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GRAPE-SEED PROANTHOCYANIDINS AND CIRCADIAN DISRUPTION: a chrononutritional approach to liver lipid metabolism in rats

BIOCHEMISTRY AND MOLECULAR BIOLOGY

FINAL DEGREE PROJECT with a distinction in MOLECULAR NUTRITION

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ABSTRACT

Circadian disruption is frequently linked to metabolic disorders, particularly affecting hepatic lipid metabolism. This study analyses the effects of grape-seed proanthocyanidin-rich extract (GSPE, 25 mg/kg/day) in Wistar rats exposed to either a standard 24-h light–dark cycle or a shortened 22-h cycle. Hepatic triglyceride levels and lipid-related proteins were analysed across four Zeitgeber times. No significant triglyceride changes were found, although total ACC expression increased in the disrupted + GSPE group. Several proteins showed diurnal variation, confirming hepatic circadian resilience. These findings highlight GSPE's limited effects under healthy conditions and the need for challenge models to assess its chrononutritional potential.

Keywords: Circadian disruption; Chrononutrition; Hepatic lipid metabolism; Grape-seed proanthocyanidins (GSPE); Polyphenols; Wistar rats.

ABBREVIATIONS

ACC: Acetyl-CoA Carboxylase

ACOX1: Acyl-CoA Oxidase 1

ACS: Acyl-CoA Synthetase

AGPAT: Acylglycerol-3-Phosphate Acyltransferase

AMPK: AMP-Activated Protein Kinase

apoB-100: Apolipoprotein B-100

BCA: Bicinchoninic Acid

BMAL1: Brain and Muscle ARNT-Like 1

BSA: Bovine Serum Albumin

CCGs: Clock-Controlled Genes

CD36 / FAT: Cluster of Differentiation 36 / Fatty Acid Translocase

CLOCK: Circadian Locomotor Output Cycles Kaput

CPT1a: Carnitine Palmitoyltransferase 1A

CRY: Cryptochrome

DGAT2: Diacylglycerol O-Acyltransferase 2

DNA: Deoxyribonucleic Acid

E-box: Enhancer box DNA sequence

ECL: Enhanced Chemiluminescence

EDTA: Ethylenediaminetetraacetic Acid

FABP1: Fatty Acid Binding Protein 1

FASN, FAS: Fatty Acid Synthase (gene, protein respectively)

FATPs (FATP2 / FATP5): Fatty Acid Transport Proteins

G3P: Glycerol-3-Phosphate

GPAT: Glycerol-3-Phosphate Acyltransferase

GSPE: Grape-Seed Proanthocyanidin-rich Extract

HDL: High-Density Lipoprotein

HPLC-MS/MS: High-Performance Liquid Chromatography

LDL: Low-Density Lipoprotein

MASLD: Metabolic Dysfunction-Associated Steatotic Liver Disease

MTTP: Microsomal Triglyceride Transfer Protein

NADPH: Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

NAFLD: Non-Alcoholic Fatty Liver Disease (previous term for MASLD)

NEFA: Non-Esterified Fatty Acids

NASH: Non-Alcoholic Steatohepatitis

PBS: Phosphate Buffered Saline

PBS-Tween: Phosphate Buffered Saline with Tween 20

PER: Period gene family

PGC1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha

pACC: Phosphorylated Acetyl-CoA Carboxylase

PPARα: Peroxisome Proliferator-Activated Receptor Alpha

PVDF: Polyvinylidene Fluoride

ROR: Retinoic Acid-Related Orphan Receptor

SCFAs: Short-Chain Fatty Acids

SCN: Suprachiasmatic Nucleus

SEM: Standard Error of the Mean

SIRT1: Sirtuin 1

SREBP-1c: Sterol Regulatory Element-Binding Protein 1c

SULTs: Sulfotransferases

TAGs: Triacylglycerols

TTFL: Transcriptional-Translational Feedback Loop

UGTs: UDP-Glucuronosyltransferases

VLDL: Very Low-Density Lipoprotein

ZT: Zeitgeber Time

1 INTRODUCTION

Metabolic homeostasis in mammals is a dynamic and meticulously regulated process that exhibits a marked temporal organisation determined by the so-called **biological rhythms**. These rhythmic fluctuations are regulated by an internal molecular clock, also known as the **biological clock**, which synchronises with cyclical changes in the environment called *zeitgeber-s* or synchronisers (Koronowski, and Sassone–Corsi, 2021; Mazzoccoli et al., 2012). It is responsible for orchestrating a wide range of physiological processes, including sleep-wake cycles, hormone release, body temperature, blood pressure and heart rate, immune function, intestinal microbiote and, crucially, energy metabolism (Rijo-Ferreira & Takahashi, 2019; Fagiani et al., 2022; Scheiermann et al., 2013). This evolutionary adaptation allows organisms to anticipate and prepare the organism for predictable environmental changes that follow a pattern, such as light-dark cycles, under the direct and indirect influence of this temporal system, optimising the efficiency of biological functions and contributing to overall health (Sharma, 2003). **Circadian rhythms** are the most studied biological rhythms, with a periodicity of approximately 24 hours. The study of these time-regulated rhythms in living organisms has been coined **chronobiology**.

In mammals, the liver plays a vital role in regulating the metabolism of carbohydrates, lipids and proteins, adapting its activity to the energy and nutritional demands that vary throughout the day and night, under the direct and indirect influence of this temporal system (Hunter & Ray, 2019). Within this complex system, **hepatic lipid metabolism is characterised by a marked rhythmicity**; metabolic pathways such as fatty acid synthesis (lipogenesis), triglyceride uptake and storage, fatty acid oxidation (β-oxidation), and very-low-density lipoprotein (VLDL) production and secretion experience diurnal fluctuations in response to hormonal cues, nutrient availability, and hepatic circadian clock activity (Zheng et al., 2016; Reinke & Asher, 2016). Key genes encoding enzymes and regulatory proteins in these metabolic pathways, such as sirtuin 1 (***SIRT1***), peroxisome proliferator-activated receptor alpha (***PPARα***), fatty acid transport proteins (***FATPs***), CD36 (***CD36***), diacylglycerol O-acyltransferase 2 (***DGAT2***), fatty acid synthase (***FASN***), acetyl CoA carboxylase (***ACC***) and carnitine palmitoyl transferase 1A (***CPT1A***) have demonstrated **circadian gene expression patterns** in studies with rodents (Masri, and Sassone–Corsi, 2014; Sukumaran et al., 2010; Paredes et al., 2014; Paredes et al., 2015). This precise time regulation ensures an efficient use of lipids as an energy source during periods of activity and adequate storage during resting and food-intake periods (Paredes et al., 2014).

However, in the modern world, a considerable proportion of the population experiences a chronic misalignment between their endogenous circadian rhythms and the light-dark environmental cycle imposed by irregular work schedules, such as night work or rotating shifts. This **circadian disruption** has been consistently **associated with an increased risk of developing various metabolic disorders**, including obesity, dyslipidaemia, insulin resistance, and metabolic dysfunction-associated steatotic liver disease (**MASLD**) (previously referred to as non-alcoholic fatty liver disease (NAFLD)) (Fatima, and Rana, 2020; Ansu, and Knutson, 2023; Green et al., 2008; Schrader et al., 2024). Although the mechanisms underlying these associations are complex and multifactorial, it is believed that the alteration of the synchronisation of metabolic processes in key organs such as the liver plays a fundamental role (Sabath, Báez-Ruíz, and Buijs, 2015; Oosterman et al., 2020; Reinke & Asher, 2016).

Emerging research suggests a potential role for dietary interventions, particularly involving **phenolic compounds**, in supporting healthy circadian rhythms and counteracting the effects of

their disruption. Certain polyphenols have demonstrated the ability to **interact with core clock components**: for instance, both *in vitro* and *in vivo* studies have shown that resveratrol can modulate the expression of key clock genes such as *CLOCK*, *BMAL1*, *PER2*, and *Rev-Erbα* (Kapar et al., 2024; Miranda et al., 2013; Park et al., 2014), hinting at a pathway through which dietary factors might influence circadian timing. Some other polyphenols, including nobiletin, tangeretin, curcumin, bavachalcone, cinnamic acid and urolithin A have also shown the potential to regulate circadian oscillators and associated metabolic processes in various types of cells, however, it has been stated that there is significant methodological heterogeneity among the studies, which makes it difficult to compare outcomes (Sulaimani et al., 2024).

Beyond direct interactions with the clock, **phenolic compounds may also alleviate some of the metabolic disturbances associated with circadian misalignment**. In this line, anthocyanins have shown promise in improving glucose metabolism and reducing inflammation in preclinical models of metabolic disturbances (Hernández-Ruiz et al., 2025; Aboonabi et al., 2020). Different studies have observed that anthocyanidins are able to reduce inflammation and improve glucose and lipid metabolism in subjects with metabolic syndrome, conditions often exacerbated by disrupted circadian rhythms (Godyla-Jabłoński et al., 2021; Chen et al., 2023; Aboonabi et al., 2020). While in many cases the link to the clock might be indirect, the impact on metabolically regulated processes suggests a potential benefit in the context of circadian disruption. As an example, there is an interplay between circadian rhythms and inflammation, with disruption often promoting a pro-inflammatory state (Xu et al., 2020; Scheiermann et al., 2013), so there are chances that, given the established anti-inflammatory and antioxidant properties of many phenolic compounds (Tsao, 2010), their consumption could offer a dietary strategy to mitigate this effect. Furthermore, proanthocyanidins from grape seeds can improve metabolic and redox status in animal models of diet-induced obesity (Fernández-Iglesias et al., 2014), suggesting a potential to counteract the redox imbalances often seen with circadian disruption.

1.1 LIPID METABOLISM IN THE LIVER

The liver stands as a central and metabolically versatile organ in mammals, orchestrating energy homeostasis and processing key nutritional substrates (Cohen & Granner, 2008). It serves as a fundamental axis in carbohydrate, lipid, and protein metabolism, coordinating an intricate network of pathways essential for survival (Cohen & Granner, 2008).

Under normal physiological conditions, the liver processes substantial quantities of fatty acids, with relatively low amounts of triglycerides (less than 5%) stored in cytoplasmic lipid droplets (Ipsen et al., 2018). It actively participates in **fatty acid uptake** from both dietary sources (via chylomicron remnants) and adipose tissue (released during lipolysis), directing them towards various metabolic fates (Tirumalasetty et al., 2020). Fatty acids can undergo **β-oxidation**, a mitochondrial and peroxisomal process that breaks them down into acetyl-CoA for energy or ketone body synthesis, especially important during fasting (Rangaraju & Grims, 2016). Conversely, if energy intake surpasses demand, the liver performs ***de novo* lipogenesis**, synthesising new fatty acids primarily from excess carbohydrates (Tirumalasetty et al., 2020). These newly synthesised or taken-up fatty acids are then esterified into **triacylglycerols (TAGs)**, the primary storage form of lipids. Hepatic TAGs can either be stored in intracellular lipid droplets or packaged into **very-low-density lipoproteins (VLDLs)** for secretion into the bloodstream, supplying lipids to other tissues (Tirumalasetty et al., 2020).

Beyond fatty acids and TAGs, the liver is central to **cholesterol metabolism**, managing its synthesis, uptake from circulating lipoproteins, esterification, and conversion into bile acids vital for fat digestion and absorption (Rangaraju & Grims, 2016).

Hepatic steatosis, characterised by excessive fat accumulation in the liver, is a hallmark of Metabolic dysfunction-associated Steatotic Liver Disease (**MASLD**) (Pourteymour et al., 2023; Musso et al., 2009; Li et al., 2024). This condition stems from an imbalance between lipid acquisition (fatty acid uptake and *de novo* lipogenesis) and removal (fatty acid oxidation and VLDL secretion) (Pourteymour et al., 2023; Musso et al., 2009).

1.1.1 Molecular Arrangement of Hepatic Lipid Metabolism

1.1.1.1 Pathways involved in intrahepatic fat accumulation.

Intrahepatic fat accumulation arises when the intake and synthesis of fatty acids surpass their oxidation and secretion. This imbalance is orchestrated through several key molecular pathways. Fatty acids contributing to hepatic triglyceride accumulation can originate from three distinct sources: dietary lipids from lipoprotein remnants, non-esterified fatty acids (NEFAs) released by adipose tissue, and *de novo* lipogenesis (Mashek, 2021). Dietary fatty acids can stem from chylomicron spillover or chylomicron remnants. NEFAs are also released from adipose tissue. In obesity, the enlarged adipose tissue increases the flux of NEFAs released from this tissue (Jung & Choi, 2014). Furthermore, in cases of insulin resistance, adipose tissue lipolysis remains unsuppressed, consequently elevating the plasma NEFA flux delivered to the liver, among other tissues and organs (Musso et al., 2009).

FATTY ACID UPTAKE

Plasma NEFAs are taken up by hepatocytes via specific transporters. Key among these are **Fatty Acid Transport Proteins (FATPs)**, with **FATP2** and **FATP5** being prominent in the liver (Ipsen et al., 2018). Additionally, **Fatty Acid Translocase (FAT/CD36)** and **Fatty Acid Binding Proteins (FABPs)**, particularly **FABP1**, are highly expressed in hepatic tissue (Ipsen et al., 2018). A study by Greco et al. identified a positive correlation between CD36 expression and liver fat content in humans (Greco et al., 2008). In murine models, silencing of FABP1 and deletion of FATP5 resulted in decreased liver weight and triglyceride accumulation (Mukai et al., 2017; Doege et al., 2006). Furthermore, the protein expression of CD36 and FABP1 was elevated in mice fed a high-fat diet developing hepatic steatosis compared to control mice (Nie et al., 2024). Nevertheless, in certain instances, a reduction in the expression of CD36 and FATP5 has been noted in steatotic rats, potentially as a compensatory biological response (Gómez-Zorita et al., 2021).

DE NOVO LIPOGENESIS

De novo lipogenesis represents another significant source of the hepatic fatty acid pool. It is, in fact, the second largest contributor to hepatic triglycerides in Metabolic dysfunction-associated Steatotic Liver Disease (MASLD), following plasma NEFAs. Donnelly et al. reported that in humans, 60% of liver triglycerides originated from plasma NEFAs, 26% from *de novo* lipogenesis, and 15% from dietary intake.

In *de novo* lipogenesis, long-chain fatty acids are newly synthesized from acetyl-CoA, primarily derived from glucose. The rate-limiting step is catalysed by **Acetyl-CoA Carboxylase (ACC),** which carboxylates acetyl-CoA to malonyl-CoA. Subsequently, **Fatty Acid Synthase (FAS)** catalyses the formation of predominantly palmitic acid from malonyl-CoA. Palmitic acid can

undergo further elongation by elongases and desaturation by desaturases (Ipsen et al., 2018; Nagle et al., 2009). *De novo* lipogenesis also necessitates the hydrogen donor, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), generated via the pentose phosphate pathway and the malic enzyme reaction (Nguyen et al., 2008).

Plasma insulin leads to the activation of many transcription factors, which leads to the upregulation of lipogenic genes, including those for Fatty Acid Synthase (FAS), a multi-enzyme complex responsible for the final steps of fatty acid chain elongation, and Acetyl-CoA Carboxylase 1 (ACC1), which catalyses the rate-limiting step in *de novo* lipogenesis by converting acetyl-CoA to malonyl-CoA (Daemen, Kutmon, and Evelo, 2013; Chacko et al., 2020). Excessive plasma glucose promotes the upregulation of most fatty acid biosynthetic genes (Ipsen et al., 2018; Postic et al., 2007). Conversely, ACC is inactivated through phosphorylation by AMP-activated protein kinase (AMPK), resulting in decreased malonyl-CoA levels and enhanced mitochondrial fatty acid oxidation (Sanders & Griffin, 2016).

Isotope labelling studies have revealed that individuals with MASLD exhibit increased *de novo* lipogenesis compared to control subjects (Lambert et al., 2014; Syed-Abdul et al., 2023). Furthermore, animal models of hepatic steatosis have shown elevated markers of *de novo* lipogenesis. For instance, in a leptin-deficient (Ob/Ob) mouse model, gene and protein expression of ACC, FAS, SREBP-1c, and desaturases were increased relative to wild-type mice (Perfield et al., 2013). Nevertheless, in certain instances, a reduction in the expression of CD36 and FATP5 has been noted in steatotic rats, potentially as a compensatory biological response (Donnelly et al., 2005).

TRIGLYCERIDE ESTERIFICATION/ASSEMBLY

Triglyceride assembly represents the primary mechanism by which the liver stores fatty acids (Ipsen et al., 2018). It involves the esterification of glycerol-3-phosphate (G3P) with a combination of NEFAs to generate triglycerides. The NEFAs incorporated into triglycerides can originate from plasma or *de novo* lipogenesis, while G3P is produced either via glycolysis or through the phosphorylation of glycerol released from adipose tissue lipolysis (Nguyen et al., 2008).

The initial and rate-limiting step in triglyceride synthesis is mediated by Glycerol-3-Phosphate Acyltransferase (GPAT), which involves the esterification of acyl-CoA chains to G3P (Chacko et al., 2020). The second enzyme involved, Acylglycerol-3-Phosphate Acyltransferase (AGPAT), leads to the formation of diacylglycerols. Finally, **Diacylglycerol Acyltransferase (DGAT)** catalyses the concluding reaction in triglyceride formation. Triglycerides can be stored in cytoplasmic lipid droplets or integrated into VLDL particles, which are then secreted into the bloodstream (Nagle et al., 2009). Specifically, **Diacylglycerol Acyltransferase 2 (DGAT2)** catalyses the final, rate-limiting step in triacylglycerol formation (Chacko et al., 2020).

In vivo studies have highlighted the role of DGAT2 in the development of hepatic steatosis. Both gene and protein expression of DGAT2 were elevated in a rat model of fructose-induced fatty liver (Zhao et al., 2015), and overexpression of hepatic DGAT2 in mice led to increased hepatic triglyceride accumulation (Monetti et al., 2007). Moreover, inhibition of DGAT2 ameliorated hepatic steatosis in mice fed a steatogenic diet, without inducing liver inflammation or fibrosis. This beneficial effect was likely due to the accompanying reduction in *de novo* lipogenesis, which prevented diacylglycerol accumulation (Gluchowski et al., 2019).

1.1.1.2 Pathways Involved in Lipid Clearance

The liver can clear triglycerides primarily through mitochondrial fatty acid oxidation or by their secretion in the form of very low-density lipoproteins (VLDLs) (Manne et al., 2018).

FATTY ACID OXIDATION

Fatty acids derived from the hydrolysis of hepatic triglyceride stores, circulating lipids, or *de novo* synthesized fatty acids can be oxidized through β-oxidation. This process predominantly occurs in mitochondria but also, to a lesser extent, in peroxisomes. Peroxisomal β-oxidation shortens very long- and branched-chain fatty acids, which are then further oxidized in mitochondria (Ipsen et al., 2018; Musso et al., 2009).

Mitochondrial β-oxidation is the main route for the oxidation of short-, medium-, and long-chain fatty acids (Ipsen et al., 2018), taking place in the mitochondrial matrix. Fatty acids are progressively shortened into acetyl-CoA subunits, which can be entirely oxidized in the Krebs cycle or used to produce ketone bodies (Musso et al., 2009).

Before entering β-oxidation, fatty acids are converted into acyl-CoA by the cytosolic enzyme **Acyl-CoA Synthetase (ACS)**. This molecule then needs to be transferred into the mitochondria, a process mediated by the carnitine shuttle. First, **Carnitine Palmitoyltransferase-1A (CPT-1A)**, located in the outer mitochondrial membrane, facilitates the conversion of acyl-CoA to acyl-carnitine and its transport across the outer mitochondrial membrane. This is the rate-limiting step and a crucial regulatory point of β-oxidation (Ponziani et al., 2015; Rangaraju & Grims, 2016). This process is negatively regulated by malonyl-CoA, the product of the first step of *de novo* lipogenesis, which acts as an allosteric inhibitor of CPT-1A (Musso et al., 2009). Short- and medium-chain fatty acids traverse the mitochondrial membranes and are activated by ACS within the mitochondrial matrix; thus, their oxidation is not controlled by CPT-1A (Nguyen et al., 2008). Subsequently, **Carnitine Palmitoyltransferase-2 (CPT-2)**, situated in the inner mitochondrial membrane, catalyses the transport of acyl-carnitine into the mitochondrial matrix, releasing carnitine and regenerating fatty acyl-CoA, which then enters β-oxidation. This process involves the sequential elimination of two-carbon fragments through oxidation, yielding acetyl-CoA. In each cycle, four enzymes participate sequentially, releasing an acetyl-CoA residue while the acyl-CoA is shortened (Musso et al., 2009). **Acyl-CoA Oxidase 1 (ACOX1)** is also involved in the initial steps of peroxisomal β-oxidation (Rangaraju & Grims, 2016).

The regulation of gene expression related to fatty acid oxidation is primarily governed by **Peroxisome Proliferator-Activated Receptor Alpha (PPARα)** (Rangaraju & Grims, 2016). Additionally, **Sirtuin 1 (SIRT1)**, a member of the sirtuin family, plays a significant role in regulating fatty acid oxidation. SIRT1, a NAD^+-dependent deacetylase, deacetylates and activates Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1 alpha (PGC1α), which in turn activates PPARα, thereby promoting fatty acid oxidation (Nassir, 2022; Houtkooper et al., 2012; Chacko et al., 2020).

A recent study observed reduced hepatic mitochondrial fatty acid oxidation, coupled with an increase in dysfunctional mitochondria, in patients with MASLD (Moore et al., 2022). Kohjima et al. found that, in a group of subjects with MASLD, the gene expression of CPT-1A and PPARα was decreased compared to healthy livers (Kohjima et al., 2007). Furthermore, SIRT1, among other sirtuins, was observed to be downregulated in MASLD subjects compared to those without the disease (Wu et al., 2014). In mice fed a high-fat diet that developed steatosis, Nie et al. described reduced hepatic CPT-1A, PPARα, PGC1α, and SIRT1 protein expression in comparison to control mice (Nie et al., 2024). However, conflicting results showing increased fatty oxidation in MASLD

have been reported. This could be attributed to a compensatory mechanism activated during the early stages of MASLD in response to excessive intrahepatic fatty acids (Arroyave-Ospina et al., 2021).

TRIGLYCERIDE-RICH LIPOPROTEIN SECRETION

Under normal physiological conditions, the liver stores a small quantity of triglycerides and exports considerable amounts as VLDL particles. These VLDLs deliver fatty acids to muscle for oxidation or to adipose tissue for storage, depending on the nutritional status (Ipsen et al., 2018).

VLDL is a triglyceride-rich lipoprotein composed of a single molecule of **apolipoprotein B-100 (apoB-100)**, which is lipidated by the incorporation of triglycerides facilitated by **Microsomal Triglyceride Transfer Protein (MTTP)** (Tirumalasetty et al., 2020). This process occurs within the lumen of the endoplasmic reticulum. During the maturation of VLDL particles, they are translocated across the endoplasmic reticulum membrane and ultimately reach the Golgi apparatus (Ipsen et al., 2018; Kawano & Cohen, 2013).

Insulin exerts its anti-lipolytic action by suppressing VLDL production, likely due to a reduction in MTTP activity. However, in MASLD, owing to increased lipid availability and hepatic insulin resistance, MTTP activity is enhanced, subsequently increasing VLDL production. Consequently, hypertriglyceridemia is frequently observed in MASLD patients. Although the triglyceride-rich VLDL secretion rate is elevated in hepatic steatosis (Fabbrini et al., 2009), it is often insufficient to counterbalance the increased triglyceride availability in the liver, leading to hepatic steatosis. Indeed, it has been observed that when hepatic triglyceride infiltration surpasses 10% (hepatic steatosis), further increases in VLDL secretion are no longer possible (Musso et al., 2009; Kawano & Cohen, 2013). As demonstrated in a study by Fujita et al., patients with Non-Alcoholic Steatohepatitis (NASH) exhibited lower VLDL secretion than patients with simple steatosis (Fujita et al., 2009).

1.2 CIRCADIAN RHYTHMS

Life on Earth has evolved under predictable environmental cycles, leading to the development of **biological rhythms** – endogenous oscillations in physiological and behavioural processes. These rhythms enable organisms to anticipate and adapt to daily, monthly, or annual changes in their environment. Among these, **circadian rhythms** are the most prominent and extensively studied, representing approximately 24-hour cycles (from Latin "circa diem," meaning "around a day") (Panda, 2016).

These rhythms govern fundamental processes such as the **sleep-wake cycle**, fluctuations in **body temperature**, **hormone secretion** (such as cortisol, melatonin), **blood pressure**, **heart rate**, **immune function**, **eating habits**, **digestion** and critically **metabolism** (Arola-Arnal et al., 2019; Dibner et al., 2010a). While circadian rhythms are intrinsically generated by an internal timing system, they are continually synchronised to external cues, known as *zeitgeber*-s (German for "time givers"), with the light-dark cycle being the most powerful one (Mohawk et al., 2012). This temporal organisation ensures that metabolic pathways are aligned optimally with the periods of activity and rest, promoting physiological efficiency and health.

1.2.1 The Molecular Basis of the Biological Clock

The internal timekeeping mechanism in mammals is primarily orchestrated by a central pacemaker located in the hypothalamus; the **suprachiasmatic nucleus (SCN)** (Mohawk et al.,

2012). The SCN, often referred to as the "master clock," synchronises peripheral clocks found (virtually) in every cell and tissue throughout the body, including the liver, muscle, and adipose tissue. While the SCN is strongly influenced by light, peripheral clocks are largely influenced by SCN outputs and local cues, particularly "feeding rhythms" (Dibner et al., 2010b).

At the molecular level, the core of the circadian clock in mammalian cells is driven by an intricate transcriptional-translational feedback loop (TTFL) involving a set of **"clock genes"** (Figure 1):

The primary activators are the proteins **CLOCK** (Circadian Locomotor Output Cycles Kaput) and **BMAL1** (Brain and Muscle ARNT-Like 1), which form a complex (heterodymer, CLOCK:BMAL1), and bind to specific DNA sequences called E-boxes in the promoters of target genes, activating their transcription (Takahashi, 2017). Among the key target genes are the ***Period* (*PER1, PER2, PER3*)** and ***Cryptochrome* (*CRY1, CRY2*)** genes. Once translated, when the PER and CRY proteins accumulate in the cytoplasm, they translocate back to the nucleus and directly inhibit the transcriptional activity of the CLOCK:BMAL1 complex, suppressing their own expression. This negative feedback leads to cyclical repression and de-repression of gene expression, creating the 24-hour rhythm. Similarly, **ROR** (Retinoic acid-related Orphan Receptors) and **REV-ERB** (such as REV-ERBα, encoded by Nr1d1) protein families, which are activated by CLOCK:BMAL1, repress Bmal1 expression, adding another regulatory loop (Takahashi, 2017). The rhythmic activity of these core clock genes leads to the rhythmic expression of thousands of downstream "clock-controlled genes" (CCGs) that mediate the diverse physiological and behavioural outputs of the circadian system.

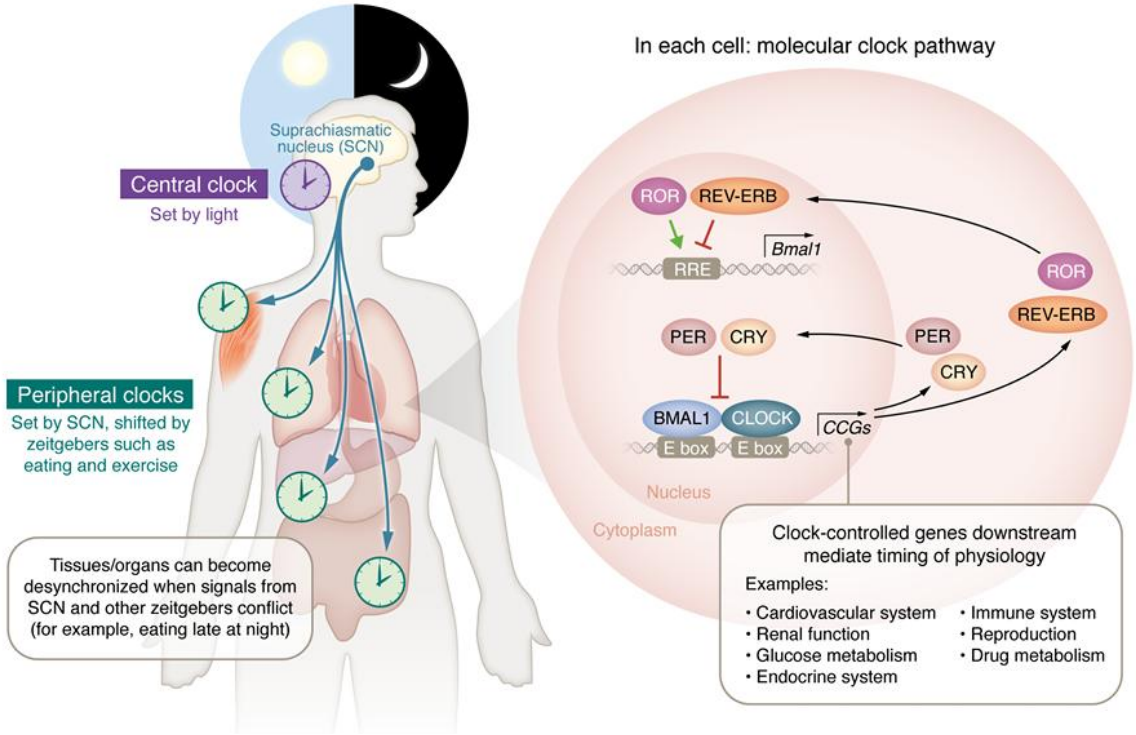

Figure 1. Represents the circadian control of molecular core clock gene signalling and physiologic regulation. The circadian clock is shown in purple and peripheral clocks in individual organs and tissue types are shown in green (right part of the image). Each cell contains transcription-translation feedback loops, the molecular clocks that drive circadian rhythms (right part of the image) (Adapted from Schrader et al., 2024).

1.2.2 Circadian Disruption and its Consequences on Lipid Metabolism

Despite the robustness of the endogenous circadian system, it is highly susceptible to disruption by modern lifestyle factors. **Circadian disruption**, or **chronodisruption**, occurs when there is a misalignment between the internal biological clock and external environmental or behavioural cues. Common causes include **irregular sleep-wake cycles**, **shift work**, chronic jet lag, and **irregular feeding patterns**, particularly eating during the biological night (Panda, 2016; Bass, and Takahashi, 2010), all of them being quite typical on these day and age.

This desynchronisation between the central SCN clock and peripheral clocks has damaging consequences for metabolic health, particularly in metabolically active organs like the liver, as it directly impacts lipid metabolism by altering the rhythmic expression of key metabolic genes, which are themselves under circadian control (Stangherlin & Barclay, 2021). For instance, irregular feeding patterns, especially common in shift workers, can lead to the inappropriate activation of lipogenic pathways in the liver during the rest phase, **increasing novo lipogenesis** and **reducing fatty acid oxidation** (Bass & Takahashi, 2010). This can result in the **accumulation of hepatic lipids**, promoting the development and progression of **Metabolic dysfunction-associated Steatotic Liver Disease (MASLD)**. Furthermore, circadian disruption is strongly associated with **dyslipidaemia**, characterised by elevated circulating triacylglycerols and altered cholesterol profiles, and impaired insulin sensitivity; and as a consequence, they all increase the risk of obesity, type 2 diabetes, and cardiovascular diseases (Stangherlin & Barclay, 2021).

1.3 GSPE: GRAPE-SEED PROANTHOCYANIDIN-RICH EXTRACT

Grape-Seed Proanthocyanidin-rich Extracts (GSPE) are natural extracts derived from the seeds of grapes (*Vitis vinifera*), recognised for its high content of **proanthocyanidins**. Proanthocyanidins, also known as condensed tannins, are a class of flavonoids that are oligomers or polymers of flavan-3-ol units, primarily **catechin** and **epicatechin** (Hümmer, and Schreier, 2008; Shi et al., 2003). Their degree of polymerization can vary significantly, ranging from dimers to more complex polymers. GSPE typically contains a diverse mixture of these proanthocyanidin oligomers and polymers, which are thought to contribute to its multifaceted biological activities.

GSPE has gained significant attention in nutraceutical and biomedical research due to its well-documented **antioxidant and anti-inflammatory properties** (Gupta et al., 2019). However, like other complex polyphenols, the bioavailability of higher molecular weight proanthocyanidins from GSPE is relatively low (Tao et al., 2019), and only the smaller oligomers present on the extract and their microbial metabolites effectively reach systemic circulation (Ou et al., 2019). Because of that, understanding the metabolism and absorption of these specific compounds is essential for knowing how to interpret their observed effects in vivo.

1.3.1 Metabolism and bioavailability of GSPE

As previously stated, the health benefits attributed to Grape-Seed Proanthocyanidin-rich Extract (GSPE) and consequently to proanthocyanidins, are largely mediated by the complex interplay between host absorption, microbial transformation, and host metabolism.

Unlike many simple phenolics, proanthocyanidins, particularly those of higher molecular weight, exhibit relatively low absorption rates in their native polymeric form (Tao et al., 2019). The primary absorption of smaller proanthocyanidin oligomers (dimers and trimers) can occur in the

small intestine, but this is a limited process (Tao et al., 2019). In fact, the crucial aspect of proanthocyanidin metabolism involves **gut microbiota** (Tian et al., 2018).

Larger proanthocyanidins, which are not absorbed in the upper gastrointestinal tract, reach the colon where they undergo extensive biotransformation by the resident microbes (Tao et al., 2019). The gut microbiota catabolises these complex polymers through various enzymatic reactions, including depolymerisation, ring-fission, and dehydroxylation. This microbial metabolization results in a diverse array of smaller and more absorbable phenolic metabolites such as **phenolic acids** (gallic acid, protocatechuic acid, dihydroxyphenyl acetic acid, hydroxyphenyl propionic acid, etc.) and **valerolactones** (such as hydroxyphenyl valerolactones), as well as **short-chain fatty acids (SCFAs)** (Tao et al., 2019). These microbial-derived metabolites are subsequently absorbed from the colon and undergo further phase I (for example, hydroxylation) and phase II (including glucuronidation, sulfation and methylation) metabolism in the liver and other tissues, facilitated by enzymes like UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) (Tao et al., 2019). The resulting conjugated metabolites, along with any parent compounds that has been absorbed, are then transported via the bloodstream to target tissues where they can manifest their beneficial effects.

1.3.2 Benefits of GSPE on Lipid Metabolism

More and more evidence from both *in vitro* and *in vivo* studies strongly supports the beneficial effects of GSPE on lipid metabolism, particularly within the liver. In various cellular models, GSPE has been shown to modulate key enzymes and transcription factors involved in lipid synthesis and degradation. For instance, GSPE can **downregulate *de novo* lipogenic genes** such as *FASN* and *ACC* and **upregulate genes involved in fatty acid oxidation**, such as *CPT1A* and *ACOX1* (Guo et al., 2012).

In **animal models** with diet-induced obesity or metabolic syndrome, GSPE supplementation has consistently demonstrated improvements in hepatic lipid profiles (Downing et al., 2015), including a **reduction in hepatic triacylglycerol accumulation (steatosis)** often accompanied by decreased body weight and fat mass (El-Alfy et al., 2019). Furthermore, GSPE has been shown to **improve systemic dyslipidaemia**, leading to lower circulating levels of total cholesterol, low-density lipoprotein cholesterol (LDL cholesterol), and triacylglycerols, while sometimes increasing high-density lipoprotein cholesterol (HDL cholesterol) (Fukuda et al., 2004). Also, many of the observed metabolic improvements are linked to GSPE's capacity to enhance **insulin sensitivity** and mitigate systemic **inflammation** and **oxidative stress**, all of which are crucial factors to hepatic lipid homeostasis (El-Alfy et al., 2019).

1.4 INTERACTION BETWEEN GSPE AND CIRCADIAN RHYTHMS

While the beneficial effects of GSPE on lipid metabolism are starting to be recognised, the potential for it to interact with and modulate circadian rhythms represents a relatively novel and underexplored area of research. In fact, this interaction is biologically realistic given the deep interconnections between the circadian clock system and metabolic pathways, which corroborates that **core clock genes directly regulate key metabolic genes**, and that metabolic signals can feedback to influence clock gene expression (Bass & Takahashi, 2010).

Experimental evidence has progressively confirmed this connection. For instance, one article of Ribas-Latre et al. demonstrated that chronic consumption of proanthocyanidins modulated peripheral clocks in both healthy and obese rats (Ribas-Latre et al., 2015a), while other showed

that they influenced melatonin levels and hypothalamic clock gene expression (Ribas-Latre et al., 2015b). At the hepatic level, proanthocyanidins have also been reported to regulate the acetylation of BMAL1, as well as Nampt expression and NAD^+ levels, indicating a direct impact on the molecular machinery of the clock (Ribas-Latre et al., 2015c). More recently, studies have highlighted that GSPE can influence the hepatic circadian system not only through transcriptional regulation but also via microRNAs (Manocchio et al., 2022), and that its effects on mitochondrial dynamics in the liver can vary depending on the time of administration (Rodríguez et al., 2022).

The extrapolation of these findings in animal models to human health is of great relevance, considering the prevalence of night work and irregular schedules in today's society. Shift workers often experience chronic chronodisruption, characterised by a lack of synchronisation between their central biological clock, regulated mainly by light, and their activity and feeding patterns (James et al., 2017). This misalignment can have significant metabolic consequences, increasing the risk of developing the complications previously mentioned. In this regard, dietary proanthocyanidins emerge as promising bioactive compounds, not only because of their antioxidant properties and effects on metabolism (Bladé et al., 2016; Bladé et al., 2019), but also due to their capacity to modulate circadian rhythmicity at multiple levels. For example, Soliz-Rueda et al. demonstrated that grape-seed flavanols may act as zeitgebers, partially resynchronising disrupted clock systems under cafeteria-diet conditions (Soliz-Rueda et al., 2025), whereas Ávila-Román et al. reviewed how polyphenols, including proanthocyanidins, can act as modulators of biological rhythms, highlighting their potential as chrononutritional agents (Ávila-Román et al., 2021).

Thus, understanding how circadian disruption specifically affects hepatic lipid metabolism at the molecular level, and how GSPE could mitigate these effects, is of great importance for developing preventive and therapeutic strategies in populations with unconventional schedules. Moreover, identifying the specific pathways through which proanthocyanidins interact with the molecular clock would open new avenues for nutritional or pharmacological interventions aimed at protecting health under irregular lifestyles. Nonetheless, the precise mechanisms remain largely unknown, and further research is required to clarify the extent to which GSPE can be considered a true chronobiotic compound.

2 HYPOTHESIS AND OBJECTIVES

The current hypothesis is that **GSPE may manifest beneficial effects by mitigating the negative consequences of circadian disruption on hepatic lipid homeostasis**. Although direct evidence specifically linking GSPE to clock gene modulation is still emerging, several lines of indirect evidence support this hypothesis (Ávila-Román et al., 2021). GSPE's potent antioxidant and anti-inflammatory properties are highly relevant, as both oxidative stress and chronic inflammation are known to disrupt circadian clock function and contribute to metabolic dysregulation (Mohawk et al., 2012). Therefore, GSPE's ability to combat these stressors could indirectly protect the integrity and functionality of the circadian clock machinery. Furthermore, studies with other polyphenolic compounds have demonstrated their capacity to modulate core clock gene expression or influence clock-controlled metabolic pathways (Ávila-Román et al., 2021; Parrish et al., 2020). Given that GSPE exerts significant effects on metabolic pathways that are themselves under robust circadian control (such as *de novo* lipogenesis, β-oxidation), it is reasonable to hypothesise that GSPE might either directly interact with components of the molecular clock or indirectly influence clock function through its effects on metabolic signalling, therefore, it is anticipated that the results of this study will provide valuable information on the potential of GSPE as a chrononutritional strategy to counteract the adverse effects of circadian desynchronisation on hepatic lipid metabolism.

In order to verify or ratify this hypothesis, three **main objectives** were proposed:

- → To evaluate the impact of circadian rhythm disruption on hepatic lipid metabolism in rats.
- → To study the ability of GSPE to modulate or alleviate the potential alterations generated as a result of the disruption of the circadian rhythm on hepatic lipid metabolism in rats.
- → To determine whether the effects of GSPE are influenced by the metabolic context.

Additionally, to achieve the previously mentioned three main objectives, the following **secondary objectives** were suggested:

- → To quantify hepatic triglyceride levels to determine whether circadian disruption induces significant changes in the hepatic lipid profile and whether GSPE exerts a therapeutic effect.
- → To analyse possible changes in the expression of key proteins involved in hepatic lipid metabolism, as well as their temporal variation across different zeitgeber times, under conditions of circadian disruption and GSPE treatment.

3 METHODOLOGY

3.1 *IN VIVO* EXPERIMENTAL DESIGN

The study was conducted on 64 male Wistar rats (Janvier Labs, France) and lasted for 6 weeks, including an initial week of adaptation. The 12-week-old rats were kept individually under the following conditions: 22 ± 1 ºC temperature and 55% ± 2 humidity. During the 5 weeks of the experimental period, all rats were fed *ad libitum* with a standard diet (2.90 kcal x g-1 Teklad Global 14% Protein Rodent Maintenance Diet, ENVIGO) and water.

The rats were randomly distributed in the following four experimental groups (n=16), depending on the light-dark cycles to which they were subjected and whether they were administered GSPE (Figure 2):

Control group (C): receiving daily a vehicle (water) and followed a cycle of 24 hours, with 12 hours of light and 12 hours of darkness.
Grape seed proanthocyanidin-rich extract group (GSPE): receiving daily GSPE (equivalent to 25 mg/kg/day,) and followed a cycle of 24 hours, with 12 hours of light and 12 hours of darkness.
Disrupted group (D): receiving daily a vehicle (water) and followed a cycle of 22 hours, with 11 hours of light and 11 hours of darkness (Campuzano et al. 1998).
Disruption + grape seed proanthocyanidin-rich extract group (DGSPE): receiving daily GSPE (equivalent to 25 mg/kg/day,) and followed a cycle of 22 hours, with 11 hours of light and 11 hours of darkness (Campuzano et al. 1998).

The cycles of both the control (24h) and the disrupted (22h) groups were restarted when the light was turned on. The GSPE or vehicle were administered 1h after the light was turned off, that is, ZT13 (13th hour of the cycle) for the control groups and ZT12 (12th hour of the cycle) for the groups with the disruption.

Throughout the study, measurements of daytime and nighttime feed intake were made, weighing the feed both when the light was turned on and when it was turned off. Additionally, temperature, activity and blood pressure were continuously monitored by telemetry through chips implanted in the aorta of all rats.

At the end of the experimental period, four animals from each experimental group were sacrificed at the following four different zeitgeber times (Zeitgeber time=ZT): ZT3 (light), ZT9 (light), ZT15 (darkness) and ZT21 (darkness); resulting in n=4 animals per ZT per experimental group. At that moment, the livers were collected and, after being quickly frozen, stored at -80°C until further analysis.

At the time that this bachelor's thesis was conducted, the experimental *in vivo* part of the present study had already been completed. This was carried out by the NUTRIGEN research group of the Universitat Rovira i Virgili, located in the province of Tarragona, in Spain.

Animal experiments were carried out according to the guidelines for care and use of animals established by the Animal Experimentation Ethics Committee from the Universitat Rovira i Virgili (reference number 12241), and in agreement with Directive 86/609EEC of the Council of the

European Union and the procedure established by the Departament d'Agricultura, Ramaderia I Pesca of the Generalitat de Catalunya.

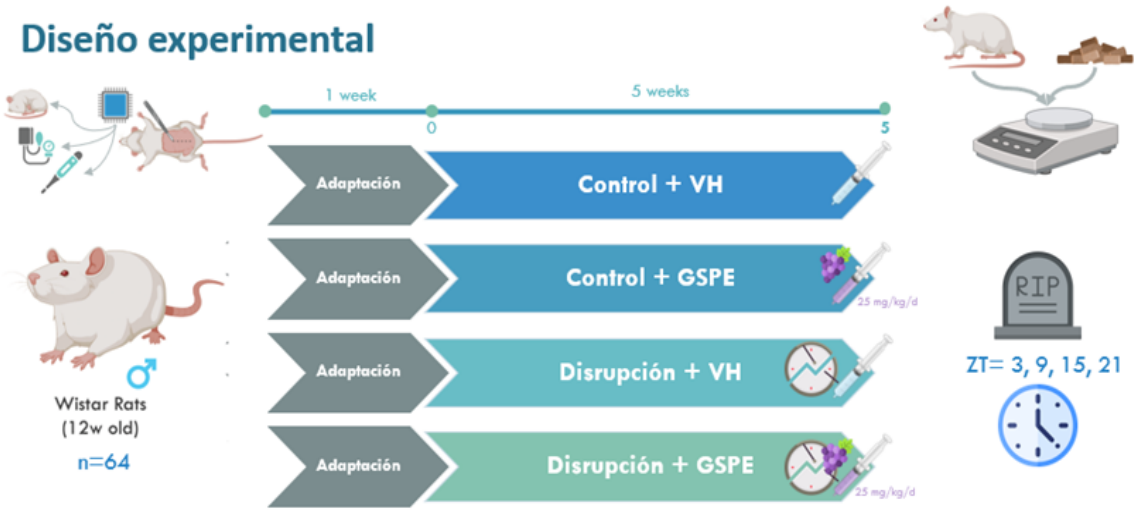

Figure 2. Summary of the *in vivo* experimental design. A total of 64 male Wistar rats (12w old) were used in this study, which was conducted over 6 weeks. The animals were euthanized at different time points (ZT=3, 9, 15, and 21) to assess diurnal rhythms. w means week; n means experimental number; ZT means zeitgeber time; VH means vehicle; GSPE means Grape-Seed Proanthocyanidin Extract.

3.2 GRAPE-SEED PROANTHOCYANIDIN EXTRACT COMPOSITION

The GSPE used in this study was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France) and, according to the manufacturer, it was obtained from white grape seed. The main phenolic compounds present in the extract were monomers (catechin, epicatechin, gallic acid and epicatechin gallate; 21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%), and oligomers (5–13 units; 31.7%) of proanthocyanidins (Schwartz et al., 2022) (Table 1).

Table 1. Main phenolic compounds of the GSPE used in this study, analysed by HPLC-MS/MS, adapted from Arreaza-Gil (2022). Concentrations are expressed as mg of compound per gram of fresh extract (mean ± standard derivation).

PHENOLIC COMPOUND	CONCENTRATION (mg/g)
Protocatechuic acid (PCA)	1.40 ± 0.25
Catechin	51.88 ± 5.56
Epicatechin	62.86 ± 8.32
3,4,5-trihydroxybenzoic acid	44.66 ± 7.76
Kaempferol-3-glucoside	0.50 ± 0.02
Naringenin-7-glucoside	0.64 ± 0.08
p-Coumaric acid	0.09 ± 0.01
Quercetin	0.05 ± 0.01
Quercetin-3-O-galactoside	0.43 ± 0.05
4-hydroxy-3-methoxybenzoic acid	0.09 ± 0.01
Procyanidin dimer	76.84 ± 15.76
Procyanidin trimer	13.04 ± 0.64
Procyanidin tetramer	5.14 ± 0.28
Dimer gallate	15.22 ± 2.72
Epicatechin gallate	14.24 ± 2.76
Epigallocatechin gallate	0.06 ± 0.01

3.3 DETERMINATION OF HEPATIC TRIGLYCERIDE CONTENT

First, 150 mg of each sample was homogenised with 2 µL of a homogenisation buffer (Tris 10mM, EDTA 2mM, Saccharose 0.25M, NaOH 1M and distilled H_2O).

Triglyceride (TG) quantification was conducted using a commercially available enzymatic colorimetric assay (Spinreact, S.A., GPO-PAP kit, no. 41031), where, upon reagent addition, the intensity of the colour produced in each sample is directly proportional to the triglyceride concentration in the sample. Absorbance was measured spectrophotometrically at a wavelength of 492 nm.

To analyse the results, a triglyceride standard curve was prepared for each assay using a known concentration standard from the aforementioned kit. Samples were analysed in parallel with the standards, and triglyceride concentration in the samples was calculated from the standard curve. Results were expressed as mg TG/g tissue, with each sample normalised by its initial tissue weight.

3.4 PROTEIN EXPRESSION

3.4.1 Tissue homogenisation

For the preparation of protein extracts for Western Blot, samples were homogenised on ice using a mechanical stirrer "COMECTA Heidolph Type RZR 1" (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), in an homogenisation buffer containing cellular Phosphate-Buffered Saline (PBS) buffer (NaCl 0.15M, KCl 3mM, NaH_2PO_4 3mM, Na_2HPO_4 7.5 mM and H_2O_{miliQ}; pH = 7.4) and protease inhibitors (PMSF 100 mM and iodoacetamide 100 mM). Then, lysates were centrifuged at 800 g for 5 minutes at 4°C to remove cellular debris, and the supernatant, containing soluble proteins, was collected. Samples were stored at -80°C until use.

3.4.2 Protein Quantification by BCA method

Pierce™ Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the protein concentration following the manufacturer's instructions.

Briefly, a standard curve of Bovine Serum Albumin (BSA) was prepared with a concentration range from 0 to 2 mg/mL. Subsequently, samples were diluted 1:5 and the freshly prepared BCA working reagent was added to each well. The plates were incubated at 37°C for 30 minutes, and the absorbances were measured at 562 nm using Agilent BioTek Synergy HTX Multi-Mode Microplate Reader (Agilent Technologies, n.d.).

3.4.3 Sample preparation and electrophoresis.

60 µg of protein of each sample was mixed with 4X Laemmli loading buffer (containing Laemmli Sample Buffer (Bio Rad 161-0737) and β-mercaptoethanol (Merck 15433.0100)), spun and denatured at 100°C for 3 minutes.

Proteins were separated by SDS-PAGE using 4-15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hércules, California), and electrophoresis was carried out at 100 V for 30-60 minutes until the dye front reached the bottom of the gel, using a freshly made running buffer (Tris 0.25 M/1.92 M glycine, SDS 10% and H_2O_{miliQ}).

3.4.4 Electrotransfer and Western Blot (Immunodetection)

Following SDS-PAGE separation, proteins were transferred to Immobilon®-P PVDF membranes (Pore size 0.45 µm; Millipore, IPVH00010), previously activated in methanol, using a Bio-Rad Trans-Blot Turbo transfer system V1.02 (Bio-Rad Laboratories, Hércules, California, n.d.) at 25V and 1A for 30. Transfer-efficiency was verified by Ponceau S staining, which was removed with one wash with transference buffer and 3 washes of 10 minutes each with PBS-Tween. Then, membranes were blocked for 1 hour at room temperature with a solution of 5% skimmed milk powder in PBS-Tween buffer (Phosphate Buffered Saline with 0.1% Tween 20).

After blocking and doing another 3 washes of 10 minutes each with PBS-Tween, membranes were incubated with the appropriate primary antibodies overnight at 4°C with gentle agitation. Primary antibodies anti- αTubulin (Ref. 2125), anti-pACC (Ref. 3661), anti-total ACC (Ref. 4190) and anti-CD36 (Ref. E8B7S) were obtained from Cell Signalling Technology (Danvers, MA, USA). Primary antibodies anti-DGAT2 (Ref. ab59493), anti-FAS (Ref. ab128870) and anti-MTTP (Ref. ab75316) were purchased from Abcam (Cambridge, UK). Primary antibodies anti-CPT-1 (Ref. NB100-53719) were obtained from Novus Biologicals (Centennial, CO, USA). Primary antibodies anti-FATP5 (SLC27A5; Ref. LS-C293846) were purchased from LifeSpan BioSciences (Seattle, WA, USA).), All antibodies were diluted 1:1000 in PBS-Tween prior to incubation.

Subsequently, membranes were washed 3 times for 10 each time with PBS-Tween and then incubated with the corresponding secondary antibodies for 1 hour at room temperature with gentle agitation. Secondary antibodies anti-Goat (Ref. SC 2354) and anti-Rabbit (Ref. SC 2357) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and were also diluted 1:1000 in PBS-Tween prior to incubation.

After another 3 thorough washes with PBS-Tween for 10 minutes each time, immunodetected proteins were visualised using the enhanced chemiluminescence (ECL) system SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and images were captured with a digital chemiluminescence detection system (ChemiDoc™ Imaging System; Bio-Rad, Hércules, California) using Image Lab Software (Bio-Rad, Hércules, California).

3.4.5 Densitometric Analysis

Band intensities of proteins of interest were quantified densitometrically using Image Lab Software (Bio-Rad Hércules, California). Protein expression levels were normalised to the expression of the loading control protein, in this case αTubulin, to ensure uniform sample loading.

3.5 STATISTICAL ANALYSIS

3.5.1 Hepatic triglyceride content

The following statistical analysis was performed using SPSS software (IBM SPSS Statistics 25, Armonk, NY, USA). A significance level of $\alpha=0.05$ was set for all statistical tests. A p-value less than 0.05 was considered statistically significant. Data is presented as the mean ± standard error of the mean (SEM).

Preliminary tests were conducted to evaluate data distribution and homogeneity of variances. The **normality** of the triglyceride concentration distribution within each of the 16 experimental subgroups (4 experimental groups x 4 ZTs) was assessed using the **Shapiro-Wilk test**. Data was

considered to follow a normal distribution as the test's p-value was greater than 0.05. The **homogeneity of variances** among the diverse groups and ZTs was checked using **Levene's Test**. Variances were determined to be homogeneous as the associated p-value was greater than 0.05.

Once assumptions were evaluated, a **two-way Analysis of Variance (ANOVA)** was run to determine the main effect of "Experimental Group" (Control, GSPE, Disruption, Disruption with GSPE), the main effect of "Zeitgeber Time" (ZT3, ZT9, ZT15, ZT21), and the potential interaction between both factors on hepatic triglyceride concentration. **Post-hoc** tests were performed to identify specific differences between groups, in cases where significant main effects or interactions were found in the ANOVA. If variances were found to be homogeneous (according to Levene's Test), the **Tukey HSD** (Honest Significant Difference) test would be used for pairwise comparisons. If variances were not homogeneous, more robust post-hoc tests that do not assume equal variances, such as the **Games-Howell test**, would be considered, if appropriate for the factorial design.

If when interpreting the ANOVA results, the interaction proved significant ($p < 0.05$), it would be interpreted that the effect of one factor was dependent on the level of the other, and estimated interaction means would then be analysed. If the interaction was not significant, the main effects would be interpreted independently.

3.5.2 Protein Quantification

Data was analysed using IBM SPSS Statistics 25 software (Armonk, NY, USA). For all analyses, a probability (p) value of <0.05 was considered statistically significant. Results are expressed as the mean ± standard error of the mean (SEM).

Prior to inferential statistical testing, **descriptive statistics** were calculated for each protein to summarize central tendency and dispersion across experimental groups and zeitgeber time points. **Normality of data distribution** within each experimental condition (combination of disruption, treatment and ZT) was assessed using the **Shapiro-Wilk test**. **Homogeneity of variances** across groups was evaluated using **Levene's test**, performed as part of the ANOVA procedure.

The primary statistical analysis for each protein was a **two-way Analysis of Variance (ANOVA)**, which was run to determine the main effect of "Experimental Group" (Control, GSPE, Disruption, Disruption with GSPE), the main effect of "Zeitgeber Time" (ZT3, ZT9, ZT15, ZT21), and the potential **interaction** between both factors. If significant interactions were detected, **simple effects analyses** were conducted to further investigate the nature of these interactions. For significant interactions, **post-hoc comparisons** were performed. **Tukey's Honestly Significant Difference (HSD) test** was used for post-hoc comparisons when the assumption of homogeneity of variances was met. If Levene's test indicated a violation of this assumption, **Games-Howell post-hoc test** was applied.

4 RESULTS

4.1 HEPATIC TRIGLYCERIDE CONTENT

As shown in Figure 3, there were no significant changes in hepatic triglyceride levels among any of the four experimental groups. Similarly, no significant differences were observed among the ZT3, ZT9, ZT15, and ZT21 subgroups (Figure 4).

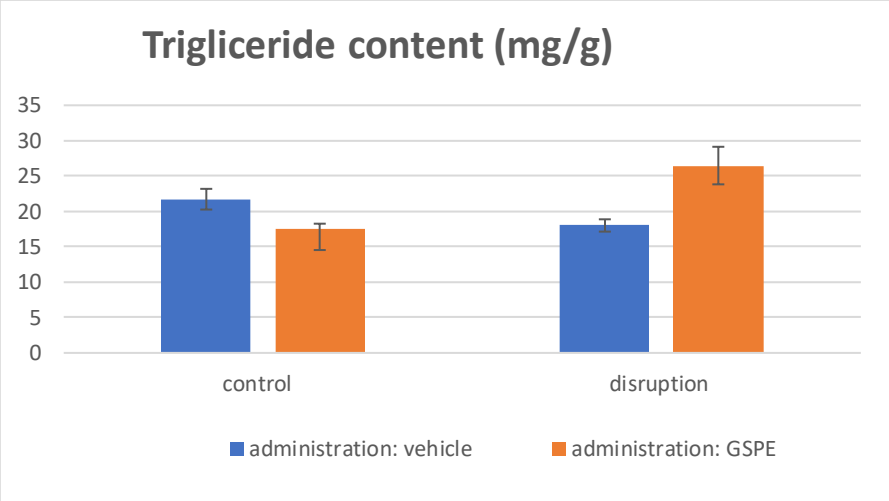

Figure 3. Representation of the hepatic lipid concentration (mg lipid/g liver) on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

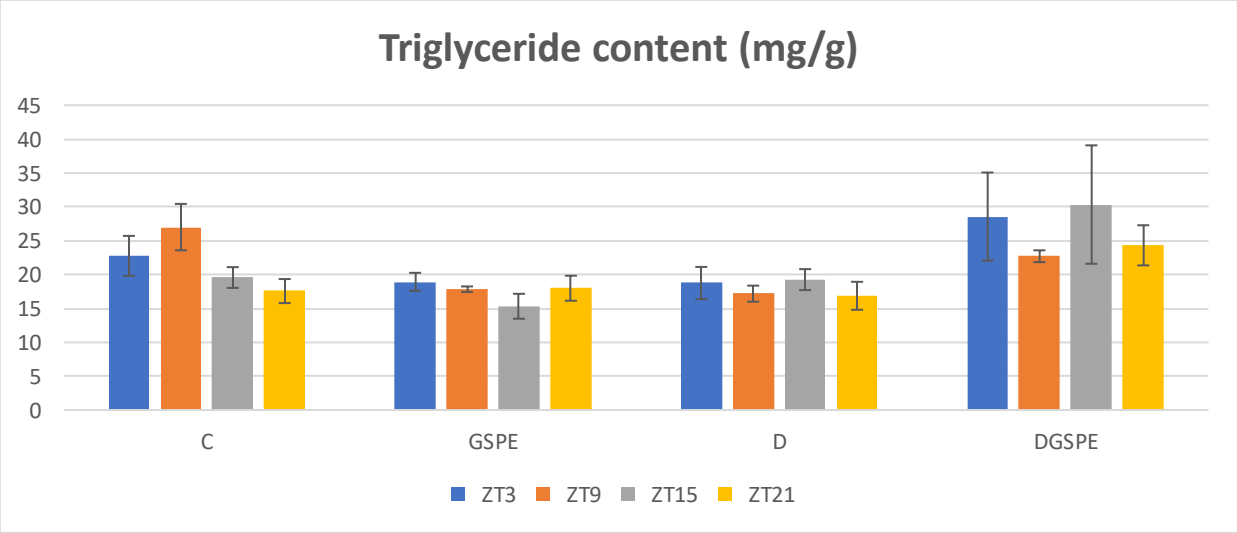

Figure 4. Representation of the hepatic lipid concentration (mg lipid/g liver) on each experimental group during the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM).

4.2 PROTEIN EXPRESSION

Significant changes were noticed in the expression of the **total** amount of the protein **ACC** (including both the phosphorylated and the no-phosphorylated forms) among the **Control** and the **Disrupted+GSPE** experimental groups (Figure 6). On the contrary, no significant differences were observed in the expression of the rest of the studied proteins (Figures 5, 7-12).

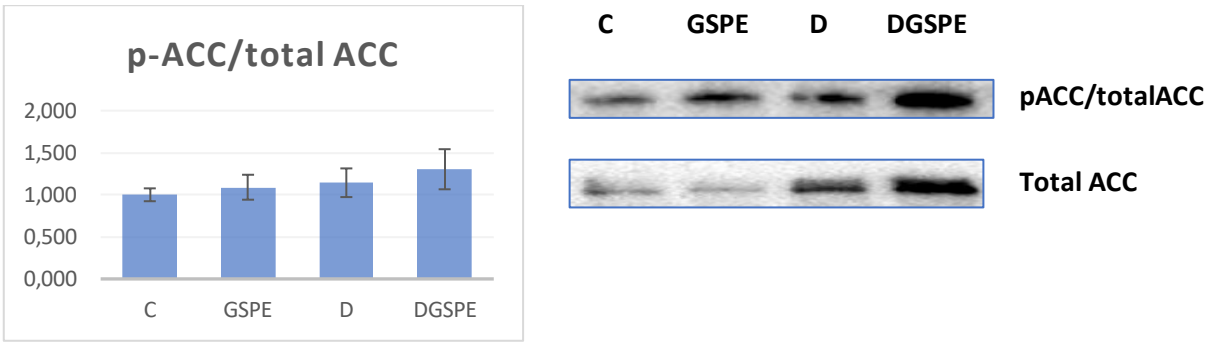

Figure 5. Representation of pACC/totalACC protein expression ratio across the four experimental groups. Results are expressed as the mean ± standard error of the mean (SEM).

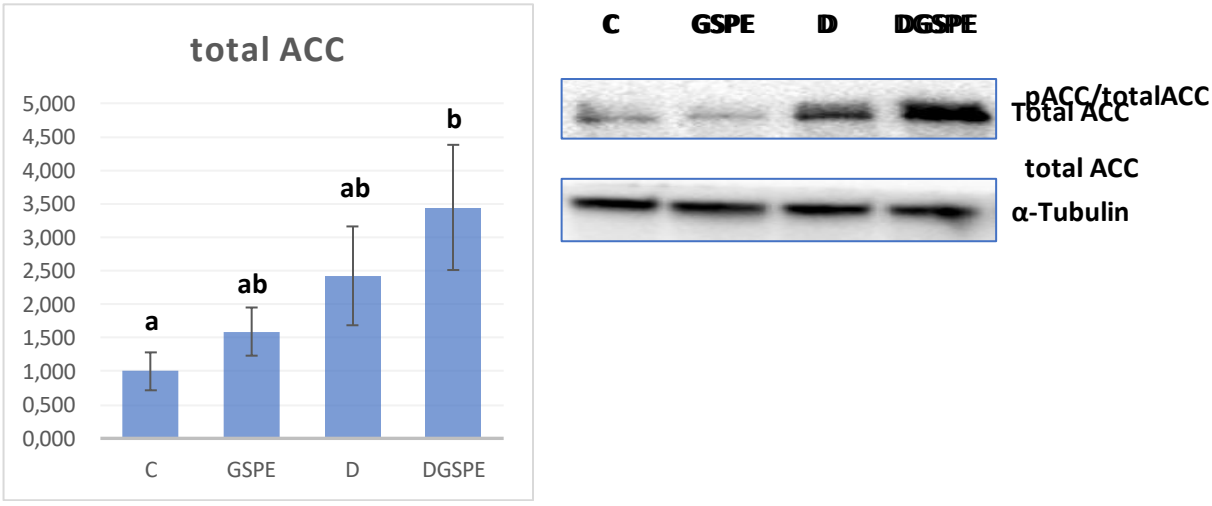

Figure 6. Representation of total ACC protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences by the one-way ANOVA test ($p<0.05$).

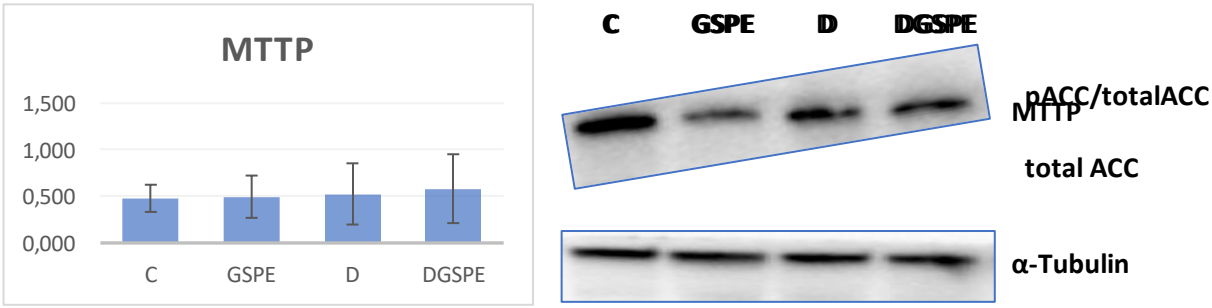

Figure 7. Representation of MTTP protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

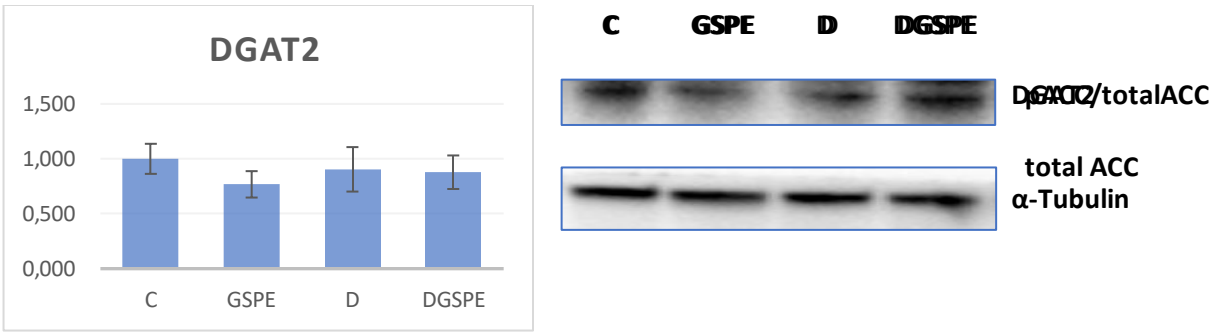

Figure 8. Representation of DGAT2 protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

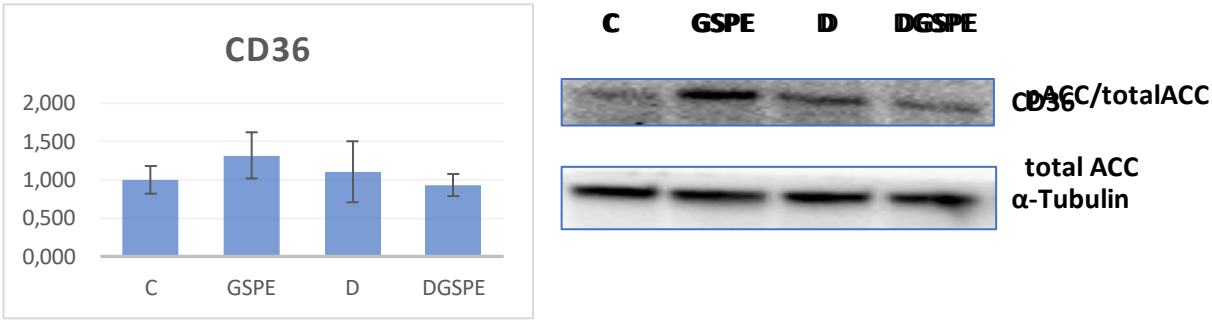

Figure 9. Representation of CD36 protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

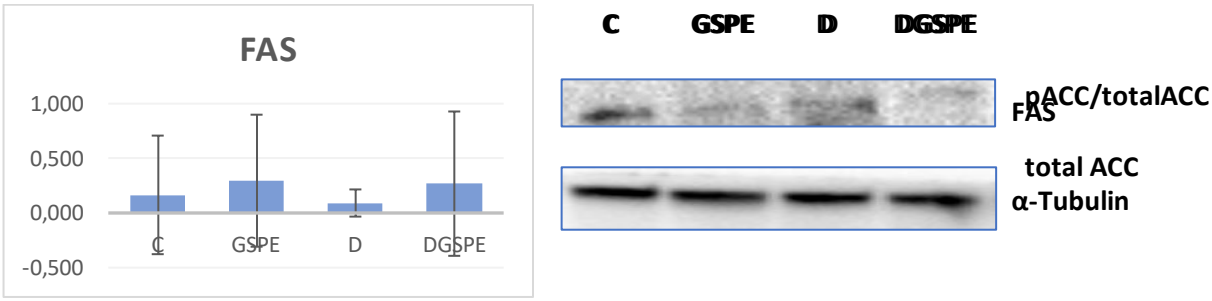

Figure 10. Representation of FAS protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

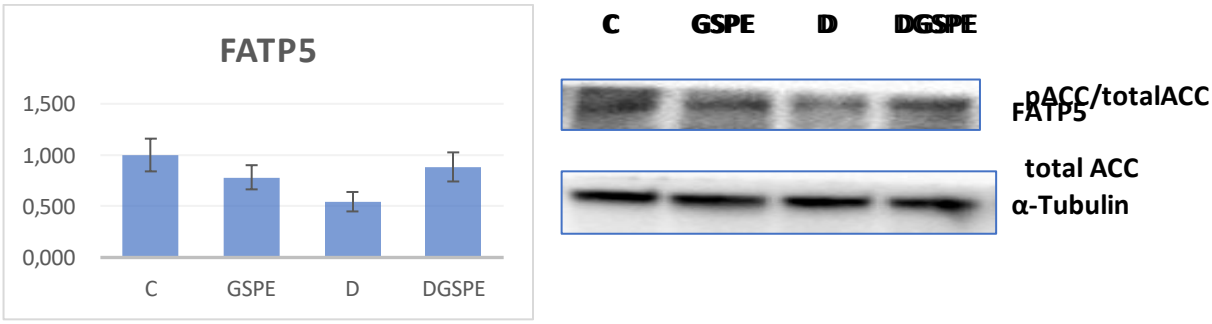

Figure 11. Representation of FATP5 protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

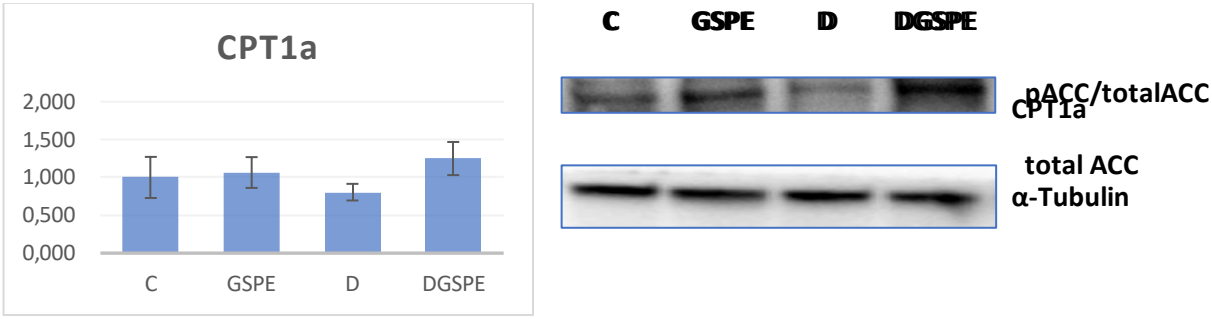

Figure 12. Representation of CPT1a protein expression levels on each experimental group. Results are expressed as the mean ± standard error of the mean (SEM).

Regarding the different **zeitgeber time points**, no significant results were found among the expression of the protein ratio pACC/totalACC, representative of the activation of ACC, neither in the protein expression of MTTP, DGAT2 and CD36 (Figures 13, 15-17). Nonetheless, results indicated not only that **total ACC** levels were significantly higher in the ZT9 group compared to the ZT3 group, but also that **FAS** levels were significantly higher in the ZT3 group compared to the other three groups (ZT9, ZT15 and ZT21). In addition, **FATP5** levels were higher in the ZT21 group compared to the other three groups (ZT3, ZT9 and ZT15) and **CPT1a** levels were significantly higher in the ZT9 and ZT21 groups compared to the ZT15 group (Figures 14, 18-20).

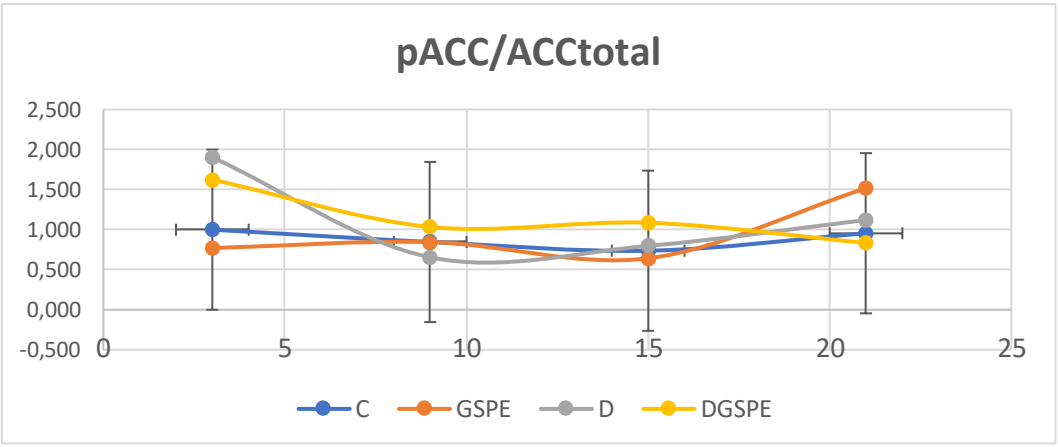

Figure 13. Time series graph illustrating the pACC/totalACC protein expression ratio across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM).

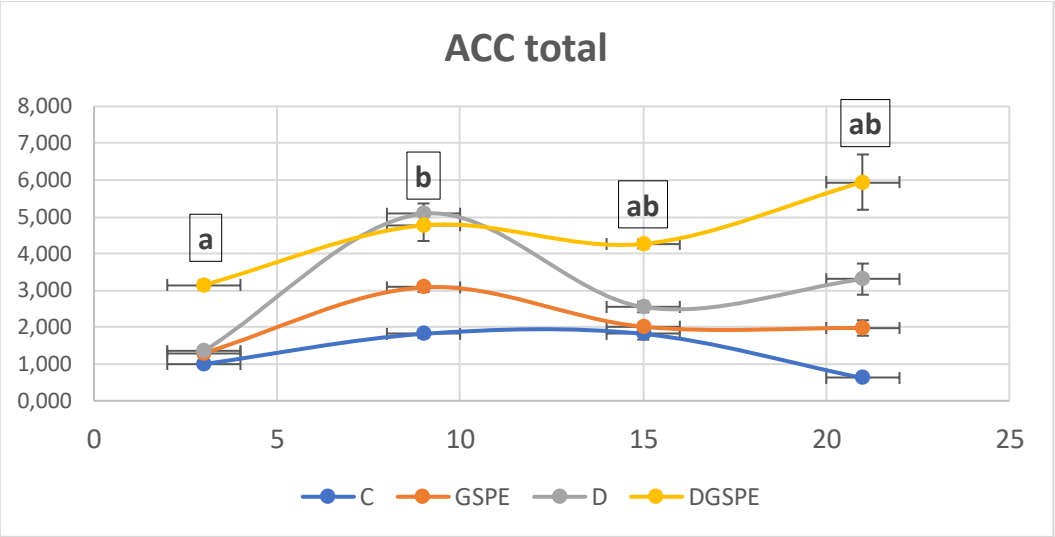

Figure 14. Time series graph illustrating the overall expression of the ACC protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences by the one-way ANOVA test ($p<0.05$).

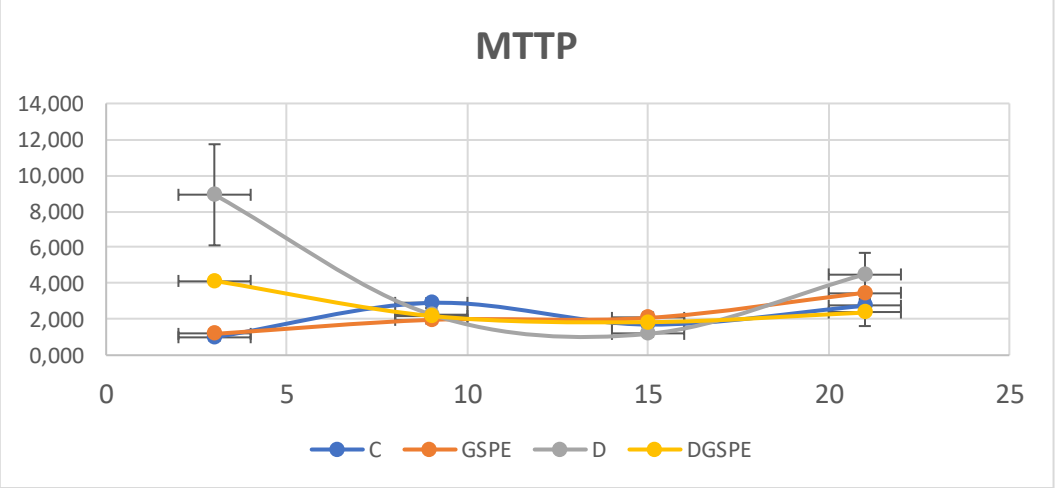

Figure 15. Time series graph illustrating the expression of the MTTP protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM).

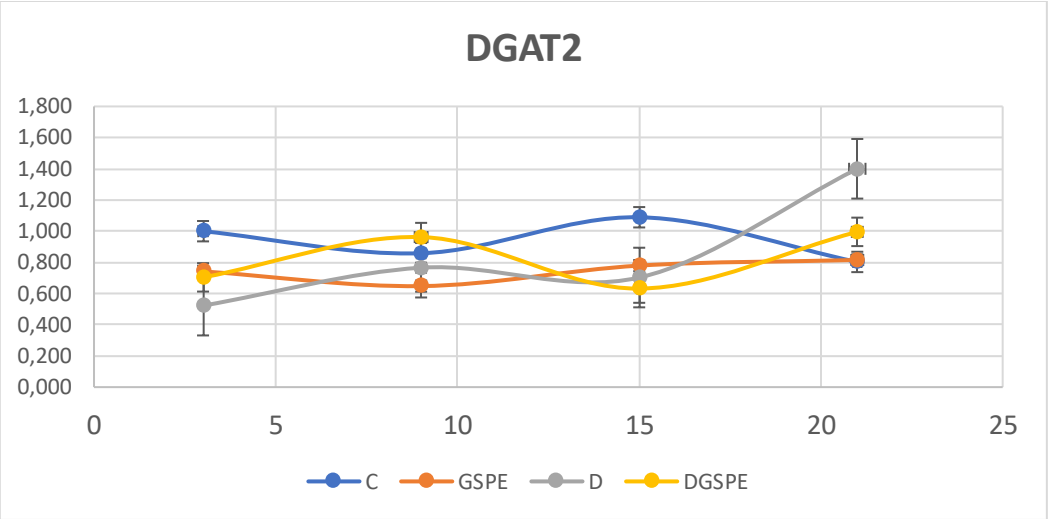

Figure 16. Time series graph illustrating the expression of the DGAT2 protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM).

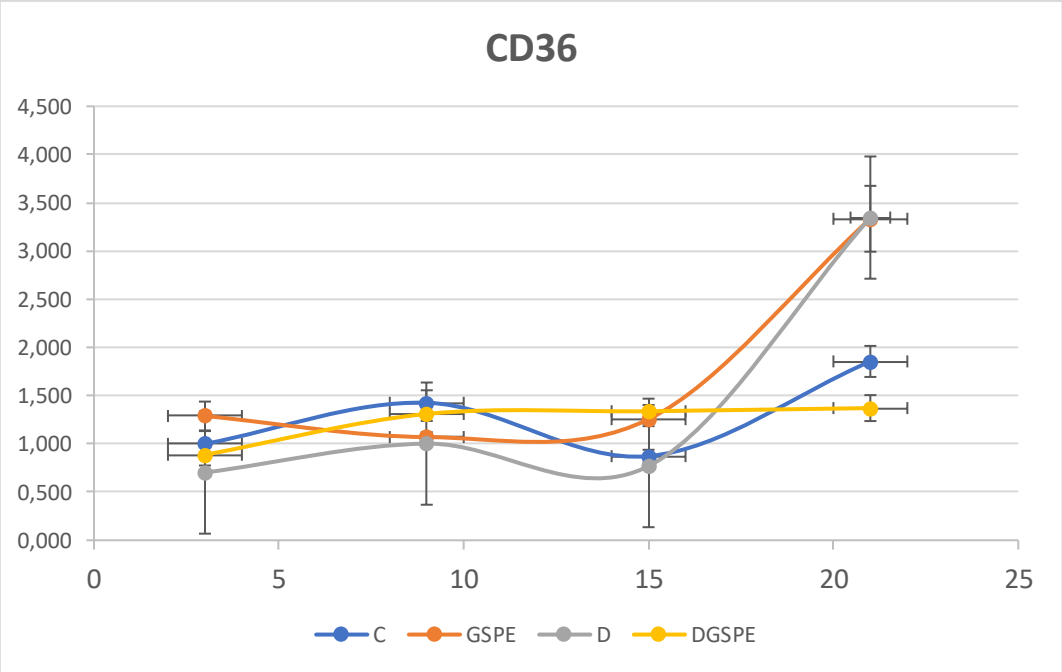

Figure 17. Time series graph illustrating the expression of the CD36 protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM).

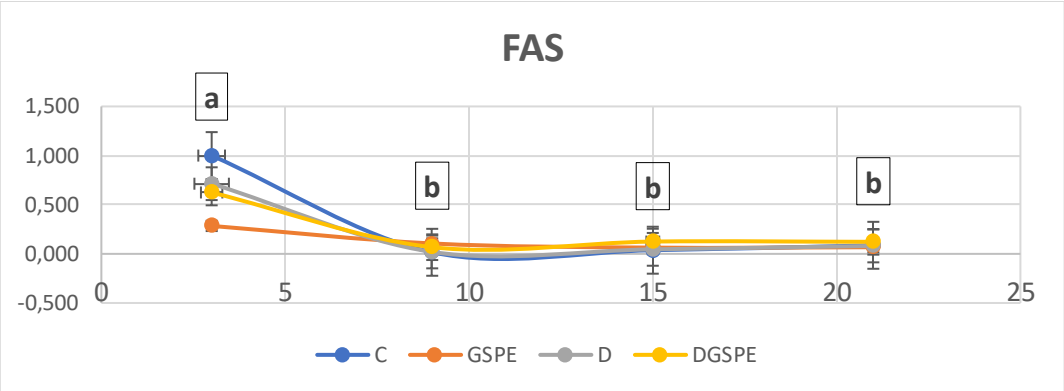

Figure 18. Time series graph illustrating the expression of the FAS protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences by the one-way ANOVA test ($p<0.05$).

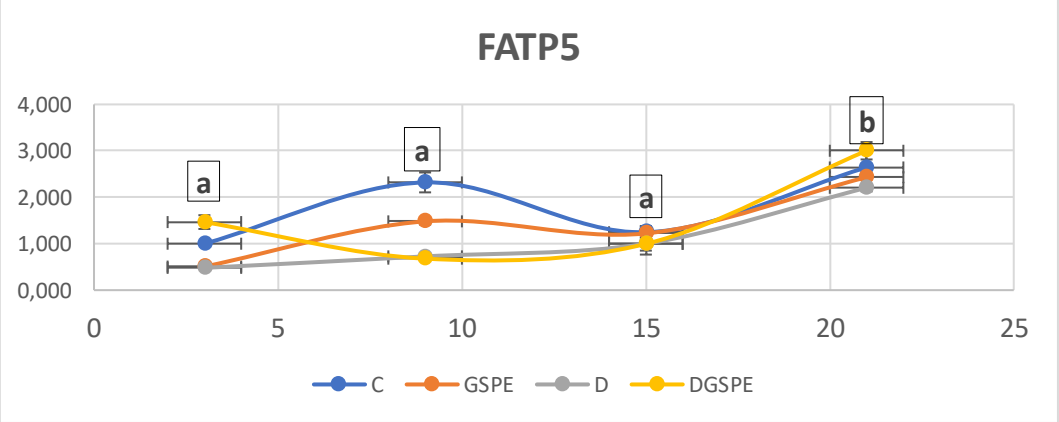

Figure 19. Time series graph illustrating the expression of the FATP5 protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences by the one-way ANOVA test ($p<0.05$).

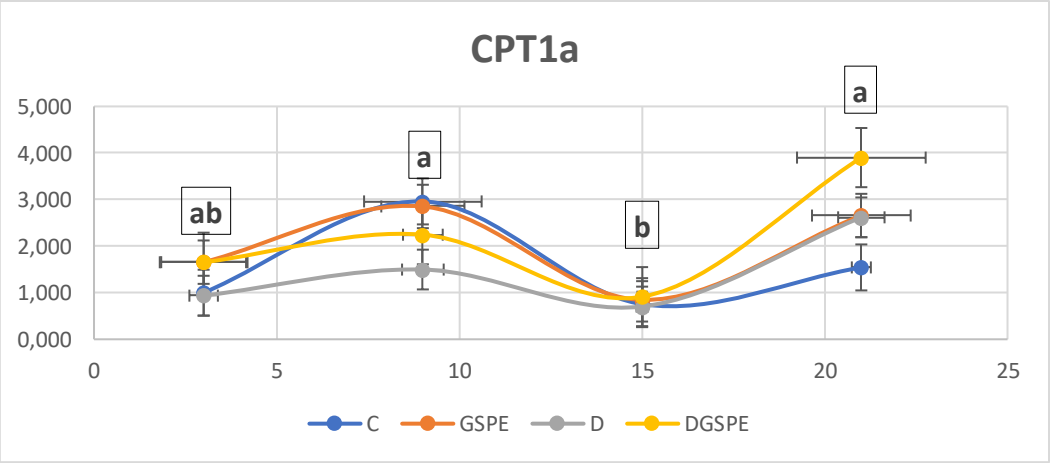

Figure 20. Time series graph illustrating the expression of the CPT1a protein across the four different zeitgeber time points (ZT3, ZT9, ZT15 and ZT21). Results are expressed as the mean ± standard error of the mean (SEM). Different letters indicate statistically significant differences by the one-way ANOVA test ($p<0.05$).

5 DISCUSSION

In today's world, where irregular lifestyles and atypical work schedules are increasingly common, circadian disruption has become a significant health risk. This misalignment, caused by factors such as irregular sleep and eating patterns, directly impacts crucial energy metabolism and is consistently linked to a higher risk of developing various metabolic disorders, including obesity and Metabolic dysfunction-associated Steatotic Liver Disease (MASLD). In this context, the idea of exploring therapeutic strategies that restore circadian rhythms is gaining interest, such as the administration of grape seed proanthocyanidin extract (GSPE), a promising bioactive compound that has been shown to modulate key enzymes involved in fat synthesis and degradation. Recent findings further suggest that GSPE can also act on circadian regulation itself, modulating peripheral clocks, melatonin secretion, and clock-gene expression in central and peripheral tissues (Ribas-Latre et al., 2015a; Ribas-Latre et al., 2015b; Ribas-Latre et al., 2015c).

The present study focused on investigating the effects of GSPE treatment on hepatic triglyceride levels and the presence and activity of key proteins involved in hepatic lipid metabolism in male rats subjected to a circadian desynchronisation regime induced by a shortened 22-hour light-dark cycle, also examining potential daily variations across different zeitgeber time points. On the one hand, including a control group and a circadian rhythm-disrupted group allowed us to assess the impact of chronodisruption on protein expression in lipid metabolism. On the other hand, the inclusion of groups treated with GSPE under both conditions (control and circadian disruption) made it possible to determine if GSPE can attenuate the alterations induced by desynchronisation. Finally, the division of each experimental group into subgroups at four distinct zeitgeber times (ZT3, ZT9, ZT15, and ZT21) allowed the analysis of the rhythmicity of protein expression under the different experimental conditions and the evaluation of whether GSPE treatment influences these temporal patterns.

Contrary to the initial hypothesis, which anticipated metabolic alterations due to chronodisruption and protective effects from GSPE, the comprehensive statistical analysis revealed **no statistically significant effects** on hepatic triglyceride levels. Nevertheless, we wanted to assess whether, under our experimental conditions, disruption affected triglyceride metabolism, which could potentially lead to steatosis in the future, and whether the extract could reverse these changes.

The **absence of differences among the four experimental groups** indicated that the chronic circadian rhythm-disruption was not sufficient to induce hepatic steatosis under our conditions, and GSPE administration could not further reduce triglyceride levels. This finding is noteworthy, as several studies in rodents have shown that circadian misalignment alone can promote lipid accumulation in the liver; for instance, Shamsi et al. demonstrated that a rotating light cycle promoted hepatic triglyceride deposition in mice even in the absence of a high-fat diet (Shamsi et al., 2018). It is important to note, however, that their work was performed in mice, which may be more sensitive to circadian disruption than rats, potentially explaining part of the discrepancy with our findings. Similarly, Escobar et al. reported that male Fischer 344 rats fed a cafeteria-style obesogenic diet exhibited disrupted hepatic circadian rhythms, highlighting that metabolic stress and diet composition are essential determinants of the outcome (Escobar et al., 2020). In line with this, recent work has demonstrated that grape-seed flavanols may act as zeitgebers, helping to resynchronise disrupted peripheral clocks in the context of obesogenic diets (Soliz-Rueda et al., 2025).

Results regarding GSPE administration also contrast with other studies' findings in models of metabolic challenge, where GSPE has been reported to ameliorate steatosis: for example, Rodríguez-Vidal et al. showed that GSPE attenuated cafeteria-diet-induced hepatic triglyceride accumulation in rats, with effects influenced by photoperiod; and Quesada et al. demonstrated that GSPE (25 mg/kg) repressed hepatic lipogenesis and various genes such as MTTP and DGAT2 in high-fat-fed rats, attenuating fatty liver (Rodríguez-Vidal et al., 2024; Quesada et al., 2009). Moreover, Rodríguez et al. recently reported time-of-day-dependent effects of GSPE on hepatic mitochondrial dynamics in cafeteria-diet obese rats, underscoring the relevance of temporal factors when evaluating its bioactivity (Rodríguez et al., 2022). Similarly, Ávila-Román et al. reviewed the growing evidence supporting polyphenols as modulators of biological rhythms, including GSPE, reinforcing the idea that its potential as a chrononutritional agent depends on context and timing (Ávila-Román et al., 2021). An important remark is that our study was conducted under standard diet conditions, whereas others' use of a high-fat cafeteria diet introduce an additional metabolic challenge that may amplify the effects of chronodisruption; in healthy rats under standard diet conditions, the substrate for change may be limited, which could explain the absence of significant effects on hepatic triglyceride levels in this study.

Taken together, these findings suggest that neither the standard diet, the circadian disruption nor the GSPE treatment triggered strong metabolic alterations, and that the absence of hepatic steatosis is consistent with the requirement of a metabolic challenge for these effects to emerge. Similarly, the **lack of significant changes in hepatic triglyceride levels during different time points** reinforce the previous point.

Regarding the common behaviour of the studied proteins, in disrupted models anabolic pathways tend to be upregulated, and catabolic ones downregulated, and GSPE often helps to restore balance by repressing anabolic pathways and upregulating catabolic ones (Rodríguez-Vidal et al., 2024; Quesada et al., 2009). In this study, the awaited effects of GSPE would be translated as the downregulation of the expression of ACC, DGAT2, CD36, FAS and FATP5 and the upregulation of MTTP and CPT1a. In contrast, the results obtained from the rats, as they were already in a state of metabolic health, showed **no statistically significant changes, with a unique exception**. This critical and unexpected finding was the **significant increase in total Acetyl-CoA Carboxylase (ACC)** protein expression, which was observed **only in the group receiving the combined treatment of circadian disruption and GSPE**; neither the disruption alone nor the GSPE alone elicited this effect.

While the lack of changes observed in most groups is consistent with the absence of significant alterations in hepatic triglyceride levels, this result is particularly intriguing because it contradicts previous findings under dyslipidaemic conditions, where GSPE typically decreases ACC expression by suppressing lipogenic regulators such as SREBP-1c (Frederico et al., 2011; Tian et al., 2021; Lu et al., 2020). This atypical upregulation suggests a complex interaction between circadian misalignment and the biochemical effects of GSPE. ACC is the rate-limiting enzyme in de novo lipogenesis and, while its activity is tightly regulated by phosphorylation (an inactivation mechanism mediated by AMPK), its total protein expression is regulated at the transcriptional level, often by transcription factors like SREBP-1c (Deng et al., 2014). The absence of a change in the phosphorylated ACC to total ACC ratio indicates that phosphorylation-mediated inactivation remained proportionate; therefore, the increase in total ACC protein expression points to a potential increase in active ACC capacity without necessarily altering net lipogenesis. Importantly, proanthocyanidins have been shown to directly modulate hepatic clock components such as BMAL1 acetylation and NAD^+ metabolism (Ribas-Latre et al., 2015c), as well

as to regulate microRNAs involved in circadian control (Manocchio et al., 2022). It is therefore plausible that the unique combination of a 22-hour light-dark cycle, acting as a discordant zeitgeber, and the administration of GSPE, acting as a weak chronobiotic agent, could have overridden the expected repressive effects and led to the observed upregulation. The mechanism behind this specific upregulation may lie in the known interplay between the circadian clock and metabolic transcription factors; the unique combination of the 22-hour light-dark cycle, which acts as a discordant zeitgeber, and the administration of GSPE, which may function as a weak chrono-biotic agent, likely modulated the expression of upstream regulatory elements, overriding the typical repressive effect of GSPE on ACC and leading to the observed abnormal upregulation.

Regarding the results on the possible temporal patterns, despite the minimal group-level effects on protein expression, a number of the studied metabolic proteins displayed pronounced diurnal variation across the four zeitgeber time points. This observation underscores the persistence of a fundamental hepatic clock influence that was not abolished by the chronic light-dark shift or GSPE administration. In terms of anabolism, **FAS**, a key enzyme in lipogenesis, exhibited a distinct peak at ZT3, which was significantly higher than the levels at ZT9, ZT15, and ZT21 (Figure 18); **FATP5**, a fatty acid transporter, showed increased expression at ZT21, which was significantly higher than the levels at the other three time points (Figure 19); and **total ACC** levels were significantly higher at ZT9 compared to ZT3 (Figure 14). In terms of catabolism, **CPT1a**, the rate-limiting enzyme in β-oxidation, showed a rhythmic pattern with significantly higher levels at ZT9 and ZT21 compared to ZT15 (Figure20). In contrast, the levels of the pACC/total-ACC ratio, MTTP, DGAT2, and CD36 proteins remained stable throughout the different time points, indicating that their expression may be less tightly coupled to the circadian clock under these conditions (Figures 13, 15-17).

On the whole, the observed diurnal patterns align well with the broader principles of chronobiology in nocturnal rodents: The **FAS** peak at ZT3 (late in the night/early light phase) is consistent with the known circadian regulation of lipogenic enzymes, since the inactive (light) phase in nocturnal rodents is typically a period of fasting, during which the liver activates *de novo* lipogenesis to convert excess carbohydrates from feeding during the active phase into fatty acids for storage. The increased **FATP5** expression at ZT21, late in the active (dark) phase is a particularly interesting and novel finding, as it suggests a capacity for the liver to take up fatty acids just as the rats' nocturnal feeding activity is beginning to taper off, preparing the liver to process the last potential intake before the fasting period begins, which could be a plausible, clock-driven mechanism that optimises nutrient utilisation and storage. The rhythmic expression of **CPT1a**, with peaks at ZT9 and ZT21, is indicative of a fluctuating β-oxidation potential aligned with the fasting-feeding cycles, as CPT1a facilitates the transport of fatty acids into the mitochondria for oxidation. The peak at ZT9, which is late in the inactive phase, is physiologically logical as the liver would be increasing its reliance on fatty acid oxidation for energy as carbohydrate reserves are depleted, whereas the second peak at ZT21 (late in the dark, active phase) could reflect the liver's response to the culmination of nocturnal feeding, preparing for a period of rest. In particular, this dual-peak pattern highlights the liver's ability to finely tune energy metabolism in anticipation of both feeding and fasting periods and reaffirms that the 22-hour light-dark shift and GSPE did not completely ablate the intrinsic rhythmicity of the peripheral hepatic clock. This suggests that the liver, as a peripheral oscillator, is resilient and retains its temporal organisation despite the desynchronising zeitgeber. Importantly, studies confirm that dietary proanthocyanidins can reinforce peripheral oscillations and strengthen clock robustness under stress conditions (Ribas-Latre et al., 2015a; Ribas-Latre et al., 2015c; Soliz-Rueda et al., 2025).

On the other side, the 22-hour light-dark cycle constitutes a different period than the natural 24-hour cycle, a condition known to induce phase shifts in biological rhythms, therefore, it is highly likely that the peaks and troughs of the FAS, FATP5, and CPT1a rhythms in the disrupted groups were shifted in time relative to those of the control group. Also, chronodisruption can often lead to a dampening of the amplitude of oscillations, resulting in less pronounced peaks, a hallmark of a less robust clock. The administration of GSPE could have modulated this effect, possibly acting as a weak zeitgeber or by strengthening the clock's robustness through its antioxidant and anti-inflammatory properties, however, the lack of significant temporal variation for proteins like DGAT2 and CD36 suggests that their expression is less regulated by the circadian clock in this model.

Therefore, the **lack of significant changes in most proteins suggests that the system's homeostatic balance between anabolic and catabolic processes was maintained, as it was not challenged to the point of dysregulation by the moderate circadian disruption or the GSPE intervention.** The stability of these protein levels is, in itself, a significant finding, as it demonstrates the resilience of the hepatic metabolic system under the experimental conditions.

6 CONCLUSIONS

According to the results obtained under our experimental conditions, **the principal conclusions are**:

- → Neither chronodisruption nor GSPE were able to modify hepatic triglyceride metabolism.
- → The circadian disruption and GSPE exerted minimal impact on lipid-related proteins and most of them retained significant diurnal variation, indicating that hepatic circadian rhythmicity was preserved despite disruption and GSPE administration.
- → The effects of GSPE appear highly context-dependent and may be more evident in dyslipidaemic models than in normolipidaemic states.

In summary, **the initial hypothesis** that grape-seed proanthocyanidins (GSPE) could mitigate the adverse effects of circadian disruption on hepatic lipid metabolism **was not supported under the present experimental conditions**. Our findings indicate that circadian disruption alone did not induce hepatic steatosis or major alterations in lipid-related proteins, and that GSPE treatment did not reverse triglyceride levels or consistently modify protein expression. The only exception was an unexpected increase in total ACC in the Disruption+GSPE group, suggesting a complex interaction rather than a protective effect.

In this line, future work should consider metabolic challenge models and direct assessment of clock- and nuclear receptor-mediated signalling to elucidate the ACC response and potential therapeutic implications of GSPE to alleviate metabolic pathologies related to the chronodisruption. Nevertheless, the observation of preserved rhythmicity in several metabolic proteins supports the resilience of the hepatic circadian clock and indicates that future studies under metabolic challenge models are needed to fully test GSPE's chrononutritional potential.

This research work suggests findings that carry significant implications for the understanding of both chrononutrition and metabolic disease, opening up useful avenues for future research into metabolic diseases related to circadian misalignment and further illuminate the details of their relationship.

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