyte. TAG are composed of a molecule of glycerol and three fatty acids. The fatty acids necessary to synthetize the TAG can be obtained from the blood or synthetized from glucose in the process called *de novo* lipogenesis. In the plasma, fatty acids can be found either bound to albumin as non-esterified fatty acids (NEFAs), or in the form of TAG, bound to lipoproteins originated in the intestine after digestion or the liver. In order to enter the cell, the TAG in the plasma must be hydrolysed by the lipoprotein lipase (**LPL**) of the endothelial cells of the tissue capillaries. The resulting fatty acids are incorporated into adipocytes by fatty acid transporter proteins such as cluster of differentiation 36 (**CD36**) and fatty acid transporter protein 1 (**FATP1**), and once inside the cytoplasm they are bound to specific transporter proteins that will transfer them for their esterification into acyl-CoA, that will be used to form TAG in the endoplasmic reticulum (7).

The *de novo* lipogenesis pathway allows the storage of excess glucose as fatty acids that will be incorporated into TAG. Glucose from the blood is absorbed in the adipocyte and after a series of reactions will generate acetyl-CoA, that will be carboxylated by the acetyl-CoA carboxylase (**ACAC**) into malonyl-CoA. From here, the fatty acid synthase (**FAS**) will take on and synthesize palmitate from the malonyl-CoA through a series of reactions, elongating the hydrocarbonic chain of fatty acid adding two carbon units in each reaction. Finally, after a series of reactions, palmitic acid is further elongated into more complex fatty acids that will be esterified with alcoholic residues of glycerol-3-phosphate (G3P), through the action of the enzymes G3P acyltransferases (**GPATs**) and ultimately forming TAG (12–14) (Figure 4).

1.1.3.2 Lipolysis

In adipocytes, the lipolysis process is activated when energy is needed in the organism, mobilizing the TAG stored in the lipid droplet into the systemic circulation in the form of free

fatty acids and glycerol. These fatty acids will be taken by other organs as an energy source for β-oxidation.

The first step in lipolysis is the generation of diacylglycerol from triglyceride hydrolysis. The principal enzyme in this reaction is the adipose triglyceride lipase (**ATGL**), however, other enzymes such as the hormone-sensitive lipase (**HSL**) have demonstrated activity against this substrate, suggesting that ATGL might not be the only protein capable of hydrolyzing triglycerides. The next step consists in the hydrolyzation of the diacylglycerol into monoacylglycerol and NEFAs, known to be controlled by **HSL**. Ultimately, monoacylglycerol will be converted by the monoacylglycerol lipase (**MGL**) into fatty acids and glycerol, which will be released from the cell and taken by other tissues (15,16) (Figure 4).

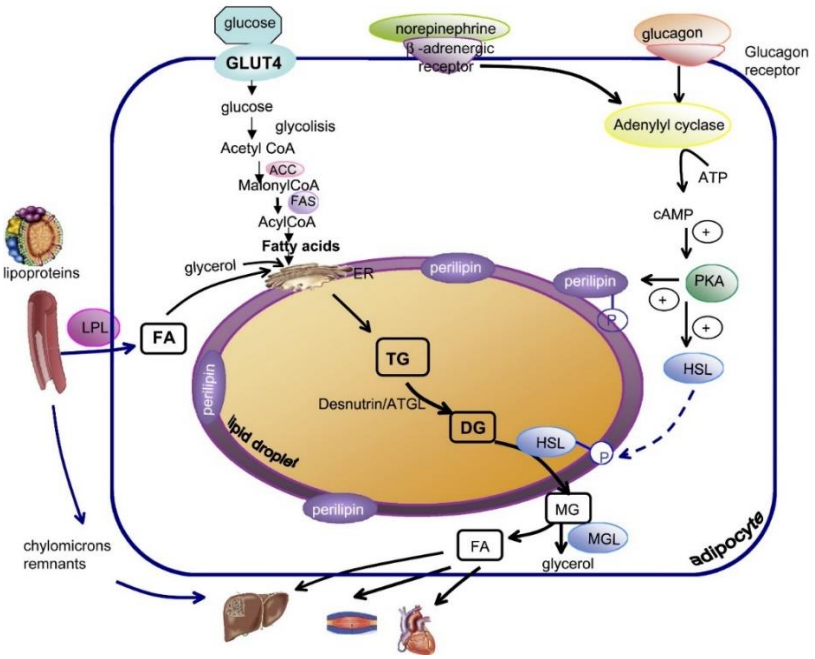

Figure 4. Lipogenesis and lipolysis pathways. Glucose excess is oxidized via glycolysis to acetyl-CoA in the adipocyte and then converted into acyl-CoA, which are then esterified in the endoplasmic reticulum (ER) to triglycerides (TG). These are then translocated into the lipid droplet. Fatty acids (FA) obtained from lipoproteins are also esterified into TG and stored. Under fasting conditions, lipolysis is activated by G-protein-coupled receptors resulting in an increase in cAMP that phosphorylates the protein perilipin located in the membrane of the lipid droplet. cAMP also phosphorylates the hormone-sensitive lipase (HSL) that triggers its translocation from the cytoplasm to the lipid droplet and induces with highest specific activity the hydrolysis of diglycerides produced by the adipocyte triglyceride lipase (ATGL) to form monoglycerides (MG). MG are then released to non-adipose tissues, mainly for energy purposes (from Vázquez-Vela et al. 2008).

1.1.4 Hypertrophy and hyperplasia

The two possible growth mechanisms of the WAT are hyperplasia, when the tissue increases the number of adipocytes, and hypertrophy, when adipocytes increase in size. Usually, hypertrophy is the first process when an increase in energy storage is needed, while hyperplasia follows after (1). Many studies have found that some fat depots are more prone to grow by one of the two mechanisms. For example, adipocytes are smaller in visceral depots than in subcutaneous fat pads in women (17), while in lean rats, it has been shown that mesenteric and epididymal pat depots grow mostly through hypertrophy, and contrarily, retroperitoneal and inguinal WAT depots grow by hyperplasia (18). Additionally, there are evidences that a high-fat diet leads to hypertrophy, while a high-sugar diet leads to hyperplasia, suggesting that the proportion of macronutrients can also influence the morphology of the WAT (19).

The growth mechanism of the WAT is important in health because it has been shown that hypertrophic subcutaneous WAT is associated with insulin resistance (20) and that it is a predictor of type 2 diabetes (21). On the other hand, hyperplastic WAT seems to be associated with a healthier metabolism in obese individuals, like an improved insulin sensitivity (22) and a decrease in the secretion of proinflammatory adipokines compared to individuals with hypertrophic WAT (23).

1.1.5 Stromal Vascular Fraction

In addition to adipocytes, the WAT includes other cell types such as mesenchymal cells, fibroblasts, vascular cells, macrophages, and preadipocytes. This heterogeneous cell population is called **stromal vascular fraction (SVF)**. From this fraction, the mesenchymal cells, also named adipose-derived stem cells (ADSC) have gained importance in the last decades because of their ability to differentiate into adipogenic, chondrogenic, myogenic and osteogenic lineages (24,25). This cell population is also important in obesity because it is directly implicated in adipogenesis and the remodelling of the WAT. For example, it has been reported that exercise inhibits the differentiation of ADSC into adipocytes (26).

ADSC cells express preadipocyte factor-1 (**PREF-1**), which is a characteristic marker of undifferentiated preadipocytes (27). **PREF-1** is a transmembrane protein that represses

adipocyte differentiation, inhibiting the expression of **PPARγ** and **C/EBPα**, and so, its levels diminish during adipocyte differentiation (28).

1.1.6 *In vitro* study of the adipose tissue

Even though primary cell culture originated from the SVF are being used to study adipogenesis and adipocyte cell function *in vitro*, their usefulness is limited due to the low percentage of preadipocytes in the adipose tissue, cell culture contamination and the low life span of these cells (29). For these reasons, immortalised cell lines are more reliable and most frequently used to study preadipocyte differentiation process and adipocyte function (30).

Two main classes of cell lines are used for *in vitro* studies, the first consists of pluripotent fibroblasts that conserve the capacity to differentiate into different cell types. Some examples are the 10T1/2 and the CHEF/18 fibroblast cell lines. The second class includes fibroblast-like preadipocytes that only retain the ability to differentiate into adipocytes, such as the 3T3-L1 cell line (28).

The 3T3-L1 cell line derives from Swiss 3T3 mouse embryonic fibroblasts and are the most used adipocyte culture model in research studies (31). This particular cell line can be differentiated into mature adipocytes using a combination of insulin, a glucocorticoid such as dexamethasone, a compound with the capacity to increase intracellular cAMP levels, such as 3-isobutyl-1-methylxanthine, and foetal bovine serum (30,31). After one round of mitosis and immediate growth arrest, preadipocytes commit to differentiation and start expressing adipocyte cell markers. After five or seven days, cells are completely differentiated into mature adipocytes (30).

1.2 Brown Adipose Tissue

1.2.1 Composition, localisation and function

Brown adipocytes have some similarities to white adipocytes, such as their capacity to store lipids, however they greatly differ in many aspects. **Brown adipocytes**, instead of accumulating lipids in one large lipid droplet, form small drops, facilitating their rapid use for heat generation. Additionally, they are smaller than white adipocytes and present a high number of mitochondria (1), which together with the high vascularization of the tissue, results in its characteristic brown coloration (32).

In humans, BAT was believed to be non-existent in adults, and to be only present in newborns. However, its presence was discovered a few decades ago, and it is now known to be **principally localised** in the supraclavicular, cervical, axillar and suprarenal areas of human adults. Nevertheless, its size and function decrease with ageing (33). On the other hand, in small mammals such as rats or mice, the largest BAT depot is found in the interscapular and dorso-cervical region, but it can also be found in the axillary area or surrounding the kidneys and other organs (Figure 5) (32).

The **BAT principal function** is to dissipate energy in the form of heat, dissociating the mitochondrial respiration from ATP production. This phenomenon is achieved through the **uncoupling protein 1 (UCP1)**, a protein found in the inner membrane of the mitochondria, which dissipates the proton gradient generated during the respiratory chain (34). Besides thermoregulation through non-shivering thermogenesis, the other important function of the BAT is to prevent excessive fat storage. Even though a few decades ago it was believed that active brown adipose tissue in adult humans was non-existent, now, given the BAT main function in whole body energy regulation, it is being focused on for its potential as an anti-obesity organ (35,36). In fact, it has been reported that the recruitment of the BAT in humans is beneficial for body fat reduction (37), and that morbidly obese humans increase their BAT mass after losing weight, suggesting that human BAT can be recruited (38).

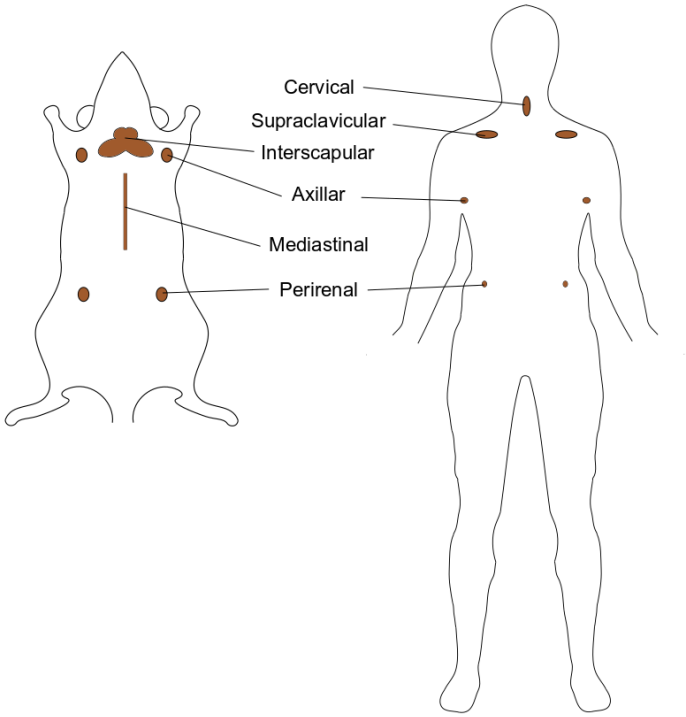

Figure 5. Drawing of the principal brown adipose tissue depots in rodents and human.

1.2.2 Thermogenesis

Thermogenesis is the most important pathway in the BAT and it consists in the generation of heat through the oxidation of fatty acids and glucose (34).

The BAT thermogenesis is activated by the sympathetic nervous system (SNS), specifically through norepinephrine, which activates the β-adrenergic receptors on brown adipocytes, stimulating lipolysis. In the lipolysis process, **ATGL**, **MGL** and **HSL** activation hydrolyse the TAG stored in the lipid droplets of the adipocyte, forming free fatty acids that will activate **UCP1** gene expression and serve as fuel for the thermogenesis through β-oxidation (39). In addition to the SNS, there are also several endocrine factors found to activate thermogenesis in the BAT, such as thyroid hormones (40), Fibroblast growth factor 21 (FGF-21) (41), BMPs (42,43) and Irisin (44).

The substrates used for the catabolism and energy expending processes in the BAT are glucose and fatty acids, and their uptake and utilization are tightly regulated (45). Glucose absorption is stimulated both by norepinephrine and insulin, and the uptake is mediated by

the GLUT family of glucose transporters (46). Norepinephrine secretion is activated during cold exposure, while insulin stimulation occurs after feeding (46). On the other hand, triglyceride clearance and absorption into the BAT are mainly carried out by **LPL** and **CD36** (47). **LPL** is synthesized by the brown adipocytes and send to the capillaries, where it will hydrolyse the fatty acids from the chylomicrons and lipoproteins (46), while **CD36** is a transmembrane receptor in adipocytes implicated in free fatty acid uptake (47).

1.2.3 Brown adipocyte determination

Even though BAT and WAT have very different roles, they share many core pathways and molecular mechanisms (48). First of all, **PPARγ** is the key regulator of both tissues, however, it seems to be indispensable for BAT, while WAT can develop normally without it (49). During the process of development, C/EBPs are also necessary in both tissues, however, **C/EBPβ** is specifically important in BAT, and is believed to be responsible for the commitment towards the brown adipocyte phenotype (50). In fact, it has been found that, in conjunction with **PRDM16** (51), **C/EBPβ** stimulates genes implicated in the determination of the brown adipocyte phenotype (52). In this sense, the specific gene found to be unique for brown adipocyte cell determination is **PRDM16**, which has shown to repress several white fat-specific genes and to stimulate mitochondrial biogenesis and uncoupled cellular respiration (52,53). Additionally, **PRDM16** not only rules over brown adipocyte development, but is also responsible for maintaining its function and identity (54).

1.2.4 Browning of the WAT

Apart from the white and brown adipocytes, there is a third type of adipocyte that is found in the WAT. This adipocyte is usually called brite (brown in white) or beige adipocyte, and the process in which these cells are generated is named browning. Even though these cells share similarities to brown adipocytes, such as the capacity to express **UCP1** and **PRDM16** and the presence of multiple lipid droplets and a high number of mitochondria, they are more closely related to white adipocytes. In fact, it has been shown that white and beige adipocytes are originated from a common precursor cell type (36), while proper brown adipocytes share a common origin with skeletal muscle cells (55–57). Additionally, there are

data supporting the idea that beige adipocytes can transdifferentiate into white adipocytes and the other way around (58,59).

Even though beige adipocytes are localised in WAT depots, they express **UCP1**, and so, have the capacity to burn fat in order to generate heat. Thus, the study of this cell type has attracted the interest of researchers because it has been shown that the increase in the thermogenic capacity of the WAT offers protection against obesity and insulin resistance, increasing energy expenditure (60,61).

1.3 The problematic of the adipose tissue: obesity

Obesity is defined by the World Health Organization (WHO) as an "abnormal or excessive **fat accumulation** that may impair health" and that can be attributed to adults with a Body Mass Index (BMI) greater or equal to 30. The WHO catalogues obesity as an epidemy that now affects high, middle and low-income countries, and estimated that in 2016 more than 1.9 billion adults were overweight (39% of the population), and 650 million obese (13% of the population) (62). The main problem of the obesity epidemy is that it is associated with many diseases, including type 2 diabetes (63,64), dyslipidaemia (65,66), hypertension (67), cardiovascular diseases (68), non-alcoholic fatty liver disease (69), and cancer (70). In fact, in 2009, the WHO classified obesity and overweight as one of the five leading global risks for mortality in the world, and determined that obesity and overweight were responsible for 2.8 million of deaths in the world (71). For this reason, obesity research is of great importance, and the scientific community is intensely studying it causes and the strategies to counteract.

Obesity is generally diagnosed using the BMI, calculated with the weight in kilograms, divided by the square of the height in metres, and depending on the value obtained, the patient is classified into overweight (BMI ≥ 25) or obese (BMI ≥ 30) (72). Even though BMI is accepted as a good measure for obesity diagnosis, it has a few limitations since it does not recognize body composition and so, it is not a useful measure for everyone, for instance, for individuals with a high muscular mass. For this reason, other measures that take into account fat quantity are also being used, including total body fat, percent body fat, waist circumference and waist-to-hip ratio, each with its advantages and limitations (73).

Since obesity is linked to many other diseases with important detrimental consequences for human health, **its prevention and treatment** is of vital importance. Obesity is a chronic disease caused by genetic and environmental factors that impair energy intake and caloric expenditure (74), leading to a positive energy balance and to an increase in weight. Even though it has been estimated that heritability explains between a 30 and 70% of the variation in body mass within the population (75,76), the main causes of the increase in obesity are the environment and lifestyle, including **diet** and exercise practice (77). In fact, the organism has more adaptations that promote energy storage than expenditure, for this reason, special care has to be taken in food intake and exercise, which have been altered by modern habits (78). Additionally, other factors are being found that explain the increase in obesity prevalence of the last decades, being the influence of **photoperiod** a promising one. In this sense, there are many studies that show that the disruption of the normal pattern of sleep-wake cycle found in shift workers (79–81) and a decrease in hours of sleep at night (82,83), are highly associated with obesity.

Thus, caloric intake reduction through diet accompanied by increasing the consumption of fruit and vegetables (84), are the principal approaches used for the treatment of obesity and energy expenditure through physical activity is also highly recommended. Furthermore, other strategies are being studied, including the utilization of **natural bioactive compounds**. Some of these ingredients have shown satiating and energy expenditure stimulating effects or the capacity to regulate the adipose tissue function and obesity-related inflammation (85,86).

1.3.1 Animal models for the study of obesity

Given the fact that obesity is a long-term disease, the unviability of the monitoring of a human lifetime or perform invasive interventions, the high variation among human individuals, and the economic costs, researchers have long time used animal models for the study of this and other diseases. Between animal models, rodents are the most generally used to study obesity, because they present metabolic pathways similar to those found in humans (87) and they are more cost-effective than other larger animals because they require little space or resources, and reproduce and grow rapidly (88).

Animal models for obesity research can usually be differentiated in two classes: monogenic and polygenic models. Monogenic models consist of animals which have a particular gene

altered or suppressed, which allows the study of an individual gene or pathway related to obesity (89). Some examples of monogenic models of obesity are the ob/ob mouse, which has an spontaneous mutation leading to a lack of leptin secretion, and the db/db mouse, that lack the leptin receptor (90). On the other hand, polygenic models are used to study the different gene interactions in disease, and so, in obesity. Polygenic models are really useful because they represent the multiple factors usually involved in human obesity (90). They comprise animals with a phenotype prone to obesity, like the New Zealand obese mouse, which possess different genes favouring the development of obesity, or the diet-induced obese (DIO) animal models, like the Sprague-Dawley, Wistar or Long-Evans rat strains, fed with high-fat diets or cafeteria diets, which favours adiposity and obesity (87,90).

In particular, the cafeteria diet is a diet model used to mimic the consumption of highly caloric and high processed foods, like those observed in obesogenic human diets. It consists of high palatable and energy dense foods that stimulate hyperphagia in experimental animals (91). This diet model is particularly useful in obesity study, since animals are allowed free access to both standard chow diet and several highly palatable foods, which rapidly increases weight gain, fat accumulation and other parameters associated with obesity (92). This model has been reported to be more robust than other typical DIO animal models, because of its resemblance to human behaviour and trends regarding diet (92).

2.BIOLOGICAL RHYTHMS AND OBESITY DEVELOPMENT

The light and dark patterns that occur during a day and the changes in this timetable observed during the year have always affected life. The changing seasons bring about new opportunities and problematics that affect most life forms, and so, evolution has developed strategies in the organism to adapt to this predictable phenomenon (93).

More specifically, the biological rhythms present in organisms that respond to daily changes in light and dark, following a period of 24 hours are called **circadian rhythms** (94). This rhythm times many processes during the day, including feeding time, sleep-wake cycle and insulin secretion (95). On the other hand, the changing pattern in light during a year is named **circannual rhythm** (96). Thus, the circannual rhythm responds to the changes in the patterns of light and dark throughout the seasons of a year, and times processes such as reproduction, body growth and activity (96).

2.1 Circadian Rhythm

The circadian rhythm refers to the molecular and physiological oscillations that follow, approximately, a 24 hours periodicity, and which are timed by the molecular clock (95). Anatomically, this timing system is ruled by the Suprachiasmatic Nucleus (SCN) in the hypothalamus, which is capable of maintaining rhythmicity on its own. However, it is entrained by external factors, principally light, that synchronise the internal clock to the external photoperiod (97).

The SCN sends information to other parts within the brain, such as the pineal gland, inducing the secretion of melatonin, a fundamental hormone to transfer the photoperiodic information (98). At the same time, the SCN uses many efferent pathways to signal rhythmicity in other tissues, including autonomic innervation, body temperature and humoral signals (99). Thus, the expression of molecular clock genes, and circadian rhythmicity in their activity, has also been found in peripheral organs (100). Furthermore, peripheral tissues are able to maintain this circadian pattern on their own, following other clues such as food intake, temperature, glucocorticoids, and other circulating parameters (100).

2.1.1 The molecular clock

The molecular clock includes the oscillating mechanisms implicated in the circadian rhythms and that generate an internal response in the cell. The rhythmicity of this molecular clock is carried out by transcription factors in the neurons of the SCN that generate a feedback loop which oscillates every 24 hours. In mammals, there are two principal genes implicated, the circadian locomoter output cycles kaput protein (CLOCK) and the brain and muscle Arnt-like protein-1 (**BMAL1**), which form a heterodimer that activates the transcription of Period (**PER** 1-3) and Cryptochrome (**CRY** 1,2). When **PER** and **CRY** proteins reach a certain concentration in the cytoplasm, they translocate into the nucleus and inhibit CLOCK and **BMAL1** (95) (Figure 6). Even though this transcriptional autoregulatory loop looks simple, additional components, post-transcriptional and post-translational mechanisms play important roles in its regulation (101).

From here, genes directly implicated in the molecular clock regulatory loop, or downstream components of the signalling cascade, will bind to different promotors implicated in the

circadian metabolism (102,103). In this sense, it has been shown that there are many metabolic pathways influenced or directly controlled by the molecular clock (104–107). For example, **PER2** directly interacts with nuclear receptors such as **PPARα** and Rev-erbα, which allow a rhythmic control of glucose and lipid metabolism (105), and **CRY** expression levels have shown to modulate gluconeogenesis, inhibiting the expression of key genes implicated in the process (106). There are also many other studies showing a circadian regulation in the secretion of many hormones, such as glucagon (108) or insulin (109), among other (110,111).

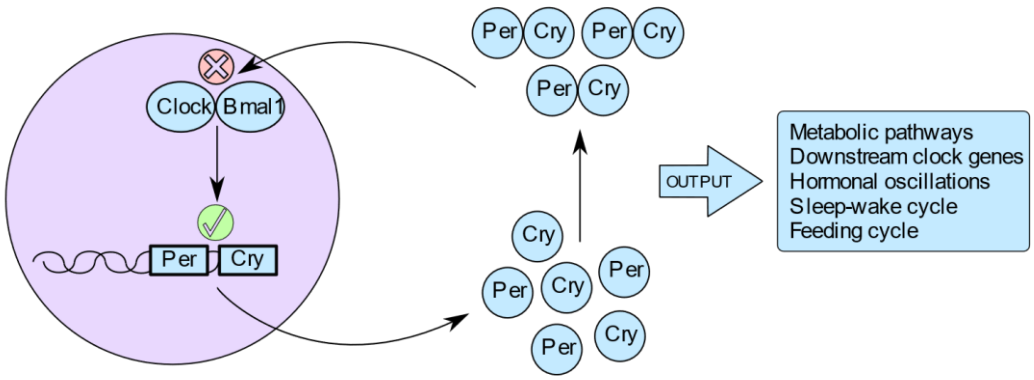

Figure 6. The molecular clock autoregulatory loop. Circadian locomoter output cycles kaput protein (CLOCK) and the brain and muscle Arnt-like protein-1 (BMAL1) heterodimer activate the transcription of PER and CRY, which accumulate into the cytoplasm and translocate into the nucleus when they reach a particular threshold, inhibiting CLOCK and BMAL1 activity.

2.2 Circannual Rhythm

Animals adapt differently to the changing seasons in order to improve their fitness, that is, their capacity to survive and reproduce. In temperate zones, the hours of light and dark change during the year in a rhythmic pattern, which affects the behaviour and metabolism of animals (112). Moreover, these changes in light allow animals to anticipate to changes in the environment associated to seasons. For example, neglecting some functions such as reproduction, and investing in survival mechanisms such as thermoregulation during winter, or engaging in other activities convenient for breeding during spring and summer (113). Seasonal rhythms are also important in humans, which display differences in body fat over the changing seasons (114) and in many other parameters such as concentration of

determinate hormones or physical activity (115–117), which are believed to be implicated in the fluctuations in total body fat observed throughout the year (118).

This set of biological processes that respond to the annual cycle of changes in light and dark is called seasonal photoperiodism (119). These changes in light are detected by the retina, which interacts with an innate timekeeping system found in many species, called the circannual clock, thus, allowing the anticipation of seasons and its associated environmental changes (120). The mechanisms by which this circannual clock is capable of maintaining an annual cycle however, are still not clear (120).

As it is already mentioned in the circadian rhythm section, melatonin plays an important role in transmitting the circadian information across the organism (98). This hormone is secreted during the dark phase, and it has been shown to be responsible in signalling the length of night (121), and so, to be critical for the physiological adaptations to the season (122). In addition to the secretion of melatonin, more recent studies have designated the *pars tuberalis*, located in the hypophysis, as another essential element for the correct circannual function (98,120). Some authors report that the expression of circadian clock genes in the *pars tuberalis* is regulated by the photoperiod (98), which might link the circadian and circannual clocks (123,124). In fact, some studies have found that clock genes are directly implicated in the duration of the melatonin secretion, which generates the seasonal adaptations (125). Thus, it is currently accepted that the circadian clock plays an important part in the circannual rhythm, however, the exact mechanism still remains to be elucidated (124).

2.2.1 Animal models for the study of photoperiodism

In order to study the photoperiodism and seasonal adaptations, researchers have been using different animal models that react to changes in the light and dark schedules in a controlled environment, such as the Siberian or the Djungarian hamsters. The photoperiod in which seasonal animals will change to a reproductive phenotype is different among species. In this sense, animals with short gestations, such as hamsters or voles, are stimulated with a long photoperiod, while animals with long gestations, like sheep, start reproductive adaptations during short photoperiod (126)

Laboratory rats are generally considered nonphotoperiodic because they do not show reproductive responses to the photoperiod. However, the Fischer 344 strain is a known

animal model that shows seasonal physiology, developing reproductive adaptations when stimulated with a long day, like increased testes size (127–129), body weight (128,129), fat content (130), and consumption of carbohydrates and proteins (130), while exposure to short photoperiods inhibits these reproductive adaptations (129,131). Thus, the Fischer 344 rat strain is a good model to study the circannual clock and seasonal adaptations to the photoperiod.

2.3 The circarhythms and obesity

2.3.1 Circadian clock dysfunction and development of obesity

As already explained above, the daily rhythms in the organism are ruled by the SCN, however, proper synchronization with the photoperiod is required. *Wyse et al.* defined circadian desynchrony as “the misalignment of endogenous circadian rhythms with the unpredictable daily photoperiodic cycles facilitated by electric lighting” and propose this phenomenon as a risk factor that predisposes humans to the increased rates in obesity prevalence of the last decades (80).

There is currently a lot of evidence that the disruption of the circadian rhythm has many negative side effects (79). For example, there are studies reporting increased risk of developing multiple sclerosis (132), cardiovascular diseases (133) or cerebrovascular insults related to alterations in the circadian rhythmicity (134). More in detail, circadian dysfunction has been found to be well correlated with an increased risk of obesity in night shift workers (81,135,136). The effects of the disruption of the circadian rhythm on obesity have also been studied in animal models, and either continuous exposure to light during the night (137,138), or altering the normal light-dark cycle (139–142) induced negative changes in the metabolism, contributing to obesity development.

Circadian desynchronization can also be induced by other factors other than light. Diet has been reported to alter the rhythm of peripheral tissues (143). For example, temporal feeding restriction has shown to reset the circadian gene expression in many tissues, leaving the rhythm in the SCN unaltered (144). Another study found that the fasting period between two meals, and the volume of food consumed, were able to influence the gene expression of molecular clock genes in the liver (145). On the other hand, not only time of feeding, but also

the type of food consumed have shown these effects, since high-fat diets have also shown to disrupt the circadian clock rhythm (146,147).

2.3.2 Circannual clock dysfunction and development of obesity

Not only daily changes in light have been studied as a potential risk factor for obesity, but also the effect of the seasonal or circannual rhythms. In humans, the fact that the changes in dark and light during the year change the secretion of melatonin and, consequently, other hormones, has been related in some individuals to fatigue, increased appetite, weight gain, and sadness, a phenomenon named Seasonal Affective Disorder (148). These affections are believed to be caused by a lack of circadian rhythm entraining, since these symptoms are alleviated with natural light, bright artificial illumination, or oral melatonin supplementation (96). Moreover, the prevalence of this disorder has been associated to a higher predisposition to the metabolic syndrome and particularly to increases in body weight (149)

On the other hand, it has been reported that the physiological changes that humans experience during the seasons can influence the risk of certain diseases. In this sense, it has been found that coronary heart disease is more prevalent during the winter (117), while the increase in subcutaneous fat that increases the insulation against the cold, elevates the risk of insulin resistance and heart disease (116), which could also influence the prevalence of other diseases such as obesity.

3.XENOHORMESIS

The word Xenohormesis comes from the Latin *xeno*, which means guest or stranger, and hormesis, a concept that refers to a benefit in health caused by a mild stressor (150). The xenohormesis theory is based in the communication of stress signals between different species (151). In this regard, consumption of components synthetized by plants, are able to signal heterotrophs through evolutionary conserved pathways (152).

More in detail, many molecules synthetized by plants, such as polyphenols, have been found to exert positive effects in health through specific and known molecular targets in the organism (153,154). These compounds have also been reported to vary in plants depending on many stresses such as light intensity, photoperiod, temperature, mild drought or season (155–158). The fact that the concentration of these secondary metabolites in plants change depending on environmental factors, makes it possible for animals or fungi to have developed and conserved mechanisms to detect changes in the concentration of these molecules. This way, animals could adapt to the availability of food, developing strategies to increase their survival and chance of reproduction, by increasing the efficiency of metabolism or decreasing heat production, for example (152). *Yun et al.* propose that "the nature of food may signal the relative stress state of the food chain" (159). In this sense, the consumption of food with a seasonal related stressor, would signal the organism about an approaching shortage in food supply, and so, animals would develop adaptations in order to survive, for example by increasing energy absorption (159).

Related to this, the expansion of the global food market has created a phenomenon where consumed food can originate from far away countries, dissociating the harvest season from the time of consumption. As an example, during 2015, 31.3% of the citrus fruits that entered the European Union were produced in South Africa, while an additional 11.2% arrived from Argentina (160). Both countries are in the southern hemisphere, and so, in a completely different season to the northern hemisphere. Alternatively, food grown locally in the natural growing season can then also be stored and eaten some months later, in a different season (161).

Altogether, the fact that food carries a molecular signature about the environment, could suppose another factor to be considered for obesity prevention and treatment, adding origin and season of growth or harvest of food in health and dietary recommendations.

4.OBESITY TREATMENT THROUGH DIET

As exposed in the first chapter of the introduction, the treatment of obesity is of vital importance due to the increasing number of cases (62), the several associated diseases (63–70), and for being responsible for a high number of deaths worldwide (71).

At present, there are several strategies to treat obesity. The most common approach is to make changes in lifestyle, principally based on a reduction of calories consumed, and if possible, accompanied by increased caloric expenditure through physical activity (162). Also, the increase in the consumption of fruits and vegetables in the diet are widely encouraged, since they have been linked to obesity prevention and improved health status due to their high content in fiber, vitamins, minerals, electrolytes and phytochemicals (84), especially, polyphenols (163). Other more specific treatments include pharmacotherapy, usually anorexiants or gastrointestinal fat blockers, however their effectivity is still limited, or present adverse side effects (164). Finally, bariatric surgery has been also applied to serious cases of obesity, but despite great results in weight loss, it has many associated risks and high costs (162).

Nevertheless, even though the reduction in caloric consumption seems to be the most plausible strategy for the reduction of obesity, the number of patients who drop out or fail to keep a long term diet are very frequent (165). For these reasons, other strategies to improve health in obese individuals through diet are being studied. One of these strategies is the use of natural bioactive compounds, substances with the capacity to affect biological processes or substrates in the body, and by doing so, are able to improve health (166). The effects of these bioactive ingredients vary from antioxidant, anti-inflammatory and anticancer activities, among others. Moreover, they are harmless, or at least, more secure than synthetic drugs, since they are found mainly in food, in the form of molecules such as polyphenols, fatty acids or peptides (85).

4.1 Polyphenols

Polyphenols are structurally diverse secondary metabolites synthetized by plants, that are not vital for growth, but with diverse functions for their survival and reproduction, including protection from infections, herbivores, UV or pollinator attractants. They are principally classified in flavonoids, which contain fifteen carbons with two aromatic rings connected by a three-carbon bridge, and nonflavonoids. From here, they are further classified depending on their biochemical structure (167). The principal sources of polyphenols are spices, herbs and fruits; among the latter, berries have the higher amount of total polyphenols, while fruits such as cherries also show a high content, followed after by other less intensely coloured fruits such as oranges or grape (163).

Even though polyphenols are not essential nutrients in the human diet, new evidences highlight their beneficial effects in different ailments when consumed in the long-term (167). In particular, studies indicate the utility of polyphenols in the prevention and treatment of obesity, and some of them associate these effects to the direct or indirect interaction of polyphenols with the adipose tissue (168,169).

4.1.1 Anthocyanins

Anthocyanins are one of the major groups in which flavonoids can be classified. The six principal anthocyanins found in diet are cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin. Usually, they are conjugated to one or more sugars in order to increase stability, the glycoside form, and so, are named anthocyanidins. In food, anthocyanins are mainly found in fruits or vegetables with red or violet colours like berries, wine and grapes (170) (Figure 7).

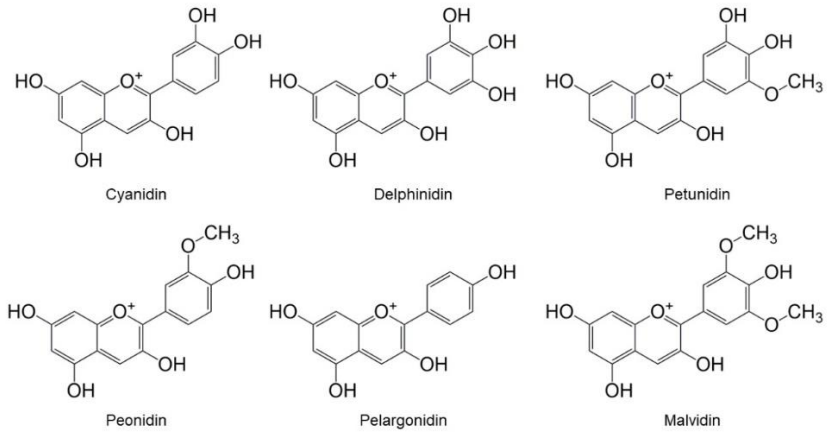

Figure 7. Structures of the principal anthocyanins found in diet.

Many studies have shown promising results on the prevention of obesity with anthocyanins (171). Sweet cherry anthocyanins supplemented to mice fed a high-fat diet (HFD) has shown to prevent obesity, reducing the body weight, and more specifically, the size of adipocytes and glucose, TAG, total cholesterol and LDL-cholesterol serum levels (172). In a similar experiment, blueberry and mulberry juice, rich in anthocyanins, were also able to protect

from obesity (173), suggesting that not only the phenolic extract, but also fruits juice rich in anthocyanins showed this protective effect.

A part from its obesity preventing action, other studies have found that anthocyanins could serve as a treatment for obese subjects. In fact, *Wu et al.* reported that 200mg/ body weight of anthocyanins extracted from *Vaccinium ashei*, supplemented to obese mice decreased body weight and the size of adipocytes (174).

Even though there is large evidence suggesting a role of anthocyanins in obesity treatment, further study of its function, metabolism and absorption is required. Additionally, more robust evidences are needed to confirm its beneficial effects on obesity treatment (171,175).

4.1.2 Proanthocyanidins

Proanthocyanidins are oligomeric and polymeric flavan-3-ols, a type of flavonoids that also are called condensed tannins (167). They can be composed of up to fifty units of flavan-3-ols, like (+)-catechin or (-)-epicatechin (Figure 8) and can be found in apples, pears, blueberries and grape seeds, among other fruits or vegetables (176). When they are exclusively composed of epicatechin units, they are named procyanidins, which are the most abundant type of proanthocyanidins found in plants (167). Proanthocyanidins are considered the most abundant phenolic compound after lignin (177).

As anthocyanins, proanthocyanidins have been extensively studied for the treatment of obesity, including for the reduction of WAT weight or beneficial effects on WAT metabolism (178). *Pascual-Serrano et al.* found that the treatment of obese rats with 25mg/ kg body weight of grape seed proanthocyanidin extract (GSPE) reduced the size of visceral adipocytes and increased their number. In this sense, the treatment favoured a healthier expansion of the WAT, which increased the capacity of adipocytes to mobilize lipids (179). In another study, obese hamsters supplemented with GSPE showed a reduction in the weights of different WAT depots and overall adiposity, accompanied by the overexpression of key genes of the β-oxidation pathway in the WAT (180).

Even with the extensive bibliography and increasing evidence, the effects of proanthocyanidins on body weight gain and fat are still not clear and need further study, since there are authors reporting clear beneficial effects, whereas others show no effects (178).

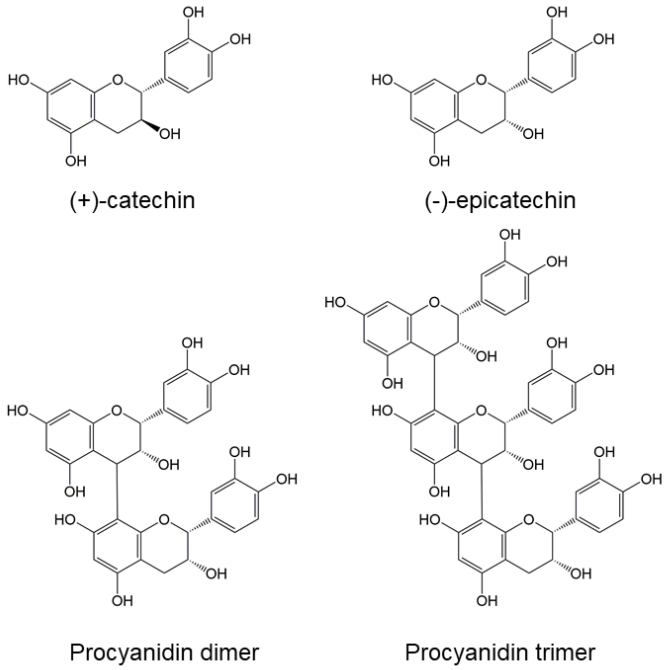

Figure 8. Structures of the flavan-3-ols (+)-catechin and (-)-epicatechin, and procyanidin dimer and trimer.

4.2 Fatty acids

Fatty acids (FA) are composed of a carboxylic acid with an added hydrocarbon chain and can be classified depending on the presence of a C-C double bond. Thus, saturated FA have no C-C double bound and unsaturated FA have one or more C-C double bonds. The latter can be divided in monounsaturated or polyunsaturated FA if they have one or more of these bonds, respectively (181).

Even though studies show that saturated fatty acids have adverse effects on health, more and more, studies report that monounsaturated and polyunsaturated fatty acids might be beneficial for the treatment of obesity (182). In this sense, ingredients such as omega-3 polyunsaturated fatty acids, α-lipoic acid or conjugated linoleic acid (CLA) have shown promising effects in the treatment of obesity and the expanded WAT through diverse mechanisms, including reduction of appetite or the improvement of energy expenditure and fat oxidation (85).

4.2.1 Conjugated Linoleic Acid

CLA is a 18-carbon polyunsaturated fatty acid that contains two conjugated double bonds. This compound is mainly found in beef and dairy products from rumen animals and comprises a high number of isomers. However, the most abundant isomer in food is the cis-9, trans-11 (c9t11). Commercially available CLA supplements are usually industrially formed from vegetal linoleic acid, and also, incorporate equal amounts of trans-10, cis-12 (t10c12) and other isomers in minor quantities (Figure 9) (183,184).

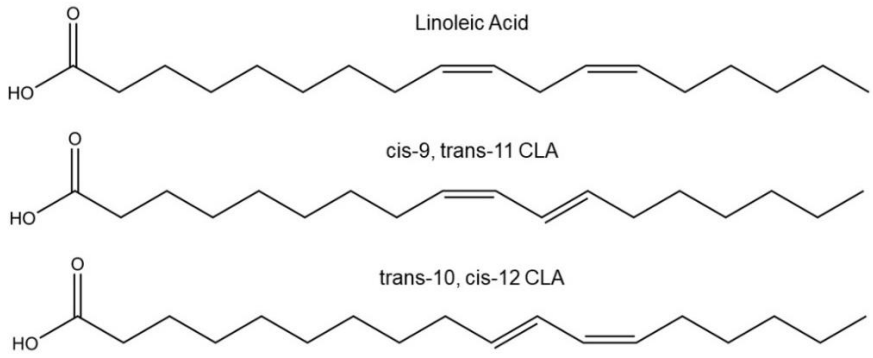

Figure 9. Structures of Linoleic acid, and the two more abundant isomers of conjugated linoleic acid (CLA).

Several researchers have shown interesting effects of CLA supplementation on the treatment of obesity, including the reduction of fat mass (184,185). For example, it has been reported that CLA supplementation is capable of increasing the thermogenesis and browning in WAT depots of obese mice, contributing to weight loss (186,187). In humans it has also been reported to reduce body fat mass of obese and overweight individuals (188). However, further studies are needed, since evidences are still weak or inconsistent, and many other studies show no weight reduction (183,184).

4.3 Peptides

Bioactive peptides are synthetized *in vitro* through enzymatic hydrolysis of proteins (189). This reaction is usually performed with pepsin, bromelin or trypsin, including other enzymes,

and it is preferable to natural synthesis, such as those in ripening or fermentation, because it is performed in tightly controlled conditions and permits the obtention of specific by-products (190). The precursor proteins used for this process are usually of animal origin, for example milk, although vegetal proteins are also used. At release from the originating protein, these peptides show a wide range of applications such as the treatment of illnesses or for cosmetic products (190).

One of the most important sources of bioactive peptides is milk, which has served as a precursor of peptides useful for different ailments related with obesity such as improving satiety or reducing hypertension (85). Other animal sources have shown promising effects like peptides from chicken feet hydrolysate, which have shown interesting anti-hypertensive effects (191), or peptides from marine organisms with satiating effects (192). Additionally, some studies have reported effects of particular peptides on the WAT, for example, casein glycomacropeptide has shown in obese Sprague-Dawley rats to decrease the weight of the adipose tissue and the activity of FAS and glycerol-3-phosphate dehydrogenase (193). Even so, the number of studies reporting the effects of dietary peptides on the adipose tissue are scarce.

4.4 Multi-ingredients approach for the treatment of obesity

Obesity is a multifactorial disease, caused by the dysregulation of several organs or mechanisms, including the metabolism of the adipose tissue and the secretion of adipokines, the gut microbiota and appetite regulatory gut-brain hormones (194). In fact, its pathogenesis results from the interactions among these and other systems and the dysregulation of their communication signals, which results in detrimental effects on energy homeostasis of the adipose tissue, affecting adipogenesis and its metabolism and differentiation (195). Its prevalence is usually accompanied by a higher risk of other co-morbidities such as type 2 diabetes (63,64), hypertension (67) and metabolic syndrome (196), already mentioned in the first chapter of the introduction. Moreover, studies show that the chronic inflammation of the WAT during obesity, and the dysregulation in the secretion of adipokines might be responsible for the rise of these abnormalities in obese individuals (197,198).

For the reasons exposed above, obesity reversal is a real challenge, and even with diets and physical activity, many patients are unable to lose weight or maintain it after losing it (195). The concept of using different natural compounds such as polyphenols or CLA, with

beneficial activities on the causes of obesity, the chronic inflammatory state and the related diseases, is an attractive idea to counteract obesity prevalence. In this sense, researchers are studying the effect of combining different bioactive compounds, in order to obtain additive or synergic effects that could tackle different pathways involved in a particular disease (199–201). Moreover, the combination of different ingredients with similar effects could increase the overall effect, guaranteeing a significant impact of the treatment and reducing the overall dosage (202).

In this aspect, the results of several studies suggest that combining different bioactive ingredients could be a suitable approach for the treatment of the variety of factors involved in obesity and associated diseases. For instance, *Rondanelli et al.* used this same concept to combine different bioactive ingredients that, when used separately have been shown to exert positive effects on the obesity treatment (203). They observed that the combination of epigallocatechin gallate (EGCG), capsaicin, piperine, carnitine, *Allium sativa* and *Fucus vesicolosus*, supplemented to overweight and obese individuals, improved insulin resistance, leptin/adiponectin ratio, LDL-cholesterol levels, and respiratory quotient. However, no differences were found in body weight or fat. They concluded that the effect of the mixture can possibly be attributed to the synergy of the components, since the observed effects were more effective than the ones observed in the scientific literature (203). A similar experiment combined other ingredients that have shown evidences of increasing thermogenesis. *Belza et al.* used EGCG, capsaicin, caffeine, tyrosine and calcium, which when separated have shown thermogenic and satiating effects throughy different mechanisms, and obtained successful results when supplemented to overweight and obese subjects, increasing thermogenesis and slightly reducing fat mass, compared to the placebo group (204). Even so, there are other studies that showed no synergic effects in the combination of ingredients to prevent obesity (205–207).

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II. HYPOTHESIS AND OBJECTIVES

This PhD thesis has been developed in three research projects of the Nutrigenomics research group of the Universitat Rovira i Virgili: “Illegitimate signalling of fruit consumption and obesity pathogenesis” (Señalización errónea del consumo de frutas y patogénesis de la obesidad – FRUITOBES - AGL2013-49500-EXP), “Development of an integrated food to maintain body weight and to prevent the risk of obesity related pathologies” (Desarrollo de un alimento integrado para el mantenimiento del peso corporal y la prevención del riesgo de patologías asociadas con la obesidad – 2.0-NORMPES - AGL2013-40707-R) and “Study of the interaction of the circadian and seasonal rhythms with the effectiveness of functional ingredients” (Estudio de la interacción de los ritmos circadianos y estacionales con la efectividad de los ingredientes funcionales – CHRONOFOOD - AGL2016-77105-R).

Obesity is principally caused by an excessive accumulation of fat, that increases the risk of many other diseases. It is a multifactorial disease, meaning that the dysregulation of many factors is implicated in its development. The increasing numbers of obesity cases in the population are attributed to changes in modern lifestyle, and new factors are being found that influence its prevalence. One of these new risk factors has been attributed to changes in the photoperiod, which synchronizes many metabolic functions in the organism through the molecular clock. In this sense, many studies report that the desynchronization of the molecular clock, through diet or changes in light and dark during the day, increase the risk of obesity. Additionally, polyphenols have been found to modulate the molecular clock in peripheral tissues, and according to the xenohormesis theory, the intake of fruits and vegetables is able to signal heterotrophs through molecules synthetized by plants, including polyphenols, about mild stresses like changes in season, temperature or drought. This information suggests that another factor should be considered when developing healthy diets for the treatment or prevention of obesity, like fruit and vegetable harvest and consumption season.

Altogether, we **hypothesized** that **photoperiod and consumption of fruit out of season, together with diet, are environmental risk factors for the increase in adipose tissue mass and the disruption of its metabolism observed in obesity**. Therefore, to study this hypothesis, we developed the following objectives:

1. To evaluate the effect of a long and short photoperiods in the adipose tissue accretion and metabolism of the Fischer 344 rat model (**Manuscript 1**).
2. To study whether an obesogenic diet affects the photoperiodic adaptations of the adipose tissue, and so, increases the risk of obesity (**Manuscript 1**).

3. To study whether the consumption of fruit out of season (orange and cherry) affects the metabolism of the adipose tissue of Fischer 344 rats, and to evaluate if these effects are worsened when feeding an obesogenic diet (**Manuscript 2 and 3**).

Moreover, the fact that obesity is a multifactorial disease and that multiple ailments are associated with its incidence, has made scientists develop new strategies for its treatment. Even though pharmacological drugs and surgical intervention are being developed and used in some severe cases, they can pose a risk for health or present side effects. For these reasons, research is also focused on natural bioactive compounds as an alternative to treat obesity, since many studies have reported to present beneficial effects on obesity treatment. Additionally, some studies have shown that the combination of several bioactive ingredients with different effects on the abnormalities observed in obesity, may have an overall synergic or additive effect, ameliorating the altered metabolic state of obese individuals.

For these reasons, we **hypothesised** that the combination of **a mix of natural bioactive ingredients** that separately, have shown favouring effects on different ailments associated with obesity, **will present beneficial effects reducing the white adipose tissue mass and improving the adipose tissue metabolism in obesity**. Thus, to this end, we proposed the following objectives:

1. To determine the effects of the supplementation of a mix of natural bioactive ingredients to obese rats on body fat mass and to determine which pathways in the adipose tissue are altered due to the treatment, and so, are implicated in the overall healthy effect of the mix of ingredients **(Manuscript 4)**

III. RESULTS

MANUSCRIPT 1

Response to the photoperiod in the white and brown adipose tissues of Fischer 344 rats fed a standard or cafeteria diet

Albert Gibert-Ramos[a], Maria Ibars[a], M. Josepa Salvadó[a], Anna Crescenti[b]

[a]Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, Tarragona, Spain

[b]Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain

Corresponding authors: albert.gibert@urv.cat, Albert Gibert Ramos; mariajosepa.salvado@urv.cat. M. Josepa Salvadó. Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Campus Sescelades, Building N4, Marcel·lí Domingo 1, 43007, Tarragona, Spain. Tel. +34 977 55 84 65

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Abstract

Researchers are identifying new factors that contribute to the obesity epidemic, with changes in the photoperiod as one promising risk factor. To study the influence of the photoperiod on adipose tissue, Fischer 344 rats were treated for fourteen weeks with a long day (18 h light:6 h dark; LD) or a short day (6 h light:18 h dark; SD) and fed a standard diet (STD). Biometric measures, postprandial triglycerides, gene expression in the retroperitoneal white adipose tissue (RWAT) and brown adipose tissue (BAT) and histology of the RWAT were analysed. A second experiment with the same conditions and analysis was performed for eleven weeks with rats fed a cafeteria diet (CAF). In the STD experiment, the SD increased triglycerides and showed a tendency to reduce fat compared to the LD. In the RWAT, genes implicated in adipogenesis, lipogenesis and lipolysis were downregulated and the histological results showed a higher percentage of small adipocytes in the SD, without changes in their total number. In the CAF experiment, lipogenesis and adipogenesis gene expression were increased in the SD, while adipocytes were smaller and their number increased. Both experiments showed in the SD a decrease in the BAT expression of lipid uptake and β-oxidation genes, while only the STD additionally showed a reduction in *Ucp1* expression. In conclusion, the RWAT morphology and the expression of key genes for lipid metabolism in RWAT and BAT were influenced by the photoperiod; however, the changes observed in the RWAT were different depending on the diet.

1. Introduction

Obesity is a major health problem, defined by the World Health Organization (WHO) as an "abnormal or excessive fat accumulation that presents a risk to health", caused by an imbalance between caloric intake and calorie expenditure [1]. Its primary consequence is the predisposition to insulin resistance, cardiovascular diseases, musculoskeletal disorders and a major risk factor for some cancers, among others [1]. The obesity epidemic has been principally attributed to modern habits, principally to a reduction in physical activity and an increase in energy-dense food intake. However, these factors alone are not enough to explain the rapid increase in the incidence of obesity, and therefore, other environmental factors are being studied [2,3]. In this sense, numerous studies show that the disruption of the circadian clock or its desynchrony can induce obesity [4,5]. For example, night-shift workers have a higher risk of developing metabolic syndrome than people who work during the day [6,7]. There is also evidence that a short photoperiod exposure decreases adiposity in Siberian hamsters [8]. These reports suggest a close relation between fat mass and the photoperiod and its importance in health.

Seasonal animals adapt to the environment in order to survive the changing conditions during the year and breed when the situation is more suitable. These animals are able to recognize the length of the day or photoperiod, which allows them to predict the season of the year in anticipation, before the environment changes [9]. The response to the photoperiod is controlled by the suprachiasmatic nucleus (SCN) in the hypothalamus [10], being the primary component of the molecular clock [11]. A the same time, an equal clock is present in other regions of the organism, named the peripheral clock, entrained principally by the central clock in the SCN [11]. Moreover, while light is critical to maintain the clock's synchronization, other factors, such as food consumption, can also entrain the molecular clock. Thus, metabolic processes can be decoupled from the SCN driven clock when food intake is desynchronized from the light/dark cycle [12]. In humans, the effects of seasonality are also observed. During the year, we can detect fluctuations in body fat mass caused by different factors that change the levels of energy intake and energy expenditure [13]. Even though these adaptations are useful in some environments, currently, they can pose a problem. For example, an increased subcutaneous adipose tissue offers insulation from the cold, with the counterproductive effect of an increase in the body's fat percentage [14].

The white adipose tissue (WAT) is the primary organ for energy storage in animals. This organ is primarily composed of adipocytes, which store triglycerides (TAG) as lipid droplets that occupy 90% of its total volume [15], and it can cause obesity when it grows excessively.

WAT has been reported to be affected by the photoperiod. Exposure of photoperiodically sensitive rodents in a long day (LD) photoperiod demonstrated an increase in fat content [16,17]. On the other hand, brown adipose tissue (BAT) is a highly oxidative tissue found in determinate areas of mammals. Specifically, BAT of rodents can be found in interscapular and peri-renal areas, among others. This tissue is specialized in thermogenesis using the uncoupling protein 1 (*Ucp1*), a protein found in the inner mitochondrial membrane that separates the oxidative phosphorylation from ATP production [18]. BAT activity has been found to be negatively correlated with obesity and diabetes [19]. While the physiological function of WAT is to store lipids, the function of BAT is thermoregulation and energy expenditure. [20]. Additionally, there is evidence that BAT is also influenced by the photoperiod since it has been shown that BAT is inactivated when light exposition is increased in mice [21], and a similar effect has been observed in Siberian and desert hamsters [22,23].

Photoperiod responsive animals have been used in research to better comprehend circadian and circannual cycles. Using different light and dark lengths, researchers can mimic the seasonal variation observed in nature and therefore the physiological effects on laboratory animals. Fischer 344 rats have been reported to display a strong response to the photoperiod [16,24–26] and therefore can be used as a model to study how animals adapt to the season.

This study was designed to better understand how the photoperiod influences adipose tissues and if the influence depends on diet, which could be of importance in relation to the human obesity epidemic. Thus, the first objective of the study was to analyse how the exposure to an LD or short (SD) photoperiods affects the BAT and WAT metabolism. Moreover, we performed a second independent experiment with rats fed a cafeteria (CAF) diet, a highly palatable diet that induces voluntary hyperphagia and that contains high quantities of fat and sugar [27], with the objective of analysing how the photoperiod affects the BAT and WAT metabolism in an obesogenic diet.

2. Methods and materials

2.1 Animals and Treatments

The twelve animals used for the first experiment were two-month-old male Fischer 344/IcoCrl rats (Charles River Laboratories, Barcelona, Spain) fed with a standard chow diet (STD) (Panlab, Barcelona, Spain) with a caloric distribution (3.2 kcal/g) of 19.3% protein, 8.4% fat and 72,4% carbohydrates. The animals were housed two animals per cage at 22ºC and 55% humidity and with free access to food and water. The animals were randomly distributed into two groups (n=6), depending on the photoperiod to which they were exposed, LD (18 h light: 6 h dark) and SD (6 h light: 18 h dark) for fourteen weeks.

For the second experiment, twenty Fischer 344/IcoCrl rats were fed a CAF ad libitum, consisting of bacon, biscuits with pâté and cheese, muffin, carrots and sweetened milk (22% sucrose w/v) in addition to the standard chow diet [27]. The caloric distribution of the CAF diet (5.28 kcal/g) was 10% protein, 31.9% fat and 58.1% carbohydrates. The food was freshly provided daily. This high-fat high-sugar diet model is based on appetizing ingredients that induce voluntary hyperphagia, increasing fat and sugar ingestion [27]. Animals were also randomly distributed into two groups (n=10) and exposed to an LD or SD photoperiod for eleven weeks.

For both experiments, body weight and food intake for the animals were recorded every week. One week prior to sacrifice, fat mass and lean mass were analysed by quantitative magnetic resonance using an EchoMRI-700™ (Echo Medical Systems, LLC., TX, USA) without anaesthesia. At the end of the experiment, all animals were sacrificed in the fed state by decapitation, and blood was collected from the neck, stored at room temperature for 45 min and centrifuged at 1200 g for 10 min to collect the serum. Retroperitoneal white adipose tissue (RWAT) and interscapular BAT were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80ºC until further analysis.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures, and the university guidelines for the use and care of laboratory animals were followed.

2.2 Plasma analysis

An enzymatic colorimetric kit was used for the determination of plasma TAG (QCA, Barcelona, Spain).

2.3 RNA extraction and quantification by real-time qRT-PCR

Total RNA from RWAT and BAT was extracted using Trizol® reagent (Thermo Fisher, Madrid, Spain) following the manufacturer's instructions. RNA yield was quantified in a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the integrity of the RNA was confirmed using agarose gel electrophoresis.

Overall, 0.5 µg of total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Madrid, Spain) in a Multigene Thermal Cycler (Labnet, Madrid, Spain), and for Q-PCR, the CFX96 real-time system C1000 Touch Thermal Cycler (Bio-Rad, Barcelona, Spain) with the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Barcelona, Spain) was used. All Q-PCR were performed with the following cycling conditions after an initial Taq activation at 95ºC for 30 s: 39 cycles of 95ºC for 5 s and 60ºC for 30 s. A melting curve was produced after the previous steps, by increasing the temperature from 65ºC to 95ºC by 0.5ºC every 5 s. Gene expression levels in RWAT were performed for Acetyl-CoA carboxylase alpha (*Acacα*), Fatty Acid Synthase (*Fasn*), Glycerol-3-phosphate acyltransferase (*Gpat*), Monoglyceride Lipase (*Mgl*), Adipose triglyceride lipase (A*tgl*), Hormone Sensitive Lipase (*Hsl*), CCAAT/Enhancer Binding Protein Alpha (*CEBP*α), and Peroxisome proliferator-activated receptor gamma (*Pparγ*) genes. In BAT, we measured gene expression levels for Cluster of differentiation 36 (*Cd36*), Fatty Acid Transport Protein 1 (*Fatp1*), Lipoprotein Lipase (*Lpl*), Carnitine Palmitoyltransferase 1B (*CPT1b*), Hydroxyacyl-CoA dehydrogenase (*Had*), and Peroxisome Proliferator Activated Receptor alfa (*Pparα*) genes. Furthermore, we measured gene expression levels for PR Domain Containing 16 (*Prdm16*) and *Ucp1* in both tissues. The primers for the different genes are described in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA was calculated as a percentage of the vehicle group using the $2^{-\Delta\Delta Ct}$ method [28], with Peptidylprolyl Isomerase A (*Ppia)*, Actin Beta (*Actb)* and Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1)* as the reference genes. Each PCR was performed at least in duplicate.

2.4 Western Blot

Ucp1 protein content in BAT was determined by Western blot. Tissues were homogenized in RIPA (Radio-Immunoprecipitation Assay lysis buffer), and the protein was extracted and stored at -20ºC. Protein content was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA), following manufacturer's instructions.

Overall, 15 µg of protein in Laemmli loading buffer was denatured and loaded into 10% acrylamide gel made with TGX™ Fast Cast™ Acrylamide Solutions (BioRad, Barcelona, Spain) and run at 90 V for 75 min. Gel transference into a PVDF membrane was done using the Trans-Blot Transfer System (Bio-Rad, Barcelona, Spain), with the Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad, Barcelona, Spain), following manufacturer's instructions. The membrane was blocked and then incubated with anti-UCP1 antibody (Abcam, Cambridge, United Kingdom) at 4ºC overnight. Afterwards, the membrane was incubated 2 h with the secondary antibody (GE Health Care Life Sciences, Barcelona) and detected with the chemiluminescent reagent ECL Select Western Blotting Detection Reagent (GE Healthcare, Barcelona, Spain); protein levels were quantified with the ImageJ open source software [29] and relativized to *Actb* protein levels.

2.5 Histology

For histological analyses, frozen RWAT samples were thawed and fixed in 4% formaldehyde. The tissue underwent successive dehydration and paraffin infiltration-immersion (Citadel 2000, HistoStar, Thermo Scientific, Madrid, Spain), and the paraffin blocks were cut into 2-µm-thick sections using a microtome (Microm HM 355S, Thermo Scientific). The sections were subjected to automated haematoxylin - eosin staining (Varistain Gemini, Shandom, Thermo Scientific)[30].

Sections were observed and acquired at x10 magnification using AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L., Madrid, Spain). The area of adipocytes was measured using the Adiposoft open source software (CIMA, University of Navarra, Spain). Six samples per group were analysed for experiments 1 and 2. Four fields per sample were measured. The adipocyte area was calculated from the average value of the area of cells in all measured fields for each sample. The total adipocyte number was calculated using the formula $(\frac{\pi}{6})\ x\ (3\sigma^2\ x\ \bar{d} + \bar{d}^3)$, where $\bar{d}$ is the mean diameter and σ is the standard deviation of the diameter, to obtain the average adipocyte volume [31]; afterwards, we converted this value to the average adipocyte weight using the adipocyte density (0.92 g/ml) and, to obtain the total adipocyte number, the weight of the RWAT depot was divided by the average adipocyte weight, as proposed by *Lemmonier* [32]. The frequencies of adipocytes were calculated by distributing all counted cells per sample into 2 groups according to their area, <5000 µm^2 or >5000 µm^2; then, the number of total counted adipocytes was used to calculate the percentage of adipocytes in both categories.

2.6 Statistical Analysis

The SPSS software (SPSS, Chicago, IL) was used for the statistical analysis. Data are expressed as the mean ± SEM, and significant differences were analysed by unpaired Student's t-test. A p value ≤0.05 was considered statistically significant. A varimax-rotated principal component analysis (PCA) was performed with XLSTAT (Addinsoft, Paris, France) to assess the relationships between our variables. Specifically, 12 samples per experiment and 27 variables were used: biometric and metabolic parameters, gene expression in RWAT and BAT, and adipocyte area and number. Gene expression levels of *Ucp1* and *Prdm16* in the RWAT were undetectable in the CAF experiment; thus, they were not included in the analysis. *Ucp1* protein levels in the BAT of STD rats and *Fasn* protein levels in the RWAT of the CAF experiment were also excluded from the PCA analysis because these parameters were not analysed in the CAF and STD groups, respectively. The Kaiser-Meyer-Olkin (KMO) index of our variables was >0.5. After data scaling, the analysis was based on the correlation matrix, and a principal component (PC) was considered to be significant if it contributed to >5% of the total variance. Only variables with an absolute loading of ±0.40 on a given PC were used to describe the PCA and interpret the results.

3. Results

3.1 Biometric and metabolic parameters

Rats fed the STD or the CAF diet showed no significant differences in body weight and food intake between the studied photoperiods (Table 1).

In rats fed the STD, although not being significant, fat (gr, %) (p=0.059, p=0.063) was decreased in the SD group compared to the LD group. Furthermore, TAG serum levels were significantly higher in the SD group than in the LD group (Table 1).

In rats fed the CAF diet, a significant increase in BAT (%) and a tendency for BAT (gr) (p=0.067) was observed in the SD group compared to the LD group. Furthermore, the lean mass (%) of rats in the SD group was increased compared to the LD group, although the increase was not statistically significant (p=0.1) (Table 1). Sugar consumption from the CAF diet was calculated; however, no differences among the groups were observed (data not shown).

3.2 Gene Expression in RWAT

In animals fed the STD, the photoperiod altered the expression of lipogenesis genes; *Fasn* expression levels were significantly lower in the SD animals than in the LD animals, and a similar effect was observed with *Acacα* expression levels; however, the differences were not significant (p=0.066). The expression levels of *Mgl*, *Atgl* and *Hsl*, which are involved in lipolysis, and C/ebp*α* and *Pparγ*, which are involved in adipogenesis, were significantly lower in the SD animals than in the LD animals. Furthermore, the expression levels of *Ucp1* and *Prdm16* genes, which are related to thermogenesis, were also significantly reduced in the SD animals compared to the LD animals (Figure 1A).

In animals fed the CAF, the expression levels of *Acacα*, *Fasn* and *C/ebpα* genes were significantly increased in the SD animals compared to the LD animals (Figure 1B). Expression levels of *Ucp1* and *Prdm16* were undetectable in both groups (data not shown).

3.3 Fasn protein levels in the RWAT

We decided to quantify *Fasn* protein levels in the RWAT of CAF animals to further study the changes already observed in gene expression. *Fasn* protein levels were significantly increased in the SD animals compared to the LD animals (Figure 2).

3.4 Histology of RWAT

Rats fed with STD and treated with the SD photoperiod showed a significant increase in the frequency of adipocytes smaller than 5000 μm^2. Adipocyte area, though not significant (p=0.06), was also smaller in the SD group. No differences between the groups were observed in the total number of adipocytes in the RWAT (Figure 3A).

Rats fed with CAF diet and treated with the SD photoperiod showed differences in adipocyte frequencies, with a higher number of small adipocytes compared to the LD animals. Moreover, they showed significantly smaller adipocyte areas and a higher total adipocyte number compared to the LD animals (Figure 3B).

3.5 Gene Expression in the BAT

In rats fed the STD, the expression levels of the genes involved in β-oxidation were significantly decreased for *CPT1b* and *Had* and significantly increased for *Pparα* in the SD animals compared to the LD animals. For genes involved in lipid uptake, the gene expression levels of *Cd36*, *Lpl* and *Fatp1* were significantly downregulated in the SD animals compared to the LD animals. For genes involved in thermogenesis, the expression levels of *Ucp1* was significantly decreased in the SD animals compared to the LD animals. *Prdm16* gene expression levels, although not significant (p=0.063), were decreased in the SD animals compared to the LD animals (Figure 4A).

In rats fed the CAF diet, we observed the expression levels significantly decreased for *Had*, *Cd36* and *Fatp1* genes and significantly increased for *Pparα* gene in the SD animals compared to the LD animals. Furthermore, *Lpl* gene expression was decreased in the SD animals compared to the LD animals, although the differences were not significant (p=0.095). No differences between the groups were observed in the expression levels of genes involved in the thermogenesis process (Figure 4B).

3.6 UCP1 protein levels on BAT

Due to significant changes on *Ucp1* gene expression levels between the groups of animals fed the STD, we decided to quantify the protein levels of UCP1 in these animals. The *Ucp1* protein levels were significantly decreased in the SD animals compared to the LD animals (Figure 5).

3.8 Principal Components Analysis

With the purpose of analysing the distribution of our data and the association between the variables, we performed a principal components analysis (PCA). In rats fed the STD, PC1 explained 44.73% of the variance and PC2 explained 17.56% (Supplemental Figure 1A). PC1 clearly discriminated between the LD and SD groups and was characterized by TAG, adipocyte number and *Pparα* gene expression on the left side and fat (gr, %), adipocyte area and the expression of all genes analysed, except *Pparα* and *Prdm16* in the BAT, in the right side. PC2 did not discriminate between the photoperiods; however, accumulated caloric intake, weight, lean mass (gr) and fat (gr, %) were positively associated. Fat (gr, %) was related to both PCs; however, its association was stronger to the PC2.

In rats fed the CAF diet, PC1 explained 31,80% of the variance and PC2 explained 22.22% (Supplemental Figure 1B). Photoperiods were separated by PC1, which associated fat (%) and adipocyte area with gene expression in the BAT of *Cd36*, *Had*, *Lpl* and *Fadp1*. At the same time, these variables were negatively associated with lean mass (%), BAT (gr, %), adipocyte number, gene expression of *Pparα* in the BAT, and gene expression of *Acacα*, *C/ebpα, Hsl*, and *Fasn* in the RWAT. PC2 did not separate the photoperiods; however, we observed a positive association between gene expression levels of *Mgl*, *Gpat* and *Atgl* in the RWAT and gene expression levels of *Ucp1* and *Prdm16* in the BAT. These variables were negatively associated with weight, accumulated caloric intake, fat (gr) and lean mass (gr).

4. Discussion

Obesity is primarily caused by an imbalance between caloric intake and expenditure, meaning that excess calories will be accumulated as lipids, especially in the WAT; however, there is an increasing tendency for new risk factors being found that contribute to obesity, including changes in the photoperiod [2–4]. Seasonal changes in the photoperiod help mammals adapt to the approaching season and augment the timing of reproduction to the changing environment [33]. Humans living far from the equator display seasonal differences in the concentration of hormones related to the molecular clock [34]. Other seasonal differences have been observed; for example, the decrease in hours of light during the winter has been associated with seasonal affective disorder a medical condition that has been associated with a predisposition to metabolic syndrome and obesity [35]. Moreover, there is a large body of evidence regarding the presence of seasonal or circannual effects in rodents [36,37]. Specifically, Fischer 344 rats have been well described as responsive to changes in the photoperiod [24,25,38], as well as the influence of the photoperiod over their body weight [16,26,39]. For instance, Fischer 344 rats grown during a short day (SD) show slower gonadal growth [24,25,39], which means that the cycles of light:dark affect their development. Although there are several published studies on the influence of the photoperiod in Fischer 344 rats, to our knowledge, none is focused on the changes in the WAT and BAT. The objective of this study was to evaluate the role of the photoperiod on the metabolism of WAT and BAT and determine its relationship to diet in order to obtain more information about how the environment affects obesity. To investigate this effect, we performed two experiments: one with rats fed an STD diet and another with rats fed a CAF diet, to study whether a highly palatable and caloric diet could influence the effects of the photoperiod, as already suggested by other authors [40].

In our study, we did not observe differences in body weight, and we only observed a trend of reduced fat in rats fed with an STD on the SD. Other studies have shown differences of fat caused by exposure to a short photoperiod in Fischer 344 [16] or in other rodents [17,21], while other authors showed differences at a body weight level but did not detect changes specifically in fat [26,41]. Observing our data, the tendency observed in the fat weight reduction did not seem to be caused by an increase or decrease in energy intake because no changes were observed in that parameter in the STD fed rats. Thus, while some authors using similar experiments detected changes in body mass through a fluctuation of food intake [16,26], other authors observed differences in body weight or fat but no changes in food intake [21,41]. *Shoemaker et al.* observed in a study with Fischer 344 rats that decreases in food intake occurred after decreases in body weight, and they explain that the

changes in body weight might be responsible for the food intake reduction [39]. Though all research to date agrees on the effect of the photoperiod on body weight, the effect on energy intake seems to remain unsettled and needs further research. *Larkin et al.* showed that rats consume most of their daily food intake during the dark phase [42], which means that even though these animals consume the same number of daily calories as SD animals, their consumption time is shorter, which would increase their accumulation as fat. Additionally, mice fed a high fat diet only during the light phase gain more weight than mice fed only during the dark phase, even though their calorie intake and locomotor activity were the same [43]. Though the authors are unable to provide the mechanism responsible for these effects, they propose body temperature, satiety hormones and sleep to be the causes. Thus, in our study, we propose that the tendency observed in fat changes might be due to other mechanisms, including an increased lipid intake by the WAT or because rats concentrate their daily caloric intake during the dark phase, influencing their accumulation as fat in the LD group. In future studies, intake levels in each light:dark phase should be measured.

The rats treated with the LD or SD photoperiods and fed the CAF diet did not show significant differences in body weight or in any of the WAT depots. However, other studies with durations of fewer weeks showed significant differences in body weight [26] and fat [16,40]. A possible reason for this could be that the CAF diet could be masking the adipose accretion, as *Ross et al.* has suggested [40]. In their study, Fischer 344 rats fed an HFD did not show the effect of the photoperiod over the fat mass, as opposed to their findings in STD rats. Specifically, while rats fed a standard chow diet increased their fat mass during the LD; rats fed an HFD showed no differences in fat accretion between photoperiods and increased lean mass in the LD compared to the SD during the 4 weeks of study. On the other hand, food intake can decouple metabolic processes from the molecular clock when desynchronized from normal activity patterns [12], meaning that the CAF diet, which is composed of highly palatable food, could be altering the normal adaptations of rats to the photoperiod.

To obtain more information about the metabolism of the WAT during both photoperiods, the expression levels of genes implicated in important pathways of adipocyte metabolism were studied in the RWAT. In STD animals, we observed a reduction of *Pparγ* and *C/ebpα* gene expression during the SD photoperiod. Both genes have been well studied and characterized as key transcription factors of adipocyte differentiation, lipid metabolism and recruitment of bone marrow-derived circulating progenitors cells to WAT [44]. Since both genes were downregulated in the SD, we could expect to observe the same effects on downstream genes or adipocyte metabolism genes. The results confirmed our hypothesis, as we detected

a downregulation of *Fasn* and a tendency in *Acacα*, which are lipogenesis genes, and at the same time *Mgl, Atgl*, and *Hsl,* which are lipolytic genes, during the SD. This general decrease in both anabolic and catabolic pathways seems to represent the general state of the tissue: either mature adipocytes are scarcer during the SD or its metabolic activity is suppressed compared to the LD.

In accordance to gene expression in RWAT, more TAG were found in the serum of the SD rats than in the LD rats. TAG from diet, after digestion and absorption in the intestine, are secreted into the lymph and shortly after enter into the blood stream where they will be transported in the form of chylomicrons. From here, TAG will be assimilated by the adipose tissue, muscle or mammary glands, mostly as fatty acids [15]. In relation to our results, no differences were detected in caloric intake, which means that the different TAG content in serum is not caused by an increased ingestion of TAG. These data suggest that the WAT of SD rats is not absorbing as much TAG as the LD rats, and therefore, it remains in the blood a longer time. Furthermore, the histology of the RWAT supports the gene expression results obtained. Thus, the animals treated with an LD photoperiod showed a higher frequency of larger adipocytes than the SD group; however, it must be taken into account that the changes were small, and therefore, no significant differences were observed in total adipocytes number and area, though the latter showed a trend to be lower in the SD group than in the LD group. Adipocyte size depends on the accumulated fat, and approximately 90% of an adipose cell is made of TAG [15], which means that adipocyte area or size is correlative with the TAG content.

On the other hand, BAT also absorbs TAG from the blood, having major responsibility for its clearance [45,46]. Data obtained from the BAT showed a general decrease in the expression of genes related to β-oxidation and lipid uptake which, jointly with the results of RWAT, could explain the higher TAG serum content during the SD. Thus, we observed a significant downregulation in the gene expression of *Lpl* and *Cd36*, which actively participate in the uptake of fatty acids by the BAT [45,46]; *Fatp1*, which translocated long-chain fatty acids into the cytoplasm [47]; and *Had*, which directly contributes to fatty acid oxidation in oxidative tissues such as muscle and BAT [48]

Additionally, *Cpt1b*, which controls the incorporation of fatty acids into the mitochondria for entry into the β-oxidation pathway [49], seemed to be downregulated in the SD photoperiod; however, the differences were not significant. Surprisingly, *Pparα*, which is a nuclear receptor protein that targets genes involved in fatty acid uptake, mitochondrial and peroxisomal β-oxidation of fatty acids [50] and also has a role activating *Ucp1* transcription

[51], was upregulated in BAT. These results seem contradictory and a possible explanation could be that even though *Pparα* gene expression was increased, its function was not performed, either because of post-transcriptional regulation or post-translational effects, or because its ligand was not present. In fact, although *Pparα* is activated when bound to fatty acids, it needs to form an heterodimer with the retinoic X receptor (RXR) to be able to bind to the DNA and activate its target genes [52]. On the other hand, RXR levels have been found to be influenced by the photoperiod in the hypothalamus of rodents and to be responsible for the changes in body weight [53,54]. Furthermore, there is evidence that RXR can control brown adipose tissue development and activation [55], that RXR is needed for adipogenesis, and that the disruption of the RXR-*Pparγ* transcription machinery reduces adipocyte formation [56]. These hypotheses are supported by studies that demonstrate that *Pparα* regulates brown adipose tissue thermogenesis, activating *Ucp1* and *Prdm16* gene expression [51], which in our study, were downregulated, together with the studied β-oxidation and lipid uptake genes. Furthermore, *Ucp1* protein levels in BAT were measured, and we obtained the same result already observed in mRNA, which supports our hypothesis of an unexpected effect of *Ppar-α* mRNA.

For the CAF fed animals, as mentioned above, no changes were detected at a biometrical level, aside from an increase in BAT weight in the SD photoperiod. For the RWAT, expression of lipogenesis genes was increased (*Acacα*, *Fasn*) and *C/ebpα* was also upregulated in the SD photoperiod, which seems to indicate higher levels of adipogenesis, though no differences were detected in *Pparγ.* Additionally, *Fasn* protein levels in the RWAT confirmed what we observed in the RNA levels. These results are quite contrary to what we observed in the STD animals, which might indicate the influence of diet on the effects of the photoperiod. The changes observed at the mRNA level are in agreement with the changes observed in the histology of the RWAT. Thus, we observed that the three parameters analysed were significantly altered by the photoperiod, and therefore, the SD rats appear to expand their adipose tissue via hyperplasia, while the LD rats suffer hypertrophy. Thus, our results suggest that although both groups accumulate the same amount of fat, the photoperiod influences the storage approach for the surplus energy. These results are of great importance, as an increase in adipocyte size has been related to insulin resistance and type II diabetes [57,58], and obese patients with healthy metabolic profiles have smaller omental adipocytes [59]. Linked to these results, it has been reported that rats fed an HFD with a voluntary intake of sucrose increased their sucrose consumption in the SD photoperiod compared to animals in the control photoperiod [60]. It has also been suggested that an increased intake of simple carbohydrates can result in an increase in de novo

lipogenesis in humans [61] and rats [62], the latter also increasing expression of fatty acid and adipogenesis genes, as in our study. Moreover, *Queiroz et al.* showed that feeding Wistar rats with a high sugar diet increased pro-adipogenic signals (Pparγ pathway) and adipocyte size and number, which means that sugar consumption can influence the morphology of the WAT [63]. All this evidence could explain why, in our study, animals in the SD group have a different morphology and gene expression profile than animals in the LD group. According to our results, the SD animals did not consume more sugar than the LD animals; however, sugar absorption or sensitivity could be altered. In future studies, more attention should be focused on this hypothesis.

Interestingly, gene expression levels in BAT of animals fed with the CAF diet were also altered between photoperiods, and the changes observed were directly relatable to the changes observed in animals fed with the STD, with both experiments showing a decrease of genes related to lipid uptake and β-oxidation produced by exposure to an SD. Furthermore, animals fed with the CAF diet during the SD photoperiod showed increased expression levels of *Pparα* gene, while other downstream genes showed a decrease in their expression levels, supporting our hypothesis that *Pparα* is not performing its expected function; nevertheless, we cannot provide a proper explanation to this. Additionally, we observed that the differences between the LD and SD groups were smaller compared to the BAT in the STD, which again supports the theory that the diet interferes with the effect of the photoperiod.

As observed in the PCA, rats fed the STD showed that the weight, lean mass (gr, %) and accumulated caloric intake were not representative of any of the photoperiods. On the other hand, fat (gr, %) was more associated with the LD than the SD photoperiod; however, two animals seemed to be resistant to these changes, which could explain why we did not observe strong differences in weight and fat (gr, %) compared to other authors [16,26,40]. *Ross et al*. also report that two animals failed to adapt to their corresponding photoperiod and gained weight independently of the light exposure [40]. Additionally, some strains of Fischer 344 rats show a stronger photoperiodic response than others [64]. Adipocyte area was well associated with the expression of all genes analysed in the RWAT and BAT, with the exception of *Pparα* and *Prdm16* in the BAT. Comparatively, CAF fed animals changed the gene expression profile of RWAT. Thus, while in the STD group, all genes analysed in RWAT and BAT, with the exception of *Pparα*, were positively associated with the LD photoperiod; in the CAF fed rats, RWAT genes related to lipogenesis (*Fasn* and *Acacα*), adipogenesis (*C/ebpα*), and lipolysis (*Hsl*) were more associated with the SD photoperiod, which points towards the effect of the diet on the photoperiod. Moreover, in the CAF fed

animals, fat (%) and lean mass (%) were negatively associated and representative of the LD and SD photoperiods, respectively, even though, as already mentioned, no differences were observed when directly comparing these data among the groups.

In conclusion, we demonstrate that the photoperiod has an evident influence on the adipose tissue and that these effects are different depending on the diet. Our data indicate that the photoperiod may affect the adipose tissue of STD fed rats, moderating lipid acquisition during the SD compared to the LD photoperiod. Our data also support the hypothesis that an increase in caloric consumption due to a highly palatable diet could alter the influence of the photoperiod over the adipose tissue. This investigation shows a more in-depth study of the WAT and BAT that could be useful for future studies related to the circannual clock and the effects of diet over the seasonal changes in the adipose tissue.

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Table 1. Biometric measures, food intake and serum parameters of Fischer 344 rats fed with a standard or cafeteria diet.

	STD		CAF	
	LD	SD	LD	SD
Weight (gr)	386.5 ± 12.66	370.33 ± 10.99	411 ± 7.77	407.1 ± 12.03
Accumulated caloric intake (Kcal)	504.79 ± 11.67	507.07 ± 0.43	1384.21 ± 38.91	1298.52 ± 48.45
Fat (gr)	55.64 ± 4.41	45.06 ± 1.29 #	89.51 ± 3.8	85.84 ± 3.27
Lean (gr)	309.74 ± 8.96	295.76 ± 8.19	291.95 ± 5.14	294.5 ± 8.32
Fat (%)	14.38 ± 0.75	12.52 ± 0.34 #	22.03 ± 0.63	21.53 ± 0.79
Lean (%)	80.71 ± 0.67	80.94 ± 1.04	72.07 ± 0.54	73.68 ± 0.76 #
BAT (gr)	0.35 ± 0.04	0.47 ± 0.16	0.521 ± 0.019	0.581 ± 0.025 #
BAT (%)	0.092 ± 0.01	0.086 ± 0.017	0.127 ± 0.005	0.142 ± 0.005 *
TAG (mg/dl)	142.06 ± 7.74	197.90 ± 14.45 *	437.02 ± 30.47	444.19 ± 42.34

Measures of Fischer 344 rats fed a standard (STD) or cafeteria (CAF) diet in a long (LD) and short (SD) day photoperiods in two independent experiments. BAT, inguinal brown adipose tissue; TAG, triglycerides. Data are presented as the mean ± SEM and both groups in the STD or CAF are compared with Student's T-test (*p<0.05; # p<0.1).

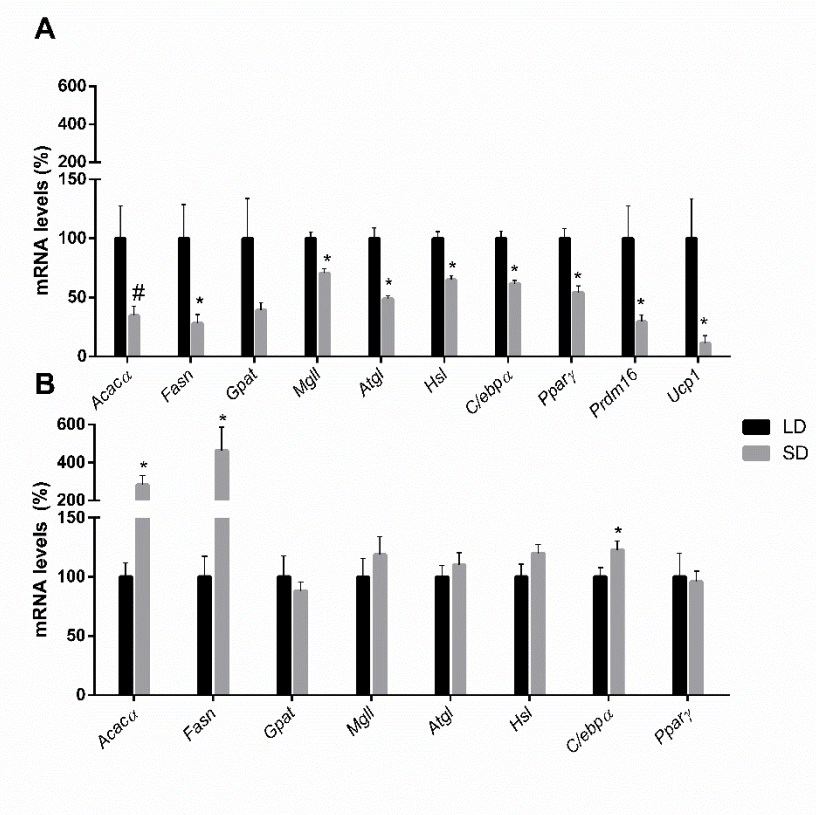


Figure 1. mRNA expression levels in RWAT of Fischer 344 rats treated with long day (LD) and short day (SD) photoperiods fed either with STD (A) or CAF (B) diets. Expression of genes related with lipogenesis, lipolysis, adipogenesis and thermogenesis. Data are presented as the ratios of gene expression, relative to *Actb*, *Ppia* and *Hprt* and expressed as a percentage of the SD group, set at 100%. The results are presented as the mean ± SEM, and the data are compared with Student's T-test (* $p<0.05$; # $p<0.1$).

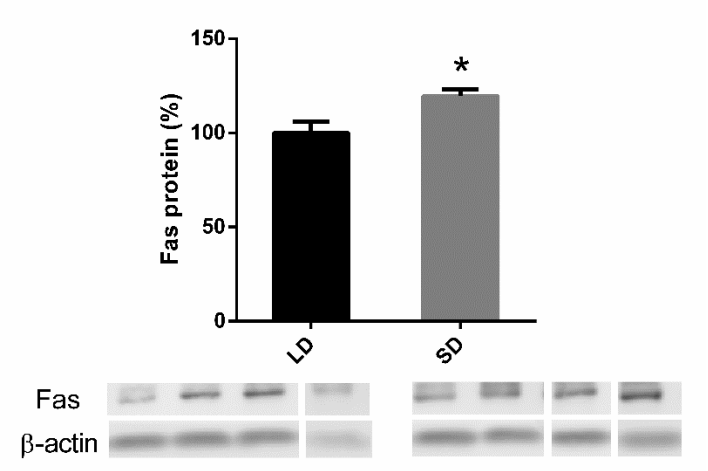


Figure 2. Fasn protein levels in RWAT measured by Western blotting of rats treated with a short day (SD) or long day (LD) photoperiods fed a CAF diet. Data are normalized to Actb and relativized to the SD group, set at 100%. The results are presented as the mean ± SEM, and the data are compared with Student's T-test (* $p<0.05$).

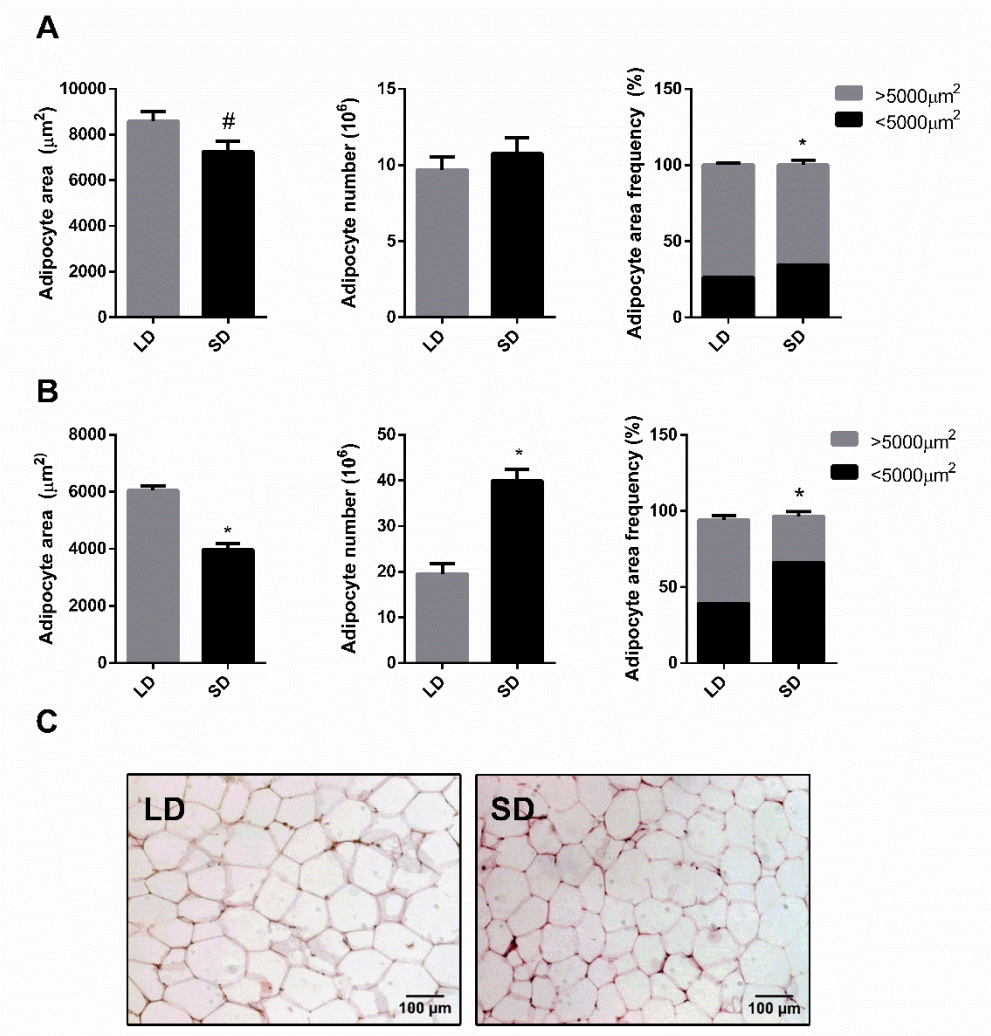


Figure 3. Adipocyte area, adipocyte number and adipocyte area frequencies of RWAT of rats treated with a short day (SD) or long day (LD) photoperiods fed either with an STD (A) or a CAF (B). Representative pictures of both CAF groups are shown (C). For frequencies, adipocytes were distributed in 2 groups depending on their areas (<5000 or >5000 µm^2). Data are presented as the mean± SEM, and statistical significance is analysed via Student's T-test (* $p<0.05$; # $p<0.1$).

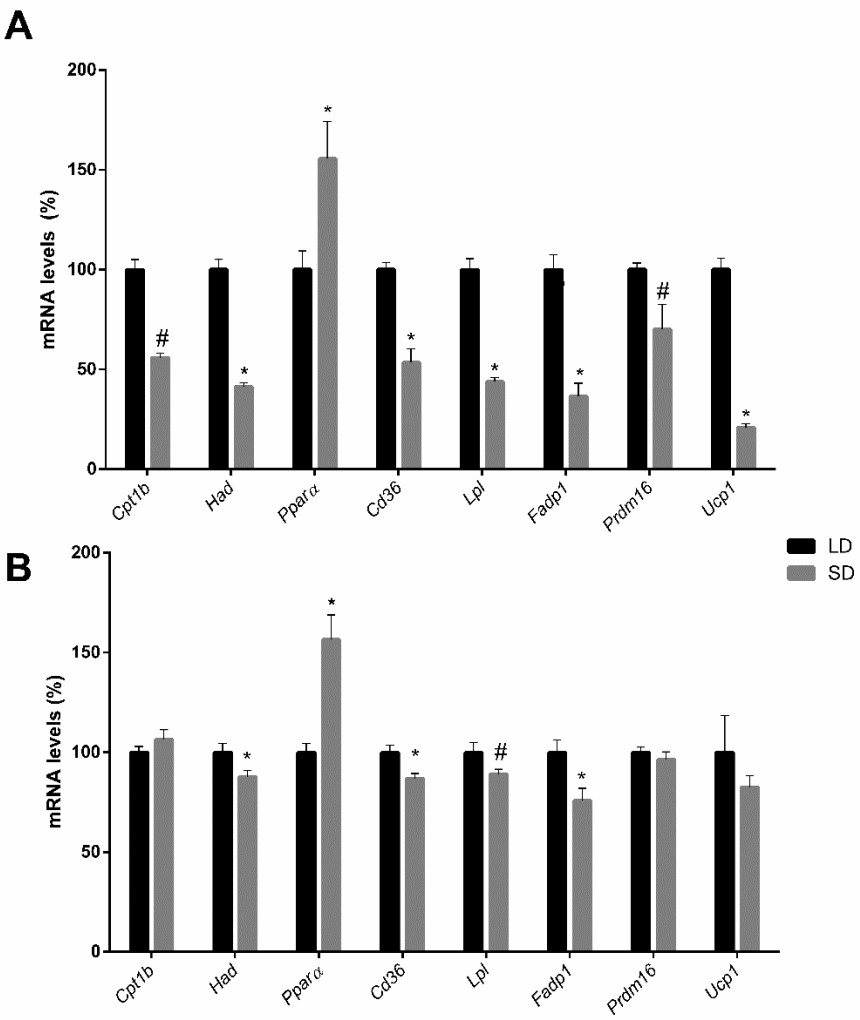


Figure 4. mRNA expression levels in BAT of genes related with β-oxidation, lipid uptake and thermogenesis in rats treated with short day (SD) or long day (LD) photoperiods fed either an STD (A) or a CAF (B) diet. Data are presented as the ratios of gene expression relative to *Actb*, *Ppia* and *Hprt* and expressed as a percentage of the LD group, set at 100%. The results are presented as the mean ± SEM, and the data are compared with Student's T-test (* $p<0.05$; # $p<0.1$).

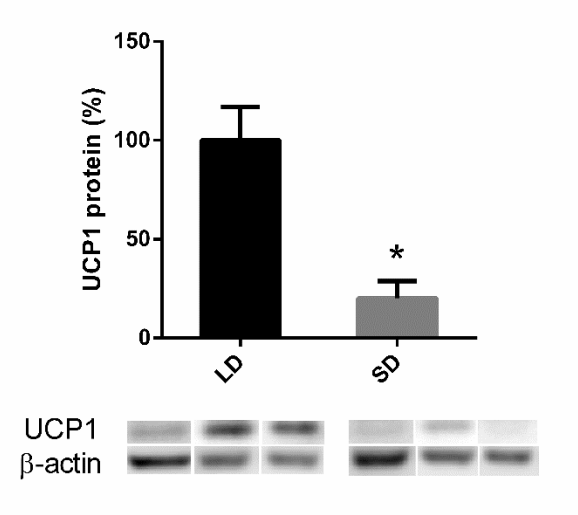
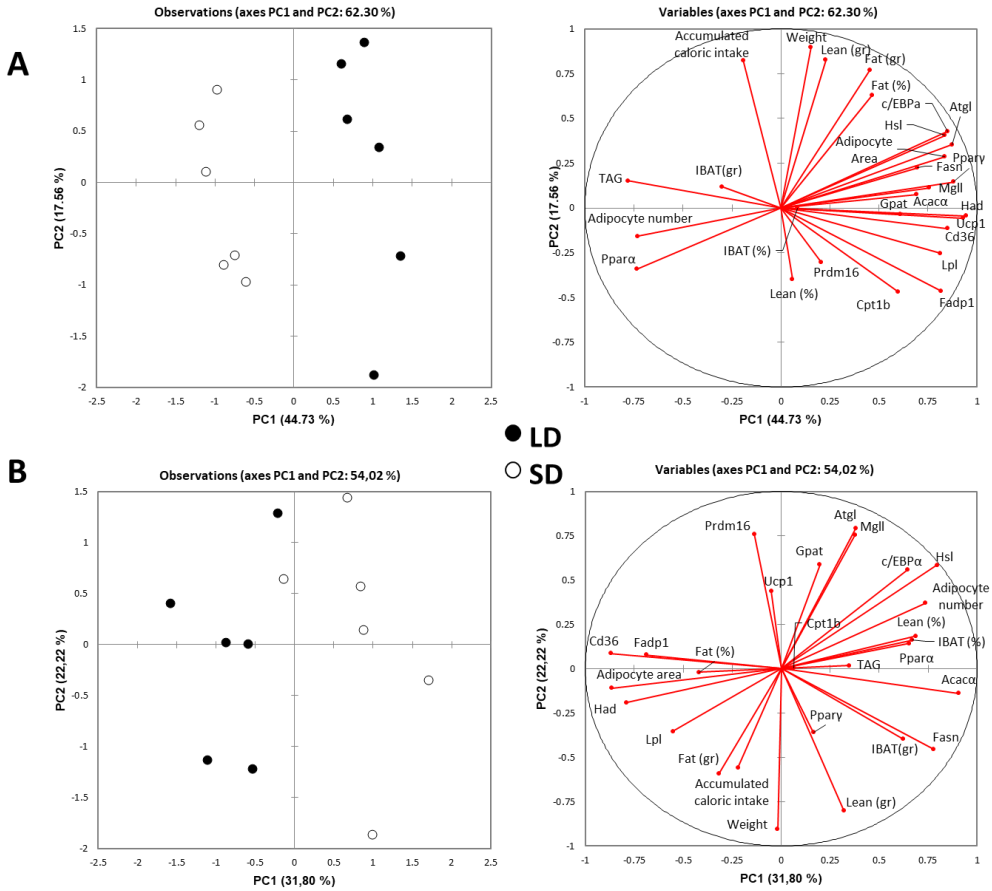


Figure 5. UCP1 protein levels in BAT measured by Western blotting of rats treated with a short day (SD) or long day (LD) photoperiods fed an STD. Data are normalized to Actb and relativized to the SD group, set at 100%. The results are presented as the mean ± SEM, and the data are compared with Student's T-test (* $p<0.05$).

Supplemental Data

Supplemental Table 1. Primers for the Q-PCR analysis.		
	Forward (5'...3')	**Reverse (5'...3')**
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG
Actb	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC
Ppia	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG
Acacα	GCGGCTCTGGAGGTATATGT	TCTGTTTAGCGTGGGGATGT
Atgl	GAAGACCCTGCCTGCTGATT	CACATAGCGCACCCCTTGAA
Fasn	TAAGCGGTCTGGAAAGCTGA	CACCAGTGTTTGTTCCTCGG
Gpat	GAATACAGCCTTGGCCGATG	GAGGCGTGCATGAATAGCAA
Hsl	AGTTCCCTCTTTACGGGTGG	GCTTGGGGTCAGAGGTTAGT
Prdm16	GTTCTGCGTGGATGCCAATC	TGGCGAGGTTTTGGTCATCA
Cebpα	TGTACTGTATGTCGCCAGCC	TGGTTTAGCATAGACGCGCA
Mgl	ATCATCCCCGAGTCAGGACA	TGACTCCCCTAGACCACGAG
Ucp1	GGTACCCACATCAGGCAACA	TCTGCTAGGCAGGCAGAAAC
Lpl	GGCCCAGCAACATTATCCAG	ACTCAAAGTTAGGCCCAGCT
Had	ATCGTGAACCGTCTCTTGGT	AGGACTGGGCTGAAATAAGG
Cpt1b	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG
Pparα	CGGCGTTGAAAACAAGGAGG	TTGGGTTCCATGATGTCGCA
Fatp1	CTACCACTCAGCAGGGAACA	GCGGCATATTTCACCGATGT
Cd36	CAGTGCAGAAACAGTGGTTGTCT	TGACATTTGCAGGTCCATCTATG
Pparγ	AGGGCGATCTTGACAGGAAA	CGAAACTGGCACCCTTGAAA



Supplemental figure 1. PCA score plot (left) and projection of the variables evaluated (right) in the plane defined by the two first PCs, of rats held in a long (LD) or short-day (SD) photoperiod, fed either a STD (A) or CAF (B) diet.

MANUSCRIPT 2

Consumption of out-of-season orange modulates fat accumulation, morphology and gene expression in the adipose tissue of Fischer 344 rats

Albert Gibert-Ramos[a], Hector Palacios-Jordan[a], M. Josepa Salvadó[a], Anna Crescenti[b]

[a]Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, Tarragona, Spain

[b]Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain

Corresponding authors: albert.gibert@urv.cat, Albert Gibert Ramos; mariajosepa.salvado@urv.cat. M. Josepa Salvadó. Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Campus Sescelades, Building N4, Marcel·lí Domingo 1, 43007, Tarragona, Spain. Tel. +34 977 55 84 65

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Abstract

Purpose: According to the xenohormesis theory, animals receive signals from plants that give clues about the changing environment, and thus, depending on the season of the year, animals develop physiological changes to adapt in advance of seasonal changes. Our objective was to study how the same fruit cultivated during two different seasons could affect the adipose tissue of rats.

Methods: Thirty-six Fischer 344 rats were acclimated for four weeks to long-day (LD) or short-day (SD) photoperiods. After adaptation, three groups (n=6) from each photoperiod were supplemented either with orange from the northern (ON) or southern (OS) hemispheres harvested the same month or a vehicle (VH) for ten weeks. Biometric measurements, postprandial plasmatic parameters, gene expression of the inguinal white adipose tissue (IWAT) and brown adipose tissue (BAT), and the histology of the IWAT were analysed.

Results: The OSSD group increased its fat content compared to the VHSD, while the ON groups showed no biometric differences. The OS groups were further studied, and the IWAT showed increased levels of *Pparγ* gene expression and a higher percentage of larger adipocytes compared to the VH group. The BAT showed down regulation of *Lpl*, *Cpt1b* and *Pparα* in the OSSD group compared to that in the VHSD group, suggesting an inhibition of BAT activity, however, *Ucp1* gene expression was up regulated.

Conclusions: We observed a different effect from both fruits, with the OS promoting a phenotype prone to fat accumulation when consumed in an SD photoperiod, which might be explained by the xenohormesis theory.

Introduction

The xenohormesis theory posits that plants synthesise small molecules or secondary metabolites when under a mild stress and that these molecules are detected by heterotrophs when ingested, activating a response that allows them to adapt to this new environment in order to survive [1, 2]. For example, this can be activating energy accumulation when food is scarce or triggering reproductive changes during good weather [3, 4]. This molecular signature can provide information about the season of the year and can be defined by different factors that modify the secondary metabolite content, including the polyphenol content of fruits, such as temperature, sun-light, and access to water [5–8].

Evolution has made animals sensitive to seasons, i.e., predictable events that change the environment in a way that affects their survival. Pre-adaptation to the coming season is achieved via the molecular clock. This mechanism generates circadian rhythms and synchronizes the external light-dark cycle with many processes using an autoregulatory feedback loop that has a periodicity of 24 h and is essential for the sleep-wake cycle of animals [9]. The central clock is the master regulator of this process, and it is found in the suprachiasmatic nucleus (SCN) of the hypothalamus and is principally entrained by ambient light [10]. Moreover, the SCN signals peripheral tissues with hormones, the autonomic nervous system and behavioural pathways, which entrain the peripheral clocks [11]. However, it has been reported that these peripheral clocks can also be synchronized independently from the central clock by food intake [12]. The disruption of the molecular clock can result in several health problems such as obesity or metabolic syndrome [13]. For example, artificial lightning has been proposed as a disruptor of circadian synchrony through misaligning the photoperiod cycle [14]. Additionally, people who usually work a night shift have been found to have a higher occurrence of metabolic syndrome [15, 16]. Furthermore, even though an excess of energy intake is marked as the principal contributor to an increase in fat accumulation in obesity, some authors disagree as to whether this is the main driver in obesity and whether other environmental factors such as climate, artificial light and day length could be involved [17, 18].

The increasing international exchange of goods allows populations to buy food from distant countries. This way, we are able to eat seasonal fruits all year long, independent of the season of the consumer or at a lower economical cost. For example, in 2015, 31.3% of citrus fruits that entered the European Union market were from South Africa and an additional 11.2% came from Argentina [19], which are both southern hemisphere countries.

Considering all of the evidence above together with the xenohormesis theory raises the question of whether the consumption of fruit coming from a different season from the current one of the consumer could somehow have a metabolic effect on an organism by giving erroneous signals of the photoperiod. The objective of the study was to test the hypothesis of whether the season of harvest for oranges could have a different effect on rats adipose tissue and metabolism, depending on the photoperiod of consumption. With this aim in mind, we selected two sweet oranges of the navelina variety from both hemispheres and harvested the same month, which were expected to have different effects on rat adipose tissue due to differences in the farming season and the photoperiod of consumption.

Methods

Animals and Treatments

The animals used were thirty-six two-month-old male Fischer 344/IcoCrl rats (Charles River Laboratories, Barcelona, Spain) fed with a standard chow diet (Panlab, Barcelona, Spain). The animals were housed singly at 22ºC and 55% humidity with free access to food and water. The animals were randomly distributed into six groups (n=6) depending on the treatment received and the photoperiod to which they were exposed.

Animals were acclimatised to two photoperiods with different light:dark cycles, long day (LD; 18 h light: 6 h dark) and short day (SD; 6 h light: 18 h dark) for four weeks. After the adaptation period to each of the photoperiods (SD and LD), three groups of animals were treated daily with 100 mg/kg body weight lyophilized sweet orange (O) (*Citrus x sinsensis*) from the northern hemisphere (ON) or from the southern hemisphere (OS) or with 20 mg/kg body weight of the vehicle (VH) for 10 weeks. The VH treatment (1:1, glucose: fructose solution) was used to match the sugar consumption of those receiving the orange treatments. Accordingly, the six animal groups of the study were: ONSD (Orange North Hemisphere Short Day), OSSD (Orange South Hemisphere Short Day), VHSD (Vehicle Short Day), ONLD (Orange North Hemisphere Long Day), OSLD (Orange South Hemisphere Long Day), and VHLD (Vehicle Long Day). Both oranges were of the navelina variety and were harvested during the same month. ON was cultivated in Spain and OS was cultivated in Argentina. Both fruits were bought from a local hypermarket, frozen in liquid nitrogen, ground with a blender and freeze dried with a liophyliser. Afterwards, the powder was stored at room temperature and protected from light until use.

The body weight and food intake for each animal were recorded every week. One week prior to sacrifice, the fat mass and lean mass were analysed by quantitative magnetic resonance using an EchoMRI-700™ (Echo Medical Systems, LLC., TX, USA) without anaesthesia. After 10 weeks of treatment, animals were sacrificed in the fed state by decapitation, and blood was collected from the neck, stored at room temperature for 45 min and then centrifuged at 1200 g for 10 min to collect the serum. Different white adipose tissue (WAT) depots - epididymal (EWAT), retroperitoneal (RWAT), inguinal (IWAT) and mesenteric (MWAT) – as well as interscapular brown adipose tissue (BAT) were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80º until further analysis. Adiposity was determined by an adiposity index computed for each rat as the sum of EWAT, IWAT, MWAT and RWAT deposit weight and expressed as a percentage of total body weight.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures, and the guidelines for the use and care of laboratory animals of the university were followed.

Polyphenol content of the orange lyophilizates

Methanol extracts of the lyophilizates were prepared following the method of *Duda et al*. [20]. First, 40 mg of the lyophilizates was diluted in 2 ml of 80% (v/v) methanol and mixed for 2 h. The solution was filtered and centrifuged for 10 min (1467 x g. 20ºC), and the supernatant was collected and stored at -20ºC until the total polyphenol content was determined. Following the method adapted from *Nenadis et al.,* we evaluated the total phenolic content of the lyophilizates using a standard curve created with gallic acid and expressed as mg gallic acid x 100 g^{-1} lyophilizate. Briefly, 500 µl of H_2O and 50 µl of sample or standard were mixed and 50 µl of Folin-Ciocalteu's reagent was added. After 3 min, 1 mL of 25% Na_2CO_3 (w/v) was added, and the solution was incubated for 1 h in dark. Finally, the absorbance of each tube was measured at 725 nm.

A higher polyphenol content was found in the oranges from the southern hemisphere, which showed 0.92 ± 0.01 total polyphenols in mg gallic acid × 100 mg^{-1} lyophilizate. Oranges from the northern hemisphere had 0.71 ± 0.01 total polyphenols in mg gallic acid × 100 mg^{-1} lyophilizate.

Plasma analysis

Enzymatic colorimetric kits were used for the determination of plasma glucose, triglyceride, cholesterol (QCA, Barcelona, Spain) and non-esterified free fatty acid (NEFA) (WAKO, Neuss, Germany) levels. Insulin and leptin levels were quantified with a rat-specific enzyme immunoassay kit (Millipore, Madrid, Spain).

RNA extraction and quantification by real-time qRT-PCR

Total RNA from IWAT and BAT tissues was extracted using Trizol® reagent (Thermo Fisher, Madrid, Spain) following the manufacturer's instructions. RNA yield was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE, USA), and the integrity of the RNA was confirmed using agarose gel electrophoresis.

Then, 0.5 µg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) in a Multigene Thermal Cycler (Labnet, Madrid, Spain), and for Q-PCR, the CFX96 real-time system C1000 Touch Thermal

Cycler (Bio-Rad, Barcelona, Spain) with the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Barcelona, Spain) was used. All Q-PCR were was performed with the following cycling conditions after an initial Taq activation at 95ºC for 30 s: 39 cycles of 95ºC for 5 s and 60ºC for 30 s. A melt curve was produced after the previous steps by increasing the temperature from 65ºC to 95ºC by 0.5ºC every 5 s. Gene expression levels in IWAT tissue were analysed for the acetyl-CoA carboxylase alpha (*Acacα*), fatty acid synthase (*Fasn*), glycerol-3-phosphate acyltransferase (*Gpat*), monoglyceride lipase (*Mgll*), adipose triglyceride lipase (A*tgl*), hormone sensitive lipase (*Hsl*), CCAAT/enhancer binding protein alpha (*CEBP*α) and peroxisome proliferator-activated receptor gamma (*Pparγ*) genes. In BAT tissue, we measured the gene expression levels for the cluster of differentiation 36 (*Cd36*), fatty acid transport protein 1 (*Fatp1*), lipoprotein lipase (*Lpl*), carnitine palmitoyltransferase 1B (*CPT1b*), hydroxyacil-CoA dehydrogenase (*Had*) and peroxisome proliferator activated receptor alpha (*Pparα*) genes. Furthermore, we measured the gene expression levels for PR domain containing 16 (*Prdm16*) and uncoupling protein 1 (*Ucp1*) in both tissues. The primers for the different genes are described in Supplementary Table 1 (Online resource 1) and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA was calculated as a percentage of the vehicle group using the $2^{-\Delta\Delta Ct}$ method [21] with *Ppia*, *Actb* and *Hprt1* as reference genes. Each PCR was performed at least in duplicate.

Western Blot

The Ucp1 protein content in BAT was determined by Western blot. Tissues were homogenized in RIPA (Radio-Immunoprecipitation Assay lysis buffer), and the protein was extracted and stored at -20ºC. The protein content was quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA) following manufacturer's instructions.

First, 15 µg of protein in Laemmli loading buffer was denatured, loaded onto 10% acrylamide gels made with TGX™ Fast Cast™ Acrylamide Solutions (Bio-Rad, Barcelona, Spain) and run at 90 V for 75 min. Gels were then transferred onto a PVDF membrane using the Trans-Blot Transfer System (Bio-Rad, Barcelona, Spain) with Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad, Barcelona, Spain) following the manufacturer's instructions. The membrane was blocked and then incubated with anti-Ucp1 antibody (Abcam, Cambridge, United Kingdom) at 4ºC overnight. Afterwards, the membrane was incubated for 2 h with the secondary antibody (GE Health Care Life Sciences, Barcelona), and the protein was detected with the chemiluminescent reagent ECL Select Western Blotting Detection Reagent (GE Healthcare, Barcelona, Spain). Protein levels were quantified with the open source software ImageJ [22] and normalized to β-actin protein levels.

Histology

For histological analyses, frozen IWAT samples were thawed and fixed in 4 % formaldehyde. The tissue underwent successive dehydration and paraffin infiltration-immersion (Citadel 2000, HistoStar, Thermo Scientific, Madrid, Spain), and the paraffin blocks were cut into 2-µm-thick sections using a microtome (Microm HM 355S, Thermo Scientific). The sections were then subjected to automated haematoxylin - eosin staining (Varistain Gemini, Shandom, Thermo Scientific) [23].

Sections were observed and acquired at x10 magnification using AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L., Madrid, Spain). The area and number of adipocytes were measured using the open source software Adiposoft (CIMA, University of Navarra, Spain). Four fields per sample and 6 samples from each group were measured. The area was calculated from the average value of the area in all measured fields for each group. The total adipocyte number was calculated using the formula: $(\frac{\pi}{6})\ x\ (3\sigma^2\ x\ \bar{d} + \bar{d}^3)$, where $\bar{d}$ is the mean diameter and σ is the standard deviation of the diameter, to obtain an average adipocyte volume [24]. Afterwards, we converted this value to the average adipocyte weight using the adipocyte density (0.92 g/ml), and to obtain the total adipocyte number, the weight of the IWAT deposit was divided by the average adipocyte weight, as proposed by *Lemonnier* [25]. Frequencies of adipocytes were obtained by distributing cells into 2 groups according to their area (<5000 µm^2 or >5000 µm^2) and calculating the percentage relative to the total number of counted cells.

Statistical Analysis

The software IBM SPSS (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Data are expressed as the mean ± SEM, and significant differences were analysed by one-way ANOVA, followed by Duncan's multiple range test with post hoc comparison between all groups, comparing separately both oranges with the vehicle. mRNA expression levels in BAT were analysed using Student's t-test. A p value ≤ 0.05 was considered statistically significant.

Results

Biometric and plasma parameters

In reference to the consumption of orange from the southern hemisphere, the OSSD group showed a significantly increased fat (gr, %) content compared to that of the VHSD group. We observed a similar effect on the adiposity index; i.e., the OSSD group showed a higher adiposity index than the VHSD group; although the differences between the groups were not significant (p=0.10). No other differences were observed among the other parameters (Table 1). Furthermore, the consumption of orange from the northern hemisphere showed no significant differences for any parameters between the orange and vehicle-treated groups in any of the photoperiods (Table 2). Neither of the parameters in serum showed significant differences between the orange and vehicle-treated groups for any of the oranges studied in any of the photoperiods. No significant differences were found in the body weight and food intake between the orange and vehicle-treated groups for any of the oranges studied in any of the photoperiods (Table 1 and Table 2).

Gene Expression in IWAT tissue

We decided to analyse the gene expression levels of genes related to adiposity and thermogenesis in the IWAT deposits of the VH and OS groups despite the few changes observed in their biometric parameters. In this sense, it should be taken into account that we observed a clear effect from OS consumption on fat weight in the SD photoperiod. Furthermore, other studies have demonstrated that, even if no differences in weight between groups are found, differences in other parameters in the tissue can occur [26, 27]. Additionally, it has been described that IWAT deposits in adipose tissue have higher levels of browning compared to those of other WAT deposits [28, 29].

PPARγ mRNA expression in OSSD rats was significantly higher than that of the VHSD rats. No other differences were observed in any other gene (Table 3).

Histology of IWAT

The OSSD group showed significantly higher levels of larger adipocytes (>5000 μm^2) than the VHSD rats, while no differences in adipocyte frequencies were observed between the LD groups. (Fig. 1).

Adipocyte area and total number were not significantly different among groups.

Gene Expression in BAT tissue

Due to the effect of OS consumption on fat weight in the SD photoperiod, we analysed the expression of several genes related to BAT activity in this photoperiod. *Lpl* mRNA expression in OSSD rats was significantly down regulated compared to that in VHSD rats. Concerning β-oxidation, the gene expression levels of *Cpt1b* and *Pparα* were significantly down regulated and those of *Ucp1* were highly up regulated in the OSSD rats compared to those in the VHSD rats (Table 4).

UCP1 protein levels on BAT

No significant differences between the OSSD and VHSD groups were observed in the BAT UCP1 protein levels (Fig. 2).

Discussion

In the globalized world, it is increasingly common to purchase consumables from distant countries. This practise has extended to food and, in particular, to fruits and vegetables due to more economical costs or preferences. This phenomenon has permitted the population to consume fruits during what is called “out of season”, which means consuming a fruit from a season that does not correspond to the current season of the consumer. According to the xenohormesis theory [1, 2], heterotrophs can recognize phenolic compounds and other chemical cues from autotrophs, which modify their molecular signatures depending on the environment, signalling animals about different stresses and conditions in the habitat, which can change their strategy for survival in order to adapt to the changing environment. This means that the consumption of fruit from a specific season or photoperiod could have different effects on the metabolism of different tissues, for example adipose tissue, depending on the season of consumption. Based on this theory, we performed this study to investigate how the same species of orange grown during two different seasons can affect adipose tissue physiology and metabolism depending on the photoperiod of consumption.

In the present study, the consumption of orange from the southern hemisphere by SD rats increased the body fat mass of rats compared to that of the control group in the same photoperiod, while it had no effect on the LD animals. These results seem to indicate that the consumption of orange harvested in the spring has the capacity to alter fat mass when consumed during a short photoperiod. These changes were dependent on the photoperiod because only the animals that ate orange in the short day had their parameters altered, but it was also dependent on the season in which the fruit was harvested because no differences were obtained with the orange from the northern hemisphere. Numerous studies in small rodents have shown that a long photoperiod increases fat accumulation or body weight, while the opposite was observed for a short photoperiod [30–34]. Our data show a similar effect on OSSD animals, which are in a short photoperiod but consuming fruit harvested in the spring. These results are in accordance with our hypothesis, suggesting that rats consuming orange out of season in a short photoperiod receive signals that a long photoperiod is approaching, meaning that they will adapt to the coming environment and adopt an advantageous phenotype, which translates into increased energy storage.

The reason why rodents increase their body mass in an LD photoperiod is not fully understood. Some authors report an increase in calorie intake or a change in diet preferences with longer photoperiods [31, 32], while other studies attribute the changes to a decrease in BAT activity [35] or to activation of WAT lipolysis and browning during the SD

[33]. In our study, the possibility of an increase in the fat content of the OSSD group due to diet was discarded because no significant changes were observed in food intake or plasmatic parameters. For this reason, the gene expression profiles of the IWAT and BAT were studied. We chose IWAT because it has been reported to present higher levels of browning than other typically studied white deposits [28, 29]. Moreover, even though no biometrical differences were detected, differences in gene expression or histology can still occur, as other authors have reported [26, 27]. Interestingly, in our study, the gene expression levels of PPARγ in IWAT tissue increased in the OSSD group compared to that in the control and VHSD groups, which would contribute to the differences detected in body fat mass. Even though no differences were detected in the other genes analysed, the PPARγ gene is known to activate the differentiation of preadipocytes into mature adipocytes, adipocyte lipid metabolism and the recruitment of bone marrow-derived circulating progenitor cells to WAT [36–38]. Therefore, this gene has a potential role in the activation of metabolic pathways related to lipid and fat accumulation in adipose tissue. The increased lipogenic capacity of the IWAT in the OSSD group was supported by the histology outcomes since the OSSD animals had a higher number of larger adipocytes than the control group. These results are important and relevant since other authors have suggested that there is a relationship between adipocyte size and the secretion of proinflammatory adipokines in subcutaneous fat deposits in healthy humans [39, 40], increasing the expression of proinflammatory adipokines with the size of the adipocytes. In fact, it is agreed that the size and number of white adipocytes is related to a metabolically healthy/unhealthy pattern. Thus, an increase in adipocyte size is related to insulin resistance and type II diabetes [41–43], while smaller adipocytes in obese patients are correlated with a healthier metabolic profile [44]. Furthermore, it is well known that chronic inflammation of adipose tissue is well correlated with metabolic syndrome and obesity [45, 46]. Thus, although the rats in our study did not show any signs of obesity, the possibility of a higher risk of obesity should be further studied. Overall, our results indicate that the consumption of oranges harvested in the spring in an SD increases fat accumulation and adipogenesis compared to the control group in the same photoperiod, and the different effects on the tissue depend on the photoperiod.

As explained before, we hypothesized that BAT, an organ with high oxidative activity that uses fatty acids as fuel [47–50], can be down regulated by consumption of orange from the southern hemisphere during an SD, decreasing lipid catabolism. Therefore, with the purpose of measuring this possible effect, we analysed the expression of genes related to thermogenesis, lipid uptake and beta-oxidation. *Lpl*, a gene implicated in lipid uptake, was down regulated in the OSSD group compared to that in the VHSD group. Accordingly, OSSD

animals also presented lower levels of *Cpt1b*, which controls the incorporation of fatty acids into the mitochondria for entry into the β-oxidation pathway [51]. These results indicate that less substrate is assimilated into the adipocytes, and thus, less fat is burned [48]. The same effect was observed for *Pparα* gene expression, a nuclear receptor found in BAT that induces several other genes for thermogenesis and lipolysis [52]. These effects indicate a down regulation of BAT catabolic activity, which could contribute to the fat accretion observed in the adipose tissue. According to our hypothesis, rats consuming OS receive signals that the days are lengthening, and thus, OS consumption could activate a combination of fat accumulation signals, preparing animals for a long photoperiod. Koojiman *et al*. [35] demonstrated that the inactivation of BAT in rats in an LD photoperiod was responsible for the increase in body fat mass and decrease in triglyceride uptake in BAT, which supports the changes that we have observed. Surprisingly, we observed a higher *Ucp1* expression level in the OSSD group than the control. These results were unexpected because BAT has been reported to uptake plasma triglycerides and to metabolize lipids as fuel for thermogenesis [48, 53, 54], so we would expect to have a similar gene expression profile as the other lipid uptake and β-oxidation genes. Thus, we assumed that *Ucp1* would be down regulated by OS in a SD. However, Western blotting did not confirm the influence of OSSD on *Ucp1*; so even though *Ucp1* expression in the OSSD group was up regulated, the protein levels were non-correlative. *Takahashi et al.*[55] demonstrated in mice fed a high-fat diet that UCP1 can be highly regulated post-transcriptionally, and other authors have reported several miRNA with suppression capabilities for BAT translation and/or metabolism [56, 57], which could be the reason for our divergent results between *Ucp1* gene expression and protein content in the OSSD group.

In conclusion, in our study we obtained evidence that the consumption of navelina orange harvested in the spring from the southern hemisphere increases the fat content of rats held in a short photoperiod (which represents the fall season), increasing lipogenesis, the percentage of big adipocytes and down regulating BAT lipid uptake and β-oxidation gene expression. We speculate that these effects are produced by the polyphenol content and/or composition of the fruit, which according to our hypothesis and the xenohormesis theory, has a different molecular signature depending on the season of growth, creating a physiological response in the rats. In our case, the OS would signal the advance of a long photoperiod, which translates to an increase in fat accumulation via a decrease in BAT activity, allowing rats to adapt beforehand to the new setting. These findings provide a new vision of diet and its influence on the organism, adding food origin and season to the numerous factors that must be taken into account in health and dietary recommendations to

prevent obesity and the metabolic syndrome. However, it must be taken into account that this is an exploratory study and more evidence is needed. Future studies should focus on other fruit, how the environment specifically affects the polyphenol content in fruits and how this directly modifies animal physiology.

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Table 1. Biometric and plasmatic measures of rats supplemented with orange lyophilizate from the southern hemisphere or vehicle in long day and short day photoperiods.

	VHLD	OSLD	VHSD	OSSD
Weight (gr)	386.5 ± 12.66	383.6 ± 4.41	370.33 ± 10.99	378.33 ± 10.9
Accumulated caloric intake (Kcal)	504.79 ± 11.67	484.27 ± 8.35	507.07 ± 0.43	496.93 ± 11.98
Fat (gr)	55.64 ± 4.41^{a}	54.78 ± 2.06^{a}	45.06 ± 1.29^{b}	53.56 ± 2.28^{a}
Lean (gr)	309.74 ± 8.96	298.7 ± 9.1	295.76 ± 8.19	299.24 ± 9.91
Fat (%)	14.38 ± 0.75^{a}	14.79 ± 0.56^{a}	12.52 ± 0.34^{b}	14.18 ± 0.41^{a}
Lean (%)	80.71 ± 0.67	80.44 ± 0.5	80.94 ± 1.04	79.22 ± 0.32
BAT (gr)	0.35 ± 0.04	0.36 ± 0.01	0.47 ± 0.16	0.4 ± 0.1
EWAT (gr)	12.02 ± 0.94^{a}	11.76 ± 0.4^{ab}	9.65 ± 0.67^{c}	9.8 ± 0.37^{bc}
MWAT (gr)	8.04 ± 0.85	7.85 ± 0.44	6.53 ± 0.82	6.75 ± 0.26
IWAT (gr)	5.82 ± 0.73	4.92 ± 0.57	4.52 ± 0.53	5.88 ± 0.77
RWAT (gr)	10 ± 0.67^{ab}	10.4 ± 0.45^{a}	8.52 ± 0.58^{b}	9.17 ± 0.48^{ab}
Adiposity Index (%)	9.22 ± 0.49^{a}	9.42 ± 0.17^{a}	7.72 ± 0.47^{b}	8.37 ± 0.38^{ab}
BAT (%)	0.092 ± 0.01	0.097 ± 0.004	0.086 ± 0.017	0.105 ± 0.023
EWAT (%)	3.09 ± 0.17^{a}	3.14 ± 0.12^{a}	2.6 ± 0.14^{b}	2.59 ± 0.07^{b}
MWAT (%)	2.06 ± 0.17	2.12 ± 0.13	1.74 ± 0.16	1.78 ± 0.05
IWAT (%)	1.5 ± 0.15	1.37 ± 0.14	1.25 ± 0.14	1.57 ± 0.22
RWAT (%)	2.58 ± 0.11^{ab}	2.79 ± 0.1^{a}	2.41 ± 0.05^{b}	2.43 ± 0.12^{b}
Glucose (mmol/L)	8.65 ± 0.47	8.16 ± 0.12	7.83 ± 0.2	8.66 ± 0.16
Triglycerides (mg/dl)	142.06 ± 7.74	152.61 ± 9.49	197.9 ± 14.45	174.57 ± 8.56
Cholesterol (mmol/L)	4.73 ± 0.07	4.26 ± 0.19	4.06 ± 0.33	4.14 ± 0.17
NEFA (mg/dl)	22.49 ± 3.21	21.09 ± 0.59	23.34 ± 1.74	25.01 ± 1.42
Insulin (ng/ml)	5.54 ± 0.73	5.22 ± 0.68	4.04 ± 0.66	4.39 ± 0.63
Leptin (ng/ml)	18.56 ± 0.31	19.93 ± 0.98	16.59 ± 1.49	17.38 ± 1.22

Fischer 344 rats supplemented with orange lyophilizate from the southern hemisphere (OS) or vehicle (VH) in long day (LD) and short day (SD) photoperiods. The adiposity index was computed as the sum of EWAT, MWAT, IWAT and RWAT deposit weights and expressed as a percentage of total body weight. BAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue; MWAT, mesenteric white adipose tissue; IWAT, inguinal white adipose tissue; RWAT, retroperitoneal white adipose tissue. Data are presented as the mean ± SEM and the four groups were compared by one-way ANOVA ($p < 0.05$) followed by Duncan's new multiple range (MRT) post hoc test.

Table 2. Biometric and plasmatic measures of rats supplemented with orange lyophilizate from the northern hemisphere or vehicle in long and short day photoperiods.

	VHLD	ONLD	VHSD	ONSD
Weight (gr)	386.5 ± 12.66	380.33 ± 11.33	370.33 ± 10.99	359,83 ± 8,68
Accumulated caloric intake (Kcal)	504.79 ± 11.67	480.64 ± 5.88	507.07 ± 0.43	492.15 ± 0,28
Fat (gr)	55.64 ± 4.41	54.14 ± 3.67	45.06 ± 1.29	49,74 ± 4,01
Lean (gr)	309.74 ± 8.96	305,24 ± 8,52	295.76 ± 8.19	284,49 ± 9,44
Fat (%)	14.38 ± 0.75	14,25 ± 0,8	12.52 ± 0.34	13,9 ± 1,19
Lean (%)	80.71 ± 0.67	80,54 ± 0,78	80.94 ± 1.04	79,26 ± 1
BAT (gr)	0.35 ± 0.04	0,29 ± 0,038	0.47 ± 0.16	0,395 ± 0,13
EWAT (gr)	12.02 ± 0.94	11,98 ± 0,89	9.65 ± 0.67	9,53 ± 0,93
MWAT (gr)	8.04 ± 0.85	7.92 ± 0.62	6.53 ± 0.82	5.95 ± 0.62
IWAT (gr)	5.82 ± 0.73	5.62 ± 0.63	4.52 ± 0.53	5.09 ± 0.6
RWAT (gr)	10 ± 0.67[a]	9.9 ± 0.36[a]	8.52 ± 0.58[b]	8.93 ± 0.6[ab]
Adiposity Index (%)	9.22 ± 0.49	9.29 ± 0.42	7.72 ± 0.47	8.21 ± 0.71
BAT (%)	0.092 ± 0.01	0.077 ± 0.011	0.086 ± 0.017	0.111 ± 0.036
EWAT (%)	3.09 ± 0.17	3.13 ± 0.16	2.6 ± 0.14	2.65 ± 0.26
MWAT (%)	2.06 ± 0.17	2,08 ± 0,15	1.74 ± 0.16	1.66 ± 0.19
IWAT (%)	1.5 ± 0.15	1.48 ± 0.16	1.25 ± 0.14	1.41 ± 0.16
RWAT (%)	2.58 ± 0.11	2.6 ± 0.05	2.41 ± 0.05	2.49 ± 0.18
Glucose (mmol/L)	8.65 ± 0.47	8.21 ± 0.15	7.83 ± 0.2	8.18 ± 0.39
Triglycerides (mg/dl)	142.06 ± 7.74	135.62 ± 8.41	197.9 ± 14.45	165.92 ± 19.86
Cholesterol (mmol/L)	4.73 ± 0.07	4.27 ± 0.09	4.06 ± 0.33	4.16 ± 0.25
NEFA (mg/dl)	22.49 ± 3.21	20.86 ± 2.91	23.34 ± 1.74	25.74 ± 2.25
Insulin (ng/ml)	5.54 ± 0.73	5.65 ± 0.08	4.04 ± 0.66	4.16 ± 0.32
Leptin (ng/ml)	18.56 ± 0.31	18.79 ± 1.52	16.59 ± 1.49	18 ± 2.75

Fischer 344 rats supplemented with orange lyophilizate from the northern hemisphere (ON) or vehicle (VH) in long day (LD) and short day (SD) photoperiods. The adiposity index was computed as the sum of EWAT, MWAT, IWAT and RWAT deposit weights and expressed as a percentage of total body weight. BAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue; MWAT, mesenteric white adipose tissue; IWAT, inguinal white adipose tissue; RWAT, retroperitoneal white adipose tissue. Data are presented as the mean ± SEM and the four groups were compared by one-way ANOVA ($p < 0.05$) followed by Duncan's new multiple range (MRT) post hoc test.

Table 3. mRNA expression levels in IWAT of rats supplemented with orange lyophilizate from the southern hemisphere (OS) or vehicle on long and short-day photoperiods.

	VHLD	OSLD	VHSD	OSSD
Acacα	122.3 6± 16.76	128.23 ± 2.73	100 ± 27.29	95.81 ± 7.78
Fasn	111.18 ± 30.69	122.82 ± 54.01	100 ± 13.85	107.21 ± 39.12
Gpat	133.31 ± 16.34	172.39 ± 20.4	100 ± 31.87	133.38 ± 47.1
Mgll	150.95 ± 25.2	191.08 ± 36.21	100 ± 37.81	158.04 ± 74.66
Atgl	172.23 ± 26.87^{ab}	207.27 ± 32.01^{a}	100 ± 35.57^{b}	109.88 ± 38.05^{ab}
Hsl	136.89 ± 18.55	167.51 ± 27.71	100 ± 28.92	108.53 ± 38.08
C/ebpα	121.23 ± 16.4	167.75 ± 27.44	100 ± 26.55	99.93 ± 37.61
Pparγ	123.91 ± 3.45^{ab}	128.55 ± 7.39^{a}	100 ± 11.46^{b}	129.23 ± 11.07^{a}
Prdm16	111.92 ± 21.46	118.25 ± 10.27	100 ± 16.65	91.53 ± 25.77
Ucp1	102.2 ± 16.47	192.46 ± 63.84	100 ± 29.72	121.71 ± 21.79

Expression of genes related with lipogenesis, lipolysis, adipogenesis and thermogenesis in Fischer 344 rats supplemented with orange lyophilizate from the southern hemisphere (OS) or vehicle (VH) held in a long day (LD) and short day (SD) photoperiods. Data is presented as the ratios of gene expression, relative to β-actin, ppia and hprt and expressed as a percentage of the VHSD group, set at 100%. Results are presented as the mean ± SEM and data compared by one-way ANOVA ($p< 0.05$) followed by Duncan's new multiple range (MRT) post hoc test.

Table 4. mRNA expression levels in BAT of rats supplemented with orange lyophilizate from the southern hemisphere or vehicle in a short day photoperiod.

	VHSD	**OSSD**
Cd36	100 ± 10	88.72 ± 10.87
Fatp1	100 ± 19.16	79.45 ± 8.76
Lpl	100 ± 6.68	59.21 ± 13.69*
Cpt1b	100 ± 10.81	57.47 ± 10.03*
Had	100 ± 5.58	85.89 ± 13.97
Pparα	100 ± 10.79	54.81 ± 6.73*
Ucp1	100 ± 5.98	210.43 ± 35.59*
Prdm16	100 ± 13.77	104.74 ± 20.49

Expression of genes in BAT related with β-oxidation, lipid uptake and thermogenesis in Fischer 344 rats supplemented with orange lyophilizate from the southern hemisphere (OS) or vehicle (VH) held in a short day (SD) photoperiod. Data are presented as the ratios of gene expression relative to *Actb*, *Ppia* and *Hprt*, and expressed as a percentage of the of the VHSD group, set at 100%. Results are presented as the mean ± SEM and data compared by Students T-test ($p<0.05$).

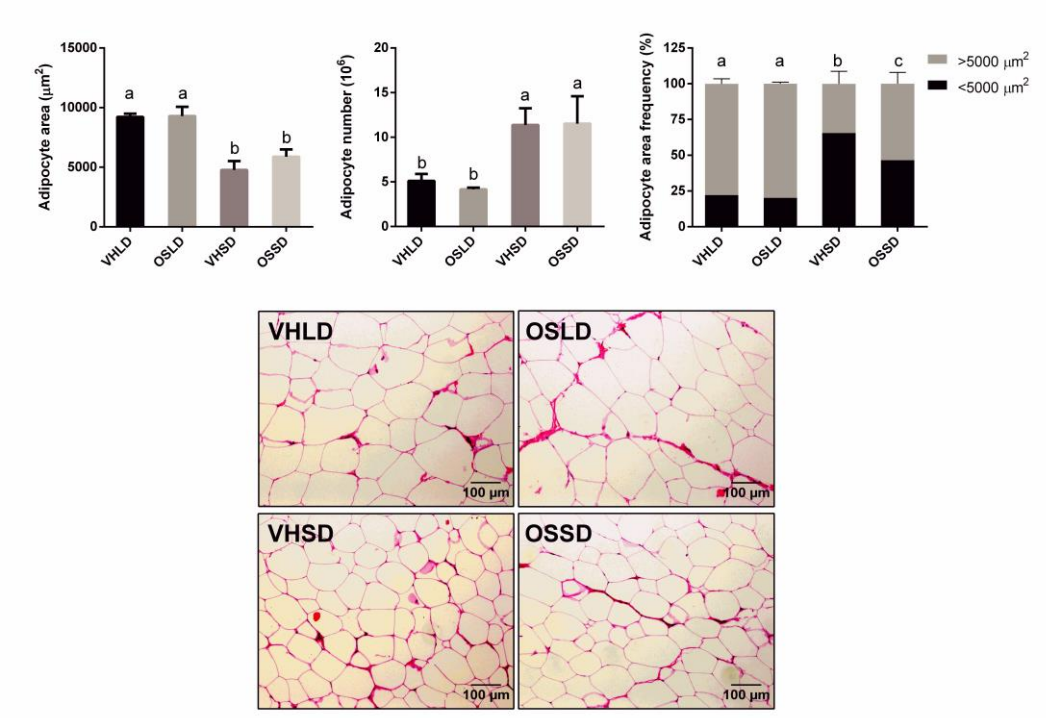

Fig. 1 Cell number, cell area and adipocyte frequencies of IWAT of Fischer 344 rats supplemented with orange lyophilizate from the southern hemisphere (OS) in short day (SD) or long day (LD) photoperiods. For frequencies, adipocytes were distributed in 2 groups according to their area (<5000 or >5000 µm^2). Data are presented as the mean ± SEM and statistical significance analysed by one-way ANOVA ($p < 0.05$) followed by Duncan's new multiple range (MRT) post hoc test.

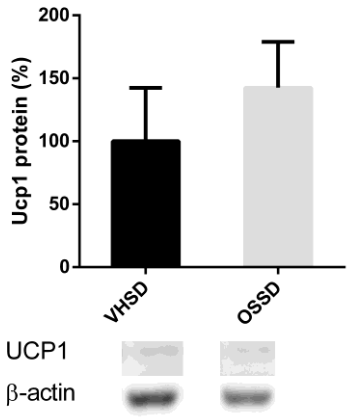

Fig. 2 UCP1 protein levels in BAT measured by western blotting of Fischer 344 rats supplemented with orange lyophilizate from the southern hemisphere (OS) or vehicle (VH) held in a short day photoperiod (SD). Data are normalized to β-actin and to the VHSD group, set at 100%. Results are presented as the mean ± SEM and data compared with Students T-test ($p < 0.05$).

Supplemental Data

Supplementary table 1. Primers for the Q-PCR analysis.		
	Forward (5'...3')	**Reverse (5'...3')**
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG
Actb	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC
Ppia	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG
Acacα	GCGGCTCTGGAGGTATATGT	TCTGTTTAGCGTGGGGATGT
Atgl	GAAGACCCTGCCTGCTGATT	CACATAGCGCACCCCTTGAA
Fasn	TAAGCGGTCTGGAAAGCTGA	CACCAGTGTTTGTTCCTCGG
Gpat	GAATACAGCCTTGGCCGATG	GAGGCGTGCATGAATAGCAA
Hsl	AGTTCCCTCTTTACGGGTGG	GCTTGGGGTCAGAGGTTAGT
Prdm16	GTTCTGCGTGGATGCCAATC	TGGCGAGGTTTTGGTCATCA
Cebpα	TGTACTGTATGTCGCCAGCC	TGGTTTAGCATAGACGCGCA
Mgl	ATCATCCCCGAGTCAGGACA	TGACTCCCCTAGACCACGAG
Ucp1	GGTACCCACATCAGGCAACA	TCTGCTAGGCAGGCAGAAAC
Lpl	GGCCCAGCAACATTATCCAG	ACTCAAAGTTAGGCCCAGCT
Had	ATCGTGAACCGTCTCTTGGT	AGGACTGGGCTGAAATAAGG
Cpt1b	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG
Pparα	CGGCGTTGAAAACAAGGAGG	TTGGGTTCCATGATGTCGCA
Fatp1	CTACCACTCAGCAGGGAACA	GCGGCATATTTCACCGATGT
Cd36	CAGTGCAGAAACAGTGGTTGTCT	TGACATTTGCAGGTCCATCTATG
Pparγ	AGGGCGATCTTGACAGGAAA	CGAAACTGGCACCCTTGAAA

MANUSCRIPT 3

Consumption of Cherry out of Season Changes White Adipose Tissue Gene Expression and Morphology to a Phenotype Prone to Fat Accumulation

Albert Gibert-Ramos[a], Anna Crescenti[b], M. Josepa Salvadó[a],

[a]Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, Tarragona, Spain

[b]Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain

Corresponding authors: albert.gibert@urv.cat (A.G.-R.); anna.crescenti@eurecat.org (A.C.); Tel.: +34-977558465 (A.G.-R.); +34-9777529-65 (A.C.)

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Consumption of Cherry out of Season Changes White Adipose Tissue Gene Expression and Morphology to a Phenotype Prone to Fat Accumulation

Albert Gibert-Ramos [1,*], Anna Crescenti [2,*] and M. Josepa Salvadó [1]

[1] Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili (URV), Tarragona 43007, Spain; mariajosepa.salvado@urv.cat

[2] Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus 43204, Spain

* Correspondence: albert.gibert@urv.cat (A.G.-R.); anna.crescenti@eurecat.org (A.C.); Tel.: +34-977558465 (A.G.-R.); +34-9777529-65 (A.C.)

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Abstract: The aim of this study was to determine whether the consumption of cherry out of its normal harvest photoperiod affects adipose tissue, increasing the risk of obesity. Fischer 344 rats were held over a long day (LD) or a short day (SD), fed a standard diet (STD), and treated with a cherry lyophilizate (CH) or vehicle (VH) (n = 6). Biometric measurements, serum parameters, gene expression in white (RWAT) and brown (BAT) adipose tissues, and RWAT histology were analysed. A second experiment with similar conditions was performed (n = 10) but with a cafeteria diet (CAF). In the STD experiment, Bmal1 and Cry1 were downregulated in the CHSD group compared to the VHSD group. Pparα expression was downregulated while Ucp1 levels were higher in the BAT of the CHSD group compared to the VHSD group. In the CAF-fed rats, glucose and insulin serum levels increased, and the expression levels of lipogenesis and lipolysis genes in RWAT were downregulated, while the adipocyte area increased and the number of adipocytes diminished in the CHSD group compared to the VHSD group. In conclusion, we show that the consumption of cherry out of season influences the metabolism of adipose tissue and promotes fat accumulation when accompanied by an obesogenic diet.

Keywords: photoperiod; seasonality; obesity; cafeteria diet; adipose tissue; fruit consumption; xenohormesis; cherry

1. Introduction

The obesity epidemic has become a worldwide problem over the last few decades caused by factors such as changes in lifestyle, including an increase in highly caloric food intake and a reduction in physical activity. However, scientists now agree that the reason for the rise in the number of obese people is much more complex, and that other factors, such as the duration of sleep at night and control over ambient temperature, may influence these numbers [1,2].

The adipose tissue of seasonal animals has been demonstrated to be directly influenced by the photoperiod or season [3,4]. Humans are also affected by seasonal changes, which affect body fat mass, activity, or the concentration of hormones all year long [5–7]. These changes are principally generated by the molecular clock [8], which is a set of autoregulatory loops generated in the suprachiasmatic nucleus (SCN) that regulates many physiological mechanisms in organisms, synchronizing the metabolism with the light–dark cycles of the environment, which are also known as the photoperiod. This mechanism is especially important in seasonal animals, which adapt in advance to the coming season to increase their chances of survival or reproduction [9,10]. Apart from the central clock in

the SCN, peripheral tissues also present circadian rhythms, which follow the signalling provided by the central clock. In fact, molecular clock gene expression has been reported to follow a coordinated expression in white adipose tissue (WAT) during the day [11], and these expression levels have been reported to change depending on the rat photoperiod [12,13]. Interestingly, the time of food intake and high-fat diets, among other factors, have been shown to disrupt the molecular and peripheral clocks from the light–dark cycle, increasing the risk of obesity and other diseases [14–18]. In this sense, it has been shown that polyphenols, secondary metabolites found in some vegetables and fruits, are capable of entraining the peripheral clocks in lean and obese rats [19].

Related to these secondary metabolites is the xenohormesis theory, which posits that some phytochemicals produced by plants or other autotrophs can have a direct effect on the enzymes and receptors of heterotrophs caused by common evolutionary conserved signalling pathways [20]. These molecules produced by plants have been demonstrated to change depending on the season, the environment, and different stresses [21–23]. Thus, this signalling could be useful for animals, in that they would be able to anticipate large changes in the environment or changing seasons and develop suitable survival adaptations [20]. In this regard, a healthy approach to counteract the obesity epidemic has been to increase the amount of fruits and vegetables in the diet [24]. However, in the globalized world, it is increasingly common to purchase seasonal fruits all year long. For example, cherry is a fruit harvested between spring and summer, but now, due to international trade, it is possible to purchase cherry produced in the other hemisphere during autumn or winter, or it can be grown locally in the natural growing season and then be stored and eaten in another season. All these factors, together with the xenohormesis theory and the disturbances on the molecular clocks described above, give rise to a new question about whether fruit from a determinate season, which contains a particular molecular signature of the environment and season, consumed at another time of the year could affect the molecular clocks and/or increase the risk of obesity.

The hypothesis of our study was that the consumption of fruit from a photoperiod different from the one an animal is acclimated to could send incorrect signals to the animal, which will either develop characteristics of another photoperiod or season or increase the risk of obesity due to desynchronization of the molecular clock. Both WAT and brown adipose tissue (BAT) are central to obesity. WAT relates to obesity mainly due to its specialization in the accumulation of a surplus of energy in the form of fat [25]. BAT is involved in energy expenditure because it expresses uncoupling protein 1 (Ucp1), which is capable of uncoupling the proton gradient from the synthesis of ATP in mitochondria, generating heat in the process [26]. For these reasons, we focused this work on the adipose tissue, and we developed this idea first with animals fed a standard diet (STD) to study how the normal adaptations of Fischer 344 rats to the photoperiod might change when cherry is consumed out of season. Furthermore, we performed a second experiment with cafeteria (CAF)-fed rats, which increases their caloric consumption with a highly palatable diet, in order to study how these changes might be modified in an obesogenic environment.

2. Materials and Methods

2.1. Treatments

Sweet cherry (*Prunus avium* L.) was of the Royal Dawn variety and purchased at Mercabarna (Barcelona, Spain). Cherry fruit was frozen in liquid nitrogen, ground with a blender, and freeze-dried with a lyophilizer. Afterwards, the powder was stored and protected from light at room temperature until use. The phenolic composition of sweet cherries was 171.42 mg/100 g fresh weight of fruit (FW) anthocyanins; 15.07 mg/100 g FW flavanols; and 87.81 mg/100 g FW phenolic acids. A more detailed description of the composition is included in the Supplemental Data. The nutritional (Table S1) and phenolic (Table S2) compositions were obtained from the U.S. Department of Agriculture [27] and Phenol Explorer [28], respectively.

We supplemented rats with an oral dose of 100 mg/kg body weight of cherry lyophilizate (CH), which in a human of 70 kg would be equivalent to 42.6 g of raw cherries or a small portion of fruit, which is below the standard daily recommendation of the World Health Organization (WHO) [29]. According to the WHO [30], the daily sugar intake for humans should not surpass 10% of their total energy intake. For this reason, we selected a dose that, in conjunction with the average daily intake of sugar included in the standard diet, would not surpass the 10% limit indicated by the WHO.

The vehicle (VH) treatment consisted of a 1:1 glucose:fructose solution in water with a concentration that ranged from 20 mg/mL to 33 mg/mL during the experiment depending on the volume to be administered and the rat weight so as not to administer an excessive volume to the animal. This solution was used to match the sugar consumption with that of the cherry (CH) treatment.

2.2. Animal Experimental Procedure

For the first experiment, we used 24 2-month-old male Fischer 344/IcoCrl rats (Charles River Laboratories, Barcelona, Spain) fed with a standard chow diet (STD) (Panlab, Barcelona, Spain) with a caloric distribution (3.2 kcal/g) of 19.3% protein, 8.4% fat, and 72.4% carbohydrates. The animals were housed two per cage at 22 °C and 55% humidity and with free access to food and water. The animals were randomly distributed into four groups (n = 6) depending on the treatment received and the photoperiod to which they were exposed. Animals were acclimated to two photoperiods with different light:dark cycles: long-day (LD, 18 h light:6 h dark) and short-day (SD, 6 h light:18 h dark) over 4 weeks. After the adaptation period, animals in each photoperiod were treated daily with 100 mg/kg body weight of CH or with 20 mg/kg body weight of the VH for 10 weeks. Accordingly, the four animal groups of the study were CHLD (cherry, long day), CHSD (cherry, short day), VHLD (vehicle, long day), and VHSD (vehicle, short day) (Figure 1A).

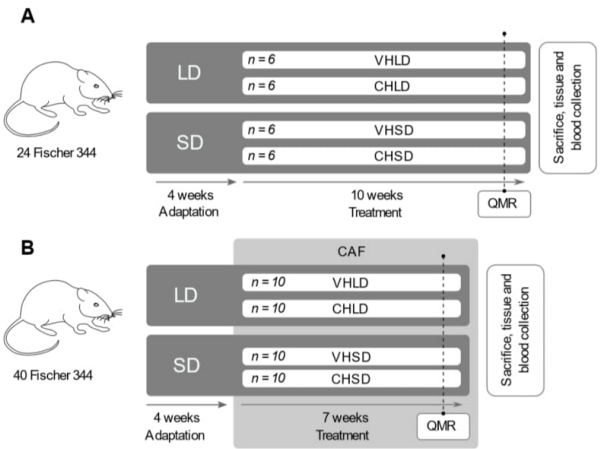

Figure 1. Experimental design of the two studies. For the first experiment (**A**), 24 Fischer 344 rats fed with a standard chow diet were randomly distributed into four groups (n = 6) and acclimated to a long (LD) or a short (SD) photoperiod (18 h light: 6 h dark; 6 h light: 18 h dark, respectively) over 4 weeks. After the adaptation period, animals were treated with 100 mg/kg body weight of cherry lyophilizate (CH) or 20 mg/kg of the vehicle solution (VH) for 10 weeks. One week prior to sacrifice, fat mass and lean mass were analysed by quantitative magnetic resonance (QMR). For the second experiment (**B**), 40 Fischer 344 rats were distributed into four groups (n = 10) and adapted in the same conditions as the first experiment. After the adaptation, animals were fed a cafeteria diet (CAF) and treated daily with the same dose and vehicle of the first experiment for 7 weeks, and 1 week prior to sacrifice, fat mass and lean mass were measured by QMR.

For the second experiment, 40 Fischer 344/IcoCrl rats were fed a CAF diet ad libitum, consisting of bacon (8–12 g), biscuits with pâté (12–15 g) and cheese (10–12 g), muffins (8–10 g), carrots (6–9 g), and sweetened milk (22% sucrose *w*/*v*; 50 mL) in addition to the same standard chow diet of the first experiment [31]. The caloric distribution of the CAF diet (5.28 kcal/g) was 10% protein, 31.9% fat, and 58.1% carbohydrates. The food was freshly provided daily. The nutritional composition of the STD and CAF diets is described in the supplementary material (Table S3). This diet model induces hyperphagia of highly caloric ingredients, which increases fat and sugar ingestion to develop the main features of metabolic syndrome and obesity [32]. Before starting the experiment, the animals were randomly distributed into four groups (n = 10) depending on the treatment received and the photoperiod to which they were exposed. As in the first STD experiment, animals were acclimated to the two photoperiods, LD and SD, for 4 weeks. After the adaptation period, animals in each photoperiod were fed a CAF diet and treated daily with 100 mg/kg body weight of CH or with 20 mg/kg body weight of the VH for seven weeks. Accordingly, as in the first experiment, the four animal groups of the study were CHLD, CHSD, VHLD, and VHSD (Figure 1B).

The body weight and food intake of animals were recorded every week for both experiments. One week prior to sacrifice, the fat mass and lean mass were analysed by quantitative magnetic resonance using an EchoMRI-700™ (Echo Medical Systems, LLC., Houston, TX, USA) without anaesthesia. Animals were then sacrificed in the fed state by decapitation, and blood was collected from the neck, stored at room temperature for 45 min, and then centrifuged at 1200× *g* for 10 min to collect the serum. Different white adipose tissue deposits—epididymal (EWAT), retroperitoneal (RWAT), inguinal (IWAT), and mesenteric (MWAT)—and interscapular BAT were rapidly removed after death, weighed, frozen in liquid nitrogen, and then stored at −80 °C until further analysis. We chose to sacrifice the animals in the fed state because this simulates more precisely the conditions found in humans, who spend the majority of the day in the postprandial state [33,34]. This allows for the study of the energetic metabolism of the adipose tissue, specifically lipid uptake and lipogenesis, as it shows higher activity levels responding to the increased glucose and lipid content in plasma [35,36].

Adiposity was determined by an adiposity index computed for each rat as the sum of the EWAT, IWAT, MWAT, and RWAT deposit weights and expressed as a percentage of total body weight.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures (reference number 4249), and the guidelines for the use and care of laboratory animals of the university were followed.

2.3. Plasma Analysis

Enzymatic colorimetric kits were used for the determination of plasma glucose (Ref. 992320) and triglycerides (Ref. 998282), (QCA, Barcelona, Spain). Insulin (Ref. EZRMI-13K) and leptin (Ref. EZML-82K) levels were quantified with a rat-specific enzyme immunoassay kit (Millipore, Madrid, Spain).

2.4. RNA Extraction and Quantification by Real-Time qRT-PCR

Total RNA from RWAT and BAT tissues was extracted using Trizol® reagent (Ambion, Life Technologies, Uppsala, Sweden) following the manufacturer's instructions. The RNA yield was quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the integrity of the RNA was confirmed using agarose gel electrophoresis.

In brief, 0.5 µg of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) in a Multigene Thermal Cycler (Labnet, Madrid, Spain), and for Q-PCR, the CFX96 real-time system C1000 Touch Thermal Cycler (Bio-Rad, Barcelona, Spain) with the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Barcelona, Spain) was used. All Q-PCRs were performed with the following cycling conditions after an initial Taq activation at 95 °C for 30 s: 39 cycles of 95 °C for 5 s and 60 °C for 30 s. A melt curve was produced after the previous steps by increasing the temperature from 65 °C to 95 °C by 0.5 °C every 5 s. Gene expression levels in

RWAT tissue were determined for the acetyl-CoA carboxylase alpha (*Acacα*), fatty acid synthase (*Fasn*), glycerol-3-phosphate acyltransferase (*Gpat*), monoglyceride lipase (*Mgll*), adipose triglyceride lipase (A*tgl*), hormone-sensitive lipase (*Hsl*), CCAAT/enhancer-binding protein alpha (*C/ebpα*), peroxisome proliferator-activated receptor gamma (*Pparγ*), brain and muscle ARNT-like1 (*Bmal1*), cryptochrome circadian clock 1 (*Cry1*), and period circadian clock 2 (Per2) genes. In BAT tissue, we measured gene expression levels for the cluster of differentiation 36 (*Cd36*), fatty acid transport protein 1 (*Fatp1*), lipoprotein lipase (*Lpl*), carnitine palmitoyltransferase 1B (*CPT1b*), hydroxyacyl-CoA dehydrogenase (*Had*), and peroxisome proliferator-activated receptor alpha (*Pparα*) genes. Furthermore, we measured the gene expression levels of PR domain containing 16 (*Prdm16*) and uncoupling protein 1 (*Ucp1*) in both tissues. The primers for the different genes are described in the Supplemental Data (Table S4) and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA was calculated as a percentage of the vehicle group using the $2^{-\Delta\Delta Ct}$ method [37] with *Ppia*, *Actb*, and *Hprt* as reference genes. Each qRT-PCR was performed at least in duplicate.

2.5. Histology

For histological analyses, frozen RWAT samples were thawed and fixed in 4% formaldehyde. The tissue underwent successive dehydration series and was afterwards embedded in paraffin (Citadel 2000, HistoStar, Thermo Scientific, Madrid, Spain). Paraffin blocks were cut into 2-µm-thick sections using a microtome (Microm HM 355S, Thermo Scientific). The sections were subjected to automated haematoxylin–eosin staining (Varistain Gemini, Shandom, Thermo Scientific) [38].

The sections were observed, and images were acquired at ×10 magnification using the AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L., Madrid, Spain). The area and number of adipocytes were measured using the open source software Adiposoft (CIMA, University of Navarra, Navarra, Spain). Four fields per sample and 6 samples from each group were measured. The area was calculated from the average value of the area in all measured fields for each group. The total adipocyte number was calculated using the formula $(\frac{\pi}{6})\ x\ (3\sigma^2\ x\ \overline{d} + \overline{d}^3)$, where $\overline{d}$ is the mean diameter and σ is the standard deviation of the diameter, to obtain the average adipocyte volume [39]. Afterwards, we converted this value to the average adipocyte weight using the adipocyte density (0.92 g/mL) and, to obtain the total adipocyte number, the weight of the IWAT deposit was divided by the average adipocyte weight as proposed by *Lemmonier* [40]. Frequencies of adipocytes were obtained by distributing cells into two groups according to their area (<5000 μm^2 or >5000 μm^2) and calculated as a percentage of the total number of counted cells.

2.6. Statistical Analysis

The software IBM SPSS (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM), and significant differences were analysed by a one-way ANOVA test followed by Duncan's new multiple range test for post hoc comparison between all groups. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Biometric Parameters

In the first experiment with rats fed the STD, there were no significant changes in weight or cumulative caloric intake between groups. Similarly, no changes were detected among the adipose deposits for fat and lean mass or in the plasma parameters between the VH and CH groups (Table 1).

Table 1. Biometric and plasmatic measurements of rats supplemented with cherry or vehicle on long- and short-day photoperiods.

	VHLD	CHLD	VHSD	CHSD
Weight (g)	386.5 ± 12.66	388 ± 5.11	370.33 ± 10.99	376.5 ± 13.58
Accumulated caloric intake (kcal)	504.79 ± 11.67	493.3 ± 10.78	507.07 ± 9.44	498.44 ± 13.38
Fat (g)	55.64 ± 4.41 ^{a}	58.38 ± 3.11 ^{a}	45.06 ± 1.29 ^{b}	50.41 ± 2.82 ^{ab}
Lean (g)	309.74 ± 8.96	307.43 ± 4.59	295.76 ± 8.19	299.16 ± 11.89
Fat (%)	14.38 ± 0.75 ^{ab}	15.17 ± 0.7 ^{a}	12.52 ± 0.34 ^{b}	13.43 ± 0.57 ^{ab}
Lean (%)	80.71 ± 0.67	79.95 ± 0.76	80.94 ± 1.04	79.69 ± 0.62
EWAT (g)	12.02 ± 0.94 ^{ab}	12.99 ± 0.7 ^{a}	9.65 ± 0.67 ^{b}	9.64 ± 0.71 ^{b}
MWAT (g)	8.04 ± 0.85	7.95 ± 0.45	6.53 ± 0.82	6.42 ± 0.65
IWAT (g)	5.82 ± 0.73	6.27 ± 0.3	4.52 ± 0.53	5.66 ± 1.03
RWAT (g)	10 ± 0.67 ^{ab}	10.33 ± 0.29 ^{a}	8.52 ± 0.58 ^{b}	8.71 ± 0.47 ^{ab}
Adiposity Index (%)	9.22 ± 0.49 ^{ab}	9.67 ± 0.34 ^{a}	7.72 ± 0.47 ^{b}	8.05 ± 0.55 ^{b}
EWAT (%)	3.09 ± 0.17 ^{a}	3.34 ± 0.16 ^{a}	2.6 ± 0.14 ^{b}	2.56 ± 0.16 ^{b}
MWAT (%)	2.06 ± 0.17	2.05 ± 0.11	1.74 ± 0.16	1.69 ± 0.13
IWAT (%)	1.5 ± 0.15	1.62 ± 0.08	1.25 ± 0.14	1.49 ± 0.25
RWAT (%)	2.58 ± 0.11 ^{ab}	2.66 ± 0.07 ^{a}	2.41 ± 0.05 ^{ab}	2.31 ± 0.09 ^{b}
Glucose (mmol/L)	136.81 ± 3.48 ^{ab}	133.61 ± 3.49 ^{b}	139.24 ± 3.51 ^{ab}	145.5 ± 2.72 ^{a}
Triglycerides (mg/dL)	142.06 ± 7.74 ^{bc}	120.03 ± 10.22 ^{c}	197.9 ± 14.45 ^{a}	181.22 ± 23.03 ^{ab}
Insulin (ng/mL)	5.54 ± 0.73	6.15 ± 0.96	4.04 ± 0.66	4.03 ± 0.79
Leptin (ng/mL)	18.56 ± 0.31 ^{ab}	22.69 ± 2.19 ^{a}	16.59 ± 1.49 ^{b}	16.21 ± 2.08 ^{b}

Fischer 344 rats supplemented with cherry (CH) lyophilizate or vehicle (VH) on long-day (LD) and short-day (SD) photoperiods. Adiposity index was computed as the sum of epididymal white adipose tissue (EWAT), mesenteric white adipose tissue (MWAT), inguinal white adipose tissue (IWAT), and retroperitoneal white adipose tissue (RWAT) deposit weights and expressed as a percentage of total body weight. BAT, interscapular brown adipose tissue. Data are presented as the mean ± standard error of the mean (SEM) and the four groups were compared with one-way ANOVA ($p < 0.05$) followed by a Duncan's new multiple range test (MRT) post hoc test. ^{abc} Mean values with unlike letters differ significantly among groups.

The second experiment with rats fed the CAF diet also showed no differences in weight between groups. However, the CHLD group showed a significantly lower caloric intake than that of the VHLD group (Table 2). Regarding the weight of the different adipose tissue deposits, no differences were observed between groups. Concerning plasmatic parameters, the CHSD group had significantly higher glucose and insulin serum levels compared to those in the VHSD group (Table 2).

Table 2. Biometric and plasmatic measurements of rats supplemented with cherry or vehicle and a cafeteria diet in long-day and short-day photoperiods.

	VHLD	CHLD	VHSD	CHSD
Weight (g)	411 ± 7.77	422.89 ± 7.14	407.1 ± 12.03	404.3 ± 9.61
Accumulated caloric intake (kcal)	1364.34 ± 37.39 ^{a}	1214.67 ± 58.02 ^{b}	1298.52 ± 48.45 ^{ab}	1198.2 ± 30.34 ^{b}
Fat (g)	89.51 ± 3.8	86.56 ± 2.23	85.84 ± 3.27	88.29 ± 5.31
Lean (g)	291.95 ± 5.14	299.07 ± 5.23	294.5 ± 8.32	296.42 ± 3.82
Fat (%)	22.03 ± 0.63	21.7 ± 0.66	21.53 ± 0.79	21.82 ± 1.04
Lean (%)	72.07 ± 0.54	72.5 ± 0.62	73.68 ± 0.76	72.33 ± 1.07
EWAT (g)	16.23 ± 0.82	15.75 ± 0.53	14.75 ± 0.72	15.61 ± 1.08
MWAT (g)	9.42 ± 0.61	9.25 ± 0.66	8.68 ± 0.47	8.28 ± 0.69
IWAT (g)	11.9 ± 1.39	11 ± 1.02	12.47 ± 1.39	10.39 ± 1.25
RWAT (g)	13.53 ± 0.59	13.41 ± 0.26	12.87 ± 0.54	12.41 ± 0.59
Adiposity Index (%)	12.23 ± 0.54	11.51 ± 0.3	11.95 ± 0.42	11.5 ± 0.58
EWAT (%)	3.93 ± 0.13	3.76 ± 0.09	3.62 ± 0.14	3.84 ± 0.21
MWAT (%)	2.28 ± 0.11	2.18 ± 0.13	2.15 ± 0.14	2.03 ± 0.14
IWAT (%)	2.87 ± 0.31	2.59 ± 0.21	3.02 ± 0.27	2.57 ± 0.31
RWAT (%)	3.28 ± 0.1	3.18 ± 0.06	3.16 ± 0.08	3.14 ± 0.06
Glucose (mmol/L)	182.87 ± 4.65 ^{ab}	180.5 ± 9.23 ^{ab}	164.13 ± 4.91 ^{b}	202.31 ± 15.46 ^{a}
Triglycerides (mg/dL)	437.02 ± 30.47	421.07 ± 12.89	444.19 ± 42.34	449.75 ± 48.06
Insulin (ng/mL)	6.59 ± 0.47 ^{ab}	7.42 ± 0.49 ^{a}	5.82 ± 0.24 ^{b}	7.31 ± 0.59 ^{a}
Leptin (ng/mL)	19.89 ± 0.55	20.08 ± 1.08	21.41 ± 1.33	20.9 ± 0.96

Fischer 344 rats supplemented with cherry (CH) lyophilizate or vehicle (VH) and fed a cafeteria diet in long-day (LD) and short-day (SD) photoperiods. The adiposity index was computed as the sum of EWAT, MWAT, IWAT, and RWAT deposit weights and expressed as a percentage of total body weight. BAT, interscapular brown adipose tissue; EWAT, epididymal white adipose tissue; MWAT, mesenteric white adipose tissue; IWAT, inguinal white adipose tissue; RWAT, retroperitoneal white adipose tissue. Data are presented as the mean ± SEM, and the four groups were compared with a one-way ANOVA test ($p < 0.05$) followed by a Duncan's new multiple range test (MRT) post hoc test. ^{ab} Mean values with unlike letters differ significantly among groups.

3.2. RWAT Gene Expression

In the STD experiment, Ucp1 expression was significantly downregulated in the CHLD group compared to that in the VHLD group. The clock genes, Bmal1 and Cry1, were significantly downregulated in the CHSD group compared to those in the VHSD group. No differences between groups were found among the other analysed genes (Figure 2A).

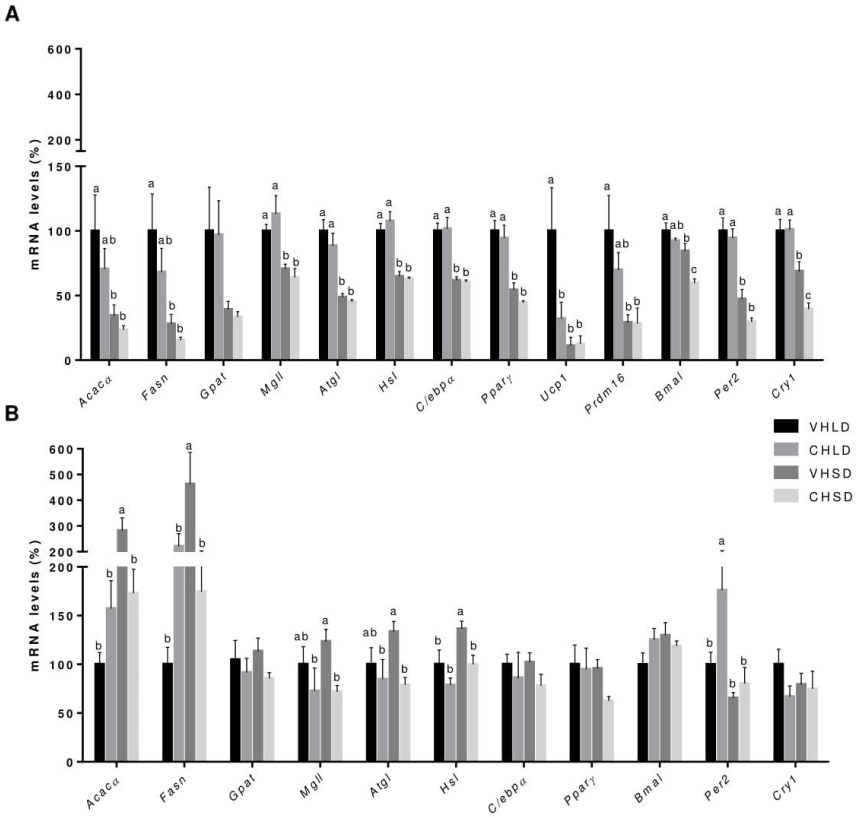

Figure 2. mRNA expression levels in RWAT of Fischer 344 rats supplemented with a cherry (CH) lyophilizate or vehicle (VH), held in long-day (LD) and short-day (SD) photoperiods, and fed either with a standard (STD) (**A**) or a cafeteria (CAF) (**B**) diet in two independent experiments. Expression of genes related to lipogenesis, lipolysis, adipogenesis, thermogenesis, and the molecular clock. Data are presented as the ratios of gene expression relative to *β-actin*, *Ppia*, and *Hprt* genes and expressed as a percentage of the LD group set at 100%. Data are presented as the mean ± SEM, and the four groups were compared with a one-way ANOVA test ($p < 0.05$) followed by a Duncan's new multiple range test (MRT) post hoc test. ^{abc} Mean values with unlike letters differ significantly among groups.

In CAF-fed rats, the expression levels of the genes Acacα and Fasn, which code for lipogenic enzymes, and of the genes *Mgll*, *Atgl*, and *Hsl*, which code for lipolytic enzymes, were significantly decreased in the CHSD group compared to those in the VHSD group. Instead, the transcriptional modulator Per2 was significantly upregulated in the CHLD group compared to that in the VHLD group. The expression levels of *Ucp1* and of *Prdm16*, the transcriptional coregulators that control the development of brown adipocytes, were undetectable on each of the studied groups (Figure 2B).

3.3. RWAT Histology

No differences were found in the morphology of the RWAT between the VH and CH groups of rats fed the STD diet (Figure 3A). However, rats fed the CAF diet and subjected to an SD photoperiod showed a significantly higher adipocyte area and a smaller number of adipocytes in the CHSD group than that in the VHSD group. In addition, the CHSD group showed a significantly increased frequency of larger adipocytes compared to that in the VHSD group (Figure 3B).

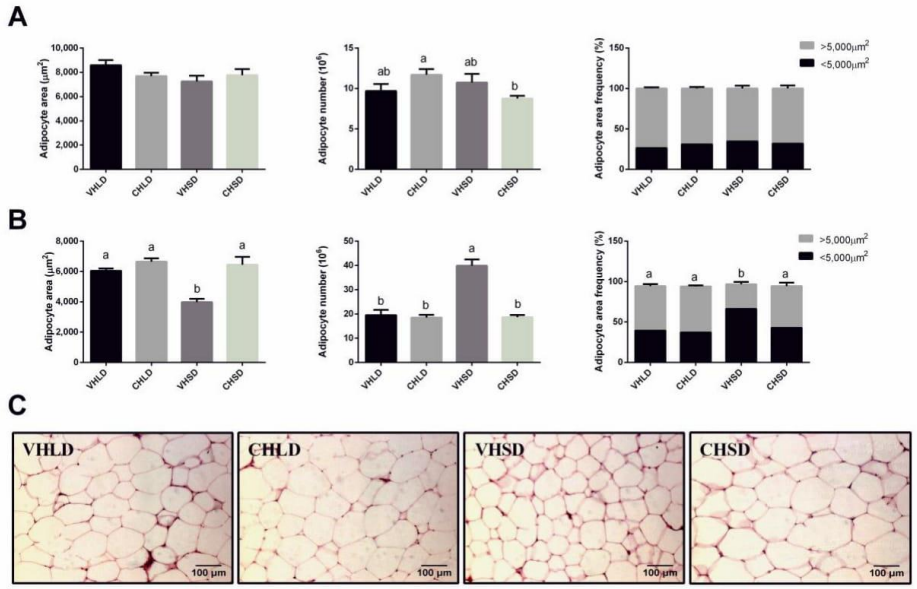

Figure 3. Adipocyte area, adipocyte total number, and adipocyte area frequencies of RWAT of Fischer 344 rats supplemented with a cherry (CH) lyophilizate or vehicle (VH), held in long-day (LD) and short-day (SD) photoperiods, and fed either with an STD (**A**) or a CAF (**B**) diet in two independent experiments. Representative pictures of all the CAF groups are shown (**C**). For frequencies, adipocytes were distributed into two groups depending on their areas (<5000 or >5000 μm^2). Data are presented as the mean ± SEM, and the four groups were compared with a one-way ANOVA test ($p < 0.05$) followed by a Duncan's new multiple range test (MRT) post hoc test. ^{ab} Mean values with unlike letters differ significantly among groups.

3.4. BAT Gene Expression

In the STD experiment, we observed a significant downregulation of the nuclear receptor involved in BAT fatty acid uptake and β-oxidation, *Pparα*, and an upregulation of *Ucp1* in the CHSD group compared to that in the VHSD group (Figure 4A).

In the CAF experiment, differences were found in the fatty acid translocase Cd36, which was significantly downregulated in both CH groups compared to that in the VH groups. *Prdm16* expression showed a statistically significant upregulation in the CHLD group compared to that in the VHLD group (Figure 4B).

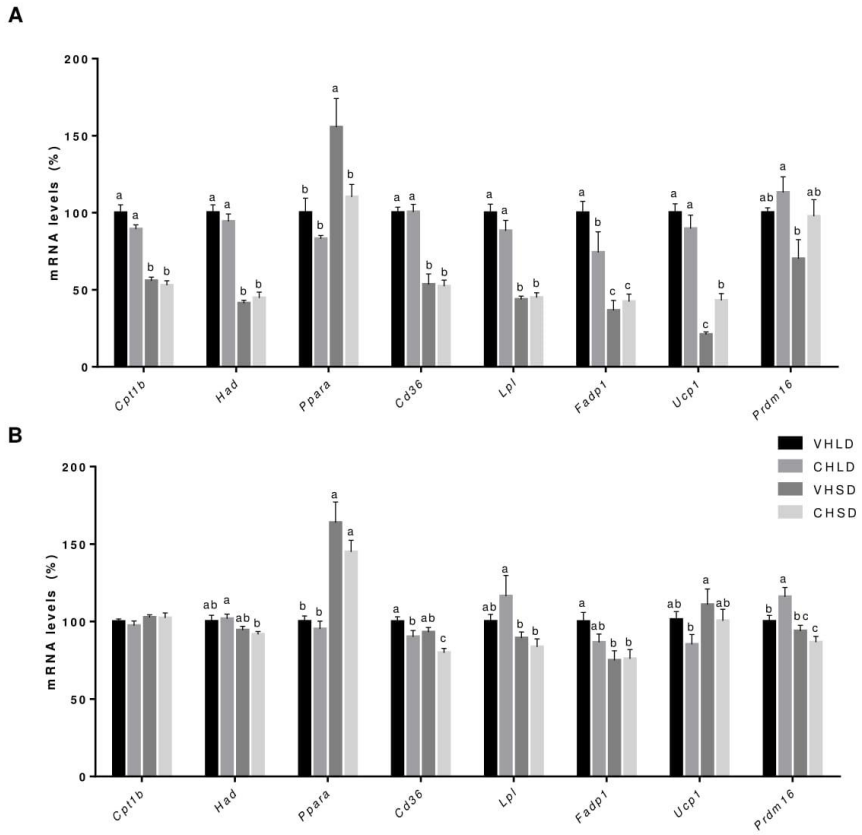

Figure 4. mRNA expression levels in BAT of Fischer 344 rats supplemented with a cherry (CH) lyophilizate or vehicle (VH), held in long-day (LD) and short-day (SD) photoperiods, and fed either with an STD (**A**) or a CAF (**B**) diet in two independent experiments. Expression of genes related to lipogenesis, lipolysis, adipogenesis, and thermogenesis. Data are presented as the ratio of gene expression relative to the *β-actin*, *Ppia*, and H*prt* genes and expressed as a percentage of the LD group set at 100%. Data are presented as the mean ± SEM, and the four groups are compared with a one-way ANOVA test ($p < 0.05$) followed by a Duncan's new multiple range test (MRT) post hoc test. ^{abc} Mean values with unlike letters differ significantly among groups.

4. Discussion

An increasing number of new factors that could contribute to the obesity epidemic are being found. For example, it is known that alterations in the photoperiod, such as those observed in night-shift workers, are well-correlated with an increased risk of obesity [15,41]. In this study, we wanted to focus on a brand-new aspect that could act as a risk factor in the development of obesity, namely the consumption of out-of-season fruit.

As observed in previous results from our group [4] and data from other authors, Fischer 344 rats adapt to changes to the photoperiod for reproductive purposes [42,43], and in an LD they tend to increase body weight by either increasing lean [42] or fat mass [44]. These characteristics are typical of seasonal animals, which detect the changes in light and dark during the seasons and adapt accordingly, generally increasing fat reserves during an LD and depleting them during an SD [3]. Furthermore,

according to the xenohormesis theory, plants synthetize secondary metabolites depending on diverse exogenous factors or stresses, such as water availability, light, and temperature [21–23], so it is understandable that this molecular signature will be different depending on the environment. Moreover, it has been found that these phytochemicals, such as polyphenols, can entrain the molecular clock [19], and thus we hypothesized that they might desynchronise or somehow modify the normal response of animals depending on the photoperiod where they were consumed.

In STD-fed rats, we did not observe biometric changes apart from those between the photoperiod groups, which have been already described by *Gibert-Ramos* et al. [4]. In the RWAT, the CHLD group showed a decrease in the expression level of *Ucp1* compared to that in the VHLD group. *Ucp1* is a good marker of browning of WAT [45], so this downregulation could mean that the ingestion of cherry in an LD photoperiod decreases the thermogenesis in WAT. Even so, these results were not conclusive, since we detected no other changes that would suggest an inhibition of the browning or the thermogenesis in the WAT, such as an increase in adiposity or in adipocyte size, among others. Furthermore, and contrary to what we found, there is evidence in the literature that some polyphenols might promote the activation of BAT [46] and the browning of WAT [47–49]. In fact, vanillic acid, a metabolite of anthocyanins, which are found in cherry, has been reported to promote the browning of the WAT in mice, increasing the gene expression of *Ucp1* and *Prdm16* [50].

In STD-fed animals, CHSD rats had decreased levels of *Pparα* and increased levels of *Ucp1* in their BAT. As we have already discussed in another manuscript [4], we have signs that *Pparα* is not carrying out its function, since we obtained contradictory results compared with other data. *Pparα* is a nuclear receptor that activates the transcription of genes related to fatty acid uptake and β-oxidation [51]. Additionally, it has been found that increased levels of *Pparα* activate *Ucp1* transcription [52], and thus we would expect higher levels in the VHSD group and not in the CHSD group. For these reasons, *Pparα* seems to incorrectly represent the metabolic state of BAT, possibly because of post-transcriptional or post-translational regulation, or because the retinoic X receptor (RXR), a ligand necessary for *Pparα*'s correct functioning, is not present [53]. Furthermore, there is evidence that RXR can control BAT development and activation [54], that RXR is needed for adipogenesis, and that the disruption of the RXR–*Pparα* heterodimer reduces adipocyte formation [55]. These hypotheses are supported by studies that demonstrate that *Pparα* regulates brown adipose tissue thermogenesis, activating *Ucp1* and *Prdm16* gene expression [52], which, in our study, were downregulated in the short-day (SD) groups versus the long-day (LD) groups together with the studied β-oxidation and lipid uptake genes. On the other hand, increased *Ucp1* levels could correspond to what we observed in the LD groups. Cherry is an LD fruit, so its consumption during an SD could erroneously signal the animal, which would develop adaptations typical of an LD.

In the CAF experiment, the ingestion of cherry in the LD showed a reduction in the accumulated caloric intake. Cherry, as with all vegetables and fruits, contains polyphenols [56,57], which have been reported to possess beneficial effects against obesity, including a reduction in food intake in some cases [58]. In order to obtain further information about the possible mechanisms by which CH ingestion could affect the physiology and seasonal adaptations of adipose tissue, it would be appropriate in future studies to characterize the polyphenolic metabolites in serum after its supplementation. The lack of caloric intake reduction in the CHSD group might be related to the consumption of cherry out of season, and thus the expected effects of cherry were not observed. Additionally, these changes were only observed in the CAF experiment and not the STD, probably because of their increased caloric intake, which makes changes more easily detected, and because of the pro-obesogenic environment of a highly palatable diet, which changes the whole system of food intake regulation in rats [59].

The CHSD group consumed cherry out of season, and according to our hypothesis, we would expect metabolic changes disassociated from the short photoperiod. These changes were observed first in the plasmatic parameters of CAF-fed rats, where insulin and glucose levels were significantly higher than those in the VHSD group. This increase in insulin circulation levels in a postprandial state is a response to increased glucose concentration, which is generally determined by the diet. However,

in our study, no differences in caloric intake between the SD groups nor in carbohydrate consumption (data not shown) were found, so other mechanisms, including nutrient absorption in the intestine or metabolization, should be considered. Moreover, insulin levels in obese rats are higher than those in lean rats [60], and postprandial hyperglycaemia has been reported to be a predictor of diabetes [61], which suggests an increased predisposition of CHSD rats to obesity or a decreased sensitivity to insulin. Interestingly, CHSD rats showed an increase in adipocyte area compared to that in the VHSD group, while the total adipocyte number was decreased. Adipocyte hypertrophy has also been linked to type II diabetes and insulin resistance [62,63] and thus appears to indicate a higher predisposition of the LD groups and CHSD groups to these diseases when fed a CAF diet.

CH consumption also altered gene expression in the RWAT in the SD groups. While the VHSD group had higher expression levels of lipogenesis genes (*Acacα* and *Fasn)* than the LD groups, consumption of CH downregulated these genes, achieving similar levels to those of the LD groups. A similar effect was observed in *Mgll*, *Atgl*, and *Hsl*, which were downregulated in the CHSD group. *Auguet* et al. found that in morbidly obese patients, the fatty acid absorption and transport in the visceral WAT were downregulated [64]. Additionally, the authors also found a decrease in the gene expression levels of *Acacα* and *Fasn*, as in our study, and a decrease in their protein levels [64]. Other studies report that adipocyte size is directly implicated in the disruption of the adipocyte functionality of obese individuals [65]. Specifically, studies have reported that hypertrophic adipocytes in the WAT suffer a dysregulation of its ability to store and mobilize lipids, which decreases the gene expression of key genes of lipogenesis and lipolysis as we observed in our study [66,67].

Considering that a CAF modifies the normal effect of the photoperiod, as observed previously with the VH groups [4], the ingestion of CH, a fruit from an LD, appears to affect somehow the photoperiodic effect of the SD in the metabolism of the adipose tissue. Altogether, data in the CAF animals appear to suggest that rats consuming cherry in an SD develop a small degree of insulin resistance that might, or might not, be related to the similar changes observed in the LD groups. This change is further observed in the BAT, where the expression of *Cd36*, a key transporter of fatty acid and lipoprotein into the cell [68], was also downregulated in accordance with what we observed in the WAT. On the other hand, *Cd36* was also downregulated in the BAT of CHLD rats and thus might indicate a direct effect of cherry on the expression levels of this gene.

According to our hypothesis, the observed changes in our study could be promoted by a desynchronization of the peripheral clocks in the CHSD group. For this reason, we quantified the gene expression levels of three key genes implicated in the autoregulatory loop of the molecular clock—*Bmal1*, *Per2*, and *Cry1* [9]—which have been found to follow a coordinated expression in the adipose tissue during the day [11]. In our study, the STD-fed animals showed a clearly different pattern of expression between the LD and SD groups, which should be taken as the standard pattern of expression. However, this pattern was altered in the CHSD group of STD rats, which showed lower expression levels of *Bmal1* and *Cry1* compared to those of the other three groups, indicating a dysregulation of the clock machinery in the WAT produced by the CH consumption out of season. Nevertheless, these changes were not translated into any biometrical or physiological effect in the parameters we analysed.

On the other hand, CAF-fed rats showed no differences in the gene expression of the molecular clock between the VH groups. To our knowledge, this is the first time that a CAF diet was used to study the gene expression of the molecular clock in rats, and thus, as already discussed in [4], we should take into account that the CAF might be playing a desynchronizing role. This high-fat diet model was composed of highly palatable ingredients that increase food consumption in comparison to that of a chow diet [31], and it has been shown that feeding time and diet can disrupt the circadian system, with consequences for the development of obesity [17,18,69–71]. For these reasons, we believe that the CAF is desynchronizing the expression of *Bmal1*, *Per2*, and *Cry1*, so we are unable to appreciate the differences between the VHLD and VHSD groups. Concerning CH consumption, we observed a significant upregulation of *Per2* in the CHLD group compared to that in the VHLD and SD groups.

However, none of the other two clock genes analysed showed significant changes, and thus we are unsure about how this result could have a significant effect on the other parameters analysed. To obtain more robust evidence of changes in the clock machinery in WAT, future studies should focus on obtaining data at different time points in order to compare the oscillations of expression levels of the clock genes during the day between groups [11].

5. Conclusions

In conclusion, we show that the consumption of cherry, a fruit that is harvested during spring or summer, and so, from a long-day season, affects the metabolism of the adipose tissue of Fischer 344 rats differently depending on the photoperiod in which it is consumed, affecting the physiology of the adipose tissue to one more prone to fat accumulation when consumed out of season. This study shows evidence about how fruit origin and seasonality might play a role in the risk of developing obesity in humans. Although more evidence is required, the results of this study could be useful for the development of obesity prevention diets.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/8/1102/s1, Table S1: Nutritional composition of sweet cherry, Table S2: Phenolic composition of sweet cherry, Table S3: Nutritional composition of the standard and cafeteria diets, Table S4: Primers for the Q-PCR analysis.

Author Contributions: Conceptualization, A.G.-R., A.C., and M.J.S.; Data curation, A.G.-R.; Formal analysis, A.G.-R.; Investigation, A.G.-R.; Project administration, A.C. and M.J.S.; Supervision, A.C. and M.J.S.; Visualization, A.G.-R.; Writing (original draft), A.G.-R.; Writing (review and editing), A.G.-R., A.C., and M.J.S.

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Supplemental Data

Supplemental Table 1. Nutritional composition of sweet cherry

Proximates	**Value per 100 g FW**
Water (g)	82.25
Energy (kcal)	63
Protein (g)	1.06
Total lipid (fat) (g)	0.2
Carbohydrate, by difference (g)	16.01
Fiber, total dietary (g)	2.1
Sugars, total (g)	12.82
Minerals	
Calcium, Ca (mg)	13
Iron, Fe (mg)	0.36
Magnesium, Mg (mg)	11
Phosphorus, P (mg)	21
Potassium, K (mg)	222
Sodium, Na (mg)	0
Zinc, Zn (mg)	0.07
Vitamins	
Vitamin C, total ascorbic acid (mg)	7
Thiamin (mg)	0.027
Riboflavin (mg)	0.033
Niacin (mg)	0.154
Vitamin B-6 (mg)	0.049
Folate, DFE (µg)	4
Vitamin B-12 (µg)	0
Vitamin A, RAE (µg)	3
Vitamin A, IU (IU)	64
Vitamin E (alpha-tocopherol) (mg)	0.07
Vitamin D (D2 + D3) (µg)	0
Vitamin D (IU)	0
Vitamin K (phylloquinone) (µg)	2.1
Lipids	
Fatty acids, total saturated (g)	0.038
Fatty acids, total monounsaturated (g)	0.047
Fatty acids, total polyunsaturated (g)	0.052
Fatty acids, total trans (g)	0
Cholesterol (mg)	0

Nutritional composition of sweet cherry expressed for 100 g fresh weight (FW). Data obtained from the U.S. Department of Agriculture [1].

Supplemental Table 2. Phenolic composition of sweet cherry.

Flavonoids		**mg per 100 g FW**
Anthocyanins	Cyanidin 3-O-glucoside	18.73
	Cyanidin 3-O-rutinoside	143.27
	Pelargonidin 3-O-rutinoside	1.24
	Peonidin 3-O-glucoside	0.76
	Peonidin 3-O-rutinoside	7.42
Flavanols	(+)-Catechin	1.5
	(-)-Epicatechin	7.78
	(-)-Epicatechin 3-O-gallate	0.09
	(-)-Epigallocatechin	0.05
	Procyanidin dimer B1	0.23
	Procyanidin dimer B2	2.1
	Procyanidin dimer B3	0.08
	Procyanidin dimer B4	0.18
	Procyanidin dimer B5	0.2
	Procyanidin dimer B7	1.01
	Procyanidin trimer C1	1.85
Phenolic acids		
Hydroxycinnamic acids	3-Caffeoylquinic acid	44.71
	3-Feruloylquinic acid	0.43
	3-p-Coumaroylquinic acid	38.43
	4-Caffeoylquinic acid	0.77
	4-p-Coumaroylquinic acid	1.27
	5-Caffeoylquinic acid	2.2

Phenolic composition of sweet cherry expressed as mg/ 100 g fresh weight (FW). Data obtained from Phenol explorer [2].

Supplemental Table 3. Nutritional composition of the standard and cafeteria diets.

	gr/100 gr of diet	
	STD	**CAF**
Proteins	16.1	5.8
Lipids	3.1	8.4
Of which saturated fatty acids	0.65	2.6
Carbohydrates	60.4	32.9
Of which total sugars	1.9	19.6
Fibres	3.9	1.8
Moisture	11.9	48.0

Nutritional composition of the standard (STD) and cafeteria (CAF). The sources of carbohydrates of the STD diet were 100% from cereals, and the protein sources were 66.7% from vegetal origin and a 33.3 % from animal origin. The ingredients of the cafeteria diet were bacon (8-12 gr), biscuits with pâté (12-15 gr) and cheese (10-12 gr), muffins (8-10 gr), carrots (6-9 gr) and sweetened milk (22% sucrose w/v; 50 mL) in addition to the standard chow diet.

Supplemental table 4. Primers for the Q-PCR analysis.

	Forward (5'...3')	Reverse (5'...3')
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG
Actb	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC
Ppia	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG
Acacα	GCGGCTCTGGAGGTATATGT	TCTGTTTAGCGTGGGGATGT
Atgl	GAAGACCCTGCCTGCTGATT	CACATAGCGCACCCCTTGAA
Fasn	TAAGCGGTCTGGAAAGCTGA	CACCAGTGTTTGTTCCTCGG
Gpat	GAATACAGCCTTGGCCGATG	GAGGCGTGCATGAATAGCAA
Hsl	AGTTCCCTCTTTACGGGTGG	GCTTGGGGTCAGAGGTTAGT
Prdm16	GTTCTGCGTGGATGCCAATC	TGGCGAGGTTTTGGTCATCA
Cebpα	TGTACTGTATGTCGCCAGCC	TGGTTTAGCATAGACGCGCA
Mgll	ATCATCCCCGAGTCAGGACA	TGACTCCCCTAGACCACGAG
Ucp1	GGTACCCACATCAGGCAACA	TCTGCTAGGCAGGCAGAAAC
Lpl	GGCCCAGCAACATTATCCAG	ACTCAAAGTTAGGCCCAGCT
Had	ATCGTGAACCGTCTCTTGGT	AGGACTGGGCTGAAATAAGG
Cpt1b	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG
Pparα	CGGCGTTGAAAACAAGGAGG	TTGGGTTCCATGATGTCGCA
Fatp1	CTACCACTCAGCAGGGAACA	GCGGCATATTTCACCGATGT
Cd36	CAGTGCAGAAACAGTGGTTGTCT	TGACATTTGCAGGTCCATCTATG
Pparγ	AGGGCGATCTTGACAGGAAA	CGAAACTGGCACCCTTGAAA
Bmal1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC
Cry1	TGGAAGGTATGCGTGTCCTC	TCCAGGAGAACCTCCTCACG
Per2	CGGACCTGGCTTCAGTTCAT	AGGATCCAAGAACGGCACAG

Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), Actin beta (*Actb*), Peptidylprolyl Isomerase A (*Ppia*), acetyl-CoA carboxylase alpha (*Acacα*), adipose triglyceride lipase (Atgl), fatty acid synthase (*Fasn*), glycerol-3-phosphate acyltransferase (*Gpat*), hormone-sensitive lipase (*Hsl*), PR domain containing 16 (*Prdm16*), CCAAT/enhancer-binding protein alpha (*C/ebpα*), monoglyceride lipase (*Mgll*), uncoupling protein 1 (*Ucp1*), lipoprotein lipase (*Lpl*), hydroxyacyl-CoA dehydrogenase (*Had*), carnitine palmitoyltransferase 1B (*CPT1b*), peroxisome proliferator-activated receptor alpha (*Pparα*), fatty acid transport protein 1 (*Fatp1*), cluster of differentiation 36 (*Cd36*), peroxisome proliferator-activated receptor gamma (*Pparγ*), brain and muscle ARNT-like1 (*Bmal1*), cryptochrome circadian clock 1 (*Cry1*) and period circadian clock 2 (*Per2*).

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MANUSCRIPT 4

A mix of natural bioactive compounds reduces fat accumulation and modulates gene expression in the adipose tissue of obese rats fed a cafeteria diet.

Albert Gibert-Ramos[a], Miguel Martín[a], Anna Crescenti[b], M. Josepa Salvadó[a],

[a]Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, Tarragona, Spain

[b]Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain

Corresponding authors: albert.gibert@urv.cat (A.G.-R.); anna.crescenti@eurecat.org (A.C.); Tel.: +34-977558465 (A.G.-R.); +34-9777529-65 (A.C.)

Waiting for patent

IV. GENERAL DISCUSSION

The adipose tissue plays an important role in the organism's energy homeostasis because its main function is lipid storage and release, though its functionality is affected during obesity (1). Obesity is a major risk factor for many other diseases and its prevalence is rapidly increasing around the world (2), being a real problem in today's society. In 2016, it was evaluated that a 39% and a 13% of the world's population was overweight and obese, respectively. In obese individuals, the WAT grows excessively through two main mechanisms named hypertrophy or hyperplasia, and incorporating triglycerides into a unilocular lipid droplet in the cytoplasm of adipocytes (3). The dysfunction of the adipose tissue is usually linked to an increased adipocyte size, and has been related to other metabolic complications, such as insulin resistance, type II diabetes or hypertriglyceridemia (1,4–6). Moreover, adipose tissue dysfunction impairs many of its main metabolic pathways such as fatty acid absorption, transport and synthesis (7–9). On the other hand, the BAT specializes in thermogenesis, burning fat reserves to generate heat, and so, it is focused on for its obesity protecting effects (10). Due to the great number of health problems associated with obesity and in particular to adipose tissue dysfunction, much focus is being put on its prevention and treatment, studying new risk factors or mechanisms that influence its prevalence and developing new drugs or natural ingredients with beneficial effects reducing the WAT accretion and associated metabolic disturbances (11–14).

Even though the rise in the number of obesity cases is generally attributed to modern lifestyle changes, such as low physical activity and increased consumption of highly caloric foods (15), other mechanisms influenced by the environment and the genetic background of each individual are being focused on (16,17). One such factor is the alterations in the circarhythms, the predictable patterns of light and dark observed during a day and a year (18). Circannual and circadian rhythms are closely related, probably through the clock genes, which have been found to be involved principally in the circadian clock, but also to influence the circannual rhythm and seasonal adaptations (19–22). Interestingly, diet is also linked to circarhythms, as it has been found to modulate or desynchronize the molecular clock. In fact, time of feeding, fasting, and diets with a high content of fat have shown to modulate the circadian rhythm in peripheral tissues (23–27), while polyphenols, such as epigallocatechin-3-gallate or proanthocyanidins have shown to regulate the expression of clock genes in peripheral clocks (28,29). There is evidence that circadian disruption is correlated with an increased risk of obesity (30–38). Furthermore, the changing seasons have shown to modulate weight, appetite, and the secretion of hormones related to circarhythms in humans (18,39–43). Furthermore, environmental light deficiency during the winter months produces, in some individuals, symptoms such as carbohydrate craving,

increased appetite and weight gain, which has been related to the metabolic syndrome (44,45). This predictable phenomenon that takes place throughout the year can be studied in the laboratory with the Fischer 344 rat, among other animal models. Seasonal animals develop physiological and behavioural adaptations to the varying annual seasons to increase their chances of survival and reproduction (46). Fischer 344 rats respond to changes in the duration of the light and dark cycle, or photoperiod, and either supress the reproductive function during a short day or develop adaptations to increase weight and reproductive maturation when exposed to long photoperiods. Thus, the Fisher rat is an appropriate animal model to study the circadian and circannual clock, or seasonal adaptations (47–51).

In this work, the first objective was to evaluate the effects of a short day and long day photoperiods on the adipose tissue metabolism of Fischer 344 rats (**Manuscript 1**). Although other studies have shown that the exposition of this rat model to different photoperiods alters fat accretion, to our knowledge, none is centred on the physiology of the adipose tissue. In order to study the relationship between diet and the circarhythms on the adipose tissue metabolism, we also studied the effects of an obesogenic diet on the effects of the photoperiod (**Manuscript 1**). Principally, we found that the exposure of Fischer 344 rats fed a standard diet to a long day photoperiod promotes fat accumulation compared to the short day photoperiod, as other authors have found in this rat strain (50) or in other long day breeders (52–55). Furthermore, the RWAT gene expression was completely opposed in both photoperiods, showing a more metabolically active WAT in long day rats than in the short day. Specifically, we observed greater levels of adipogenesis, lipogenesis and lipolysis gene expression in rats held on the former photoperiod. Accordingly, the RWAT morphology showed a higher frequency of larger adipocytes in the long day photoperiod. However, no differences in total adipocyte number or area were found between both photoperiods, which suggests that differences in morphology were small. Additionally, the BAT gene expression also showed a higher capacity for lipid absorption and utilisation in a long day photoperiod, compared to the short day photoperiod, which might contribute to the increased levels of TAG in serum observed in animals held on the short day photoperiod.

On the other hand, the effects of the obesogenic diet on the metabolism of the adipose tissue showed notable differences compared to the effects observed in the study of rats fed with the standard diet. In fact, rats fed with the cafeteria diet and held in different photoperiods did not show differences in fat accretion. Furthermore, it was found that in the RWAT, key genes of lipogenesis and adipogenesis where upregulated in the short day compared to the long day, unlike what we observed in the standard experiment. Moreover, even though no

differences were found in the amount of fat, the morphology of the RWAT was completely different between both photoperiods. Thus, rats fed with the cafeteria diet and held in a long day expanded their adipose tissue increasing the size of its adipocytes, also known as hypertrophy, compared to the short day. These differences in histology were similar to what we found with the standard fed rats and are important because although we have shown that both groups of animals were equally affected by the cafeteria diet regarding fat accretion, the approach in energy storage was different. In this sense, adipocyte hypertrophy has been associated with insulin resistance and type II diabetes (5,6,56), while hyperplasia is linked to a healthier metabolic profile in obese subjects (4,57,58). Regarding BAT tissue, as in the standard experiment, rats held in the long day photoperiod and fed with the cafeteria diet showed increased levels of lipid uptake and β-oxidation gene expression in this tissue compared to animals held in the short day photoperiod. However, the differences observed in cafeteria diet fed animals were smaller than in the standard diet experiment, probably due to the effect of the obesogenic diet intake in these animals. In fact, in **Manuscript 3** we observed that animals in a long day or short day photoperiods showed clearly different patterns of expression of key genes of the molecular clock during a standard diet, expressing higher levels of *Bmal1*, *Per2* and *Cry1* in the long day photoperiod, compared to the short day photoperiod. It has been already reported that *Bmal1*, *Cry1* and *Per2* follow a coordinated expression level during the day in the adipose tissue (59), and that these expression levels change depending on the photoperiod of the rat (60,61). Moreover, in our study with rats fed the cafeteria diet, the expression levels of these genes showed no significant differences between long and short day groups. These results, together with the lack of differences in the fat content in the cafeteria diet fed rats, and the alterations in the gene expression of lipid metabolism genes suggests that the cafeteria diet disrupts the normal pattern of molecular clock expression, disassociating the normal adaptations linked to the photoperiod. In this sense, as already exposed before, other authors have shown that the volume of food, time of consumption and the caloric contribution of the meal, collaborate in disrupting the circadian clock rhythm (23–27).

Altogether, in our study we have shown that, in rats fed a standard diet, the long day or short day photoperiod modulates the molecular clock of the white adipose tissue in a different pattern. Furthermore, related with this or not, both groups of animals showed a different physiological response of the adipose tissue, favouring the accumulation of fat, the activation of adipogenesis, lipogenesis and lipolysis gene transcription in the WAT and activation of the genes implicated in the BAT metabolism in rats held on a long day photoperiod, compared to a short day. Also, even though further evidences are needed, our results show

that the cafeteria diet, a highly caloric meal, is capable of desynchronising the molecular clock, altering the differences that are normally observed between long and short days photoperiods, which might affect fat accumulation and the mechanisms by which the adipose tissue accumulates the excess of fat. These results are relevant because humans also show differences in many parameters during seasons, such as body fat (41), concentration of hormones (42,43,62) and in other factors linked to the risk of obesity (39). The study of the relation between diet and circarhythms could help to comprehend the rise in the number of cases of obesity in humans and develop proper strategies to counteract it.

We next studied the consumption of fruit out of season as a potential risk factor for the development of obesity, focusing on the adipose tissue. We worked with oranges from the northern (ON) and southern (OS) hemisphere harvested the same month (**Manuscript 2**), and cherry (**Manuscript 3**), which is typically harvested during a long day season. Each fruit was consumed during two different photoperiods, one was the photoperiod in which the fruit was harvested and so, the appropriate photoperiod, and the other, to represent the consumption of fruit out of season, corresponds to the photoperiod in which the fruit was not harvested. Long day breeders tend to undergo a combination of physiological adaptations and changes in adipose tissue metabolism during long days, as explained above. Thus, according to the results obtained in our Manuscript 1 and in line with our hypothesis and the xenohormesis theory, the consumption of a fruit from a different season could erroneously signal the photoperiod, desynchronizing the molecular clock and seasonal adaptations from the light and dark patterns of the environment, developing adaptations typical of another season and increasing the risk of obesity. ON are typically harvested in autumn and winter months, while in the southern hemisphere the seasons are changed comparatively, and so, according to the xenohormesis theory, their content of secondary metabolites or signalling about the environment will change. Accordingly, we found that animals adapted to a short day photoperiod, supplemented with OS, a fruit that in this photoperiod is consumed out of season, increased their fat mass in a similar way than the animals held in a long day, while no differences were found when this fruit was consumed in a long day. Furthermore, the consumption of OS out of season increased the gene expression of *Pparγ* in the IWAT, implicated in adipogenesis and lipid metabolism (63,64), and incremented the percentage of big adipocytes, suggesting an overall activation of fat accumulation. Thus, these results suggest that the consumption of OS out of season could increase the risk of obesity. Furthermore, the downregulation of *Pparα, Lpl* and *Cpt1b* gene expression in the BAT, on the animals that consumed OS out of season showed that fat incorporation into the cell and the mitochondria might be altered, diminishing the oxidation of fat. The BAT is implicated in

overall energy homeostasis and the upregulation or downregulation of its activity can influence fat accumulation in the organism (65–67). Unexpectedly, we found a significant increase in *Ucp1* gene expression that does not follow the same pattern of expression of the lipid uptake and β-oxidation genes. However, UCP1 protein levels were unaltered. In this sense, UCP1 has been shown to be tightly regulated post-transcriptionally (68–70), which could explain our discrepancies between mRNA and protein expression.

We further deepened in the concept of fruit consumption out of season and its effects on the adipose tissue with cherries (**Manuscript 3**), adding the study of the effect of an obesogenic diet intake. The cherry is a fruit that is harvested from June to mid-July (71), and according to our hypothesis, will signal animals about a long day photoperiod. We found that rats fed a standard diet, acclimated to a short day and supplemented with cherry, increased the gene expression levels of *Ucp1* in the BAT compared to the group supplemented to the vehicle, showing similar levels to those in the long day groups. According to our hypothesis, the cherry, a long day fruit, could incorrectly signal the animal about a long day when it is consumed during a short day photoperiod, and so, the BAT could develop adaptations typical of long day acclimated rats, like increased *Ucp1* expression in the BAT. Moreover, the expression of the molecular clock genes in the RWAT was altered compared to those in the vehicle groups which, as commented above, showed important differences among photoperiods. In fact, the short day group showed lower expression levels of *Bmal1* and *Cry1* compared to the vehicle in the same photoperiod, indicating that cherry consumption out of season could alter its expression. Even though the molecular clock was affected, no other biometrical changes or effects on the adipose tissue were found. On the other hand, when the experiment was done with rats fed a cafeteria diet, we found that cherry consumption out of season increased postprandial glucose and insulin levels, which might indicate decreased sensitivity to insulin, or insulin resistance (72,73). Additionally, in the RWAT, cherry consumption out of season changed the morphology of adipocytes, increasing their area and decreasing the total number of cells, known as hypertrophy, and also linked to insulin resistance and type II diabetes (5,6,56). More in detail, the RWAT showed a downregulation of key lipogenesis and lipolysis genes, which has been related to a loss of adipocyte function of hypertrophic adipocytes in obese subjects (1,8,9). Interestingly, in the BAT, *Cd36*, a fatty acid transporter found in oxidative tissues (74), was also downregulated, suggesting a lower capacity of brown adipocytes to absorb fat, and so, a lower lipid oxidation (75).

Even though we hypothesised that the changes affecting normal adaptations of the adipose tissue to the photoperiod might stem from alterations in the clock machinery due to

consumption of out of season fruit, the cafeteria diet seems to already alter their transcription machinery, and we found no relevant differences between groups with either the supplementation of cherry or vehicle in the key clock genes expression. In this sense, we found some evidences that cherry consumption out of season might generate alterations on the molecular clock of lean animals. On the other hand, we observed that the cafeteria diet disrupted the expression pattern of the molecular clock genes and we were unable to detect differences caused by the supplementation of the cherry lyophilizate in the experiment with the obesogenic diet. From these results, we suggest that in rats fed a cafeteria diet, cherry supplementation could favour the prevalence of determinate factors such as increased glucose and insulin levels in blood, adipocyte hypertrophy, and a downregulation of general lipid metabolism in the WAT, linked to obesity and metabolic disease, when the fruit is consumed out of season.

After evaluating this new risk factor for the development of obesity, we worked on the treatment of pre-established obesity (**Manuscript 4**). Obesity treatment is of vital importance since it is linked to endocrine, cardiovascular, and many other dysfunctions or diseases (76–82). However, current treatments like caloric restriction, bariatric surgery, or pharmacotherapy are either difficult to maintain during a long term, are costly, unreliable, may cause side effects, or more knowledge is needed for its correct application (83–86). Additionally, since obesity is a multifactorial disease, and so, many organs, hormones and pathways are involved in its pathogenesis (87,88), other approaches are being studied, one interesting example is the use of natural bioactive ingredients and its combination with positive effects on different pathways or mechanisms related with obesity (89,90). Through this kind of treatment, researchers expect to obtain an overall higher effect than the supplementation with the individual compounds, through additive or synergic effects of the ingredients (91–93).

With this aim in mind, the Nutrigenomics research group of the Universitat Rovira i Virgili has designed a mix of natural bioactive compounds that, individually, have shown a positive effect on the treatment of obesity or its related diseases . This mix is composed of . We evaluated the effects of this mix with the aim to redress the effects of obesity induced by a cafeteria diet, focusing on the adipose tissue.

In our study, the treatment with the mix notably reduced the body weight of animals, specifically, through a reduction of fat mass accretion. More precisely, the IWAT and EWAT depots mass were greatly reduced, while the RWAT seemed more resistant to the fat

reduction and the MWAT was totally unaffected. In accordance with this, the IWAT and EWAT depots also showed an improved gene expression profile, specifically, they showed a reduced gene expression of *Fasn*, while no differences were found in RWAT. Also, *Hsl* and *Atgl*, showed different degrees of upregulation in the three studied depots, following the opposed pattern observed with the weights and the *Fasn* gene expression levels. *Pparγ* gene expression was also found to be inhibited by the treatment, but only in the EWAT. Overall, these results suggest that the mix produced an inhibition in lipogenesis and adipogenesis, and an increase in lipolysis. The increase in lipolysis might explain the increase in fat oxidation and energy expenditure observed with the indirect calorimetry analysis, since lipolysis principal function is the release of fatty acids for their use as an energetic substrate (98). Even though thermogenesis in the white adipose tissue in the form of browning or brite adipocytes can be linked to increased rates of lipolysis (99), no differences were found in the expression of *Ucp1* or *Prdm16* in the IWAT, and so, we discard this mechanism as a possible explanation for the decrease in fat mass and increased energy expenditure or fat oxidation. Furthermore, in our study, the results showed the heterogeneity of the mix over the fat depots, as the body fat regulation mechanisms induced by the mix were different depending on fat depot localisation.

The IWAT morphology was also studied and although not significant, the treated group showed a tendency to present fewer total adipocytes in the IWAT deposit, without differences in adipocyte area compared to untreated group. Even though some WAT depots show a higher predisposition to grow through hypertrophy or hyperplasia (100,101), adipocytes tend to grow first in size, and when they reach a certain threshold, adipogenesis is stimulated to supply the storage demand (102). In this sense, we propose that histologic differences in the IWAT are due to the properties of the mix to stimulate lipolysis and downregulate lipogenic genes, which leads to a decreased generation of adipocytes. On the other hand, we discard changes on the number of preadipocytes in the IWAT because the stromal vascular fraction (SVF), which includes a population of preadipocytes and mesenchymal stem cells, among other cell types, showed no differences in the expression of genes related with adipocyte differentiation or *Pref-1*, a marker of preadipocytes. Additionally, the BAT showed no differences in key genes implicated in its metabolism or thermogenesis, implying that the mix does not increase its metabolism.

We believe that the combination of increased the overall effect of each ingredient. In fact, research on the individual ingredients have shown sometimes conflicting results regarding their individual effects on lowering fat accretion.

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For example, it has been reported in obese mice, that significantly reduced fat mass, in a slightly higher proportion than our three weeks treatment. On the other hand, in a recent review explaining the principal mechanisms by which exert their positive effects on obesity, an increase in energy expenditure or lipid oxidation in the WAT is not among them. For this reason, we attribute the differences that we found in energy expenditure and lipid oxidation to the other compounds or to a synergy of . Concerning , there is evidence that it can increase energy expenditure and fat oxidation

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. Furthermore, has shown to induce thermogenesis and browning in the WAT of obese mice , however, in our study there were no differences found in UCP1 and PRDM16, which are indicators of browning in the WAT. All in all, these results support the idea that further study is needed of .

In order to further study the mechanisms by which the mix has its effects on the adipose tissue, we analysed the effects of each individual compound on the 3T3-L1 cell line, which allows the study of adipocyte differentiation and metabolism (112). We used and individually during the fibroblast differentiation into adipocyte, and found that decreased the quantity of fat accumulated into the mature 3T3-L1 cells, probably through a reduction in the gene expression of *Fasn*, which was downregulated, while also reduced *Pparγ.* Even though has shown positive results on 3T3-L1 cell culture in other studies , unexpectedly we found no differences among treatments, which we attribute to . On the other hand, other studies have reported similar results to ours with in the 3T3-L1 cell culture . These results could imply that the effects observed directly in the rat WAT could be related to , while might affect the WAT indirectly or through other mechanisms.

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V. CONCLUSIONS

1. **White and brown adipose tissues of lean Fischer 344 rats adapt to the photoperiod. A long day promotes fat accumulation and stimulates lipid metabolism in relation to a short day.** Thus, a long day photoperiod, increases the expression of key genes involved in adipogenesis, lipogenesis and lipolysis, as well as the frequency of larger adipocytes in the white adipose tissue. In addition, the brown adipose tissue also increases the expression of key genes in lipid uptake and β-oxidation in a long day photoperiod. Altogether, these changes may contribute to lowering the circulating triglycerides.

2. **The modulation of the molecular clock genes is synchronized by the photoperiod, and seems to stimulate the seasonal changes in the adipose tissue.** The differences of biometric and physiological parameters observed in the adipose tissue seem to be stimulated by changes in the expression levels of the clock genes, considering that their expression levels change depending on the photoperiod.

3. **The cafeteria, a high caloric diet, masks some of the effects of the photoperiod on the adipose tissue, however, it causes different phenotypes regarding fat accumulation in Fischer 344 rats depending on the photoperiod.** Thus, animals in a short day, and fed a cafeteria diet, showed a stimulation of the transcription of lipogenesis and adipogenesis genes, as well as an expansion of the adipose tissue through hyperplasia, compared to the long day. These results suggest that the photoperiod, together with diet, could be a risk factor for the development of obesity.

4. **The desynchronization of the molecular clocks in the adipose tissue could be an underlying factor for the observed effects of the cafeteria diet.** Thus, contrarily to what we observed in animals fed with an standard diet, rats fed with the cafeteria diet in different photoperiods showed no differences biometrically nor in the gene expression of *Bmal1*, *Per2* and *Cry1*.

5. **Consumption of fruit out of season could be a novel risk factor for the development of obesity.** Our results show that**:**

a) **Supplementation of orange from the south hemisphere, with a molecular signature of a long day, stimulates fat accumulation when consumed in a short day**, increasing fat accretion, adipocyte size and *Pparγ* gene expression in the WAT, and downregulating the gene expression of key genes implicated in the BAT metabolism.

b) **Supplementation of cherry, a long day photoperiod fruit, to lean animals in a short day desynchronises key genes of the molecular clock**. In addition, cherry consumed out of season increases *Ucp1* gene expression in the BAT, in a similar way to what is observed during a long day.

c) **Supplementation of cherry to animals fed a cafeteria diet in a short day stimulates changes linked to obesity and related diseases**, increasing glucose and insulin circulating levels, adipocyte hypertrophy in the WAT, and downregulating adipocyte function on the WAT and BAT tissues.

6. **A mix of natural bioactive compounds supplemented to obese Wistar rats, and composed of**

   , shows beneficial effects on the adipose tissue, reducing fat accretion, stimulating energy expenditure, increasing the expression of lipid mobilization genes and decreasing the expression of lipid accumulation genes.

7. **The observed beneficial effects of the mix are not caused by an increase in the browning process or a modification in the pool of preadipocytes in the IWAT. Nor does the mix show evidences of an induction of the BAT thermogenesis.**

8. **The included in the mix, inhibit lipogenesis and adipogenesis on the 3T3-L1 cell line.** reduced lipid accumulation and *Fasn* gene expression levels and protein content, while also downregulated the expression of *Pparγ*. Contrarily to what we would expect according to the existing bibliography, showed no effects.

LIST OF PUBLICATIONS

Full Papers

Gibert-Ramos A., Crescenti A., Salvadó MJ. *Consumption of Cherry out of Season Changes White Adipose Tissue Gene Expression and Morphology to a Phenotype Prone to Fat Accumulation.* Nutrients. 2018. 10, 1102.

Ibars M., Aragonès G., Ardid-Ruiz A., **Gibert-Ramos A.**, Arola-Arnal A., Suárez M., Bladé C. *Seasonal consumption of polyphenol-rich fruits affects the hypothalamic leptin signaling system in a photoperiod-dependent mode*. Scientific Reports. 2018. 11;8 (1).

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Gibert-Ramos A., Martín, M., Crescenti A., Salvadó MJ. *A mix of natural bioactive compounds reduces fat accumulation and modulates gene expression in the adipose tissue of obese rats fed a cafeteria diet*. (Waiting for patent).

Abstracts

Gibert-Ramos A., Salvadó MJ., Crescenti A. *Consumption of fruits out of season modify body fat mass and lean mass in rats.* Mechanisms of a long-life health – NuGOweek – Barcelona, 2015.

Gibert-Ramos A., Salvadó MJ. Crescenti A., *A mix of natural compounds decreases fat accumulation and modulates lipogenic and lipolysis gene expression in rats consuming a high fat diet*. IX Seminario sobre Alimentación y Estilos de Vida Saludables. Tarragona, 2017.

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Martín M., Mas-Capdevila A. **Gibert-Ramos A.**, Ardid-Ruiz A., Aragonès G., Muguerza B. *Reduction of hypertriglyceridemia and improvement of triglycerides homeostasis in cafeteria diet-fed rats by chronic administration of a natural extract*. The annual Congress of the SFBBM FEBS3+XL SEBBM Congress – Barcelona, 2017.